STT Publications

30

Biotechnology: a Dutch Perspective

Edited by J.H.F. van Apeldoorn

Biotechnology:
a Dutch Perspective

STT Publications 30

Stichting Toekomstbeeld der Techniek
(Netherlands Study Centre for Technology Trends)

Biotechnology:

a Dutch Perspective

Edited by J.H.F. van Apeldoorn

Delft University Press/1981

The Netherlands Study Centre for Technology Trends (STT) was established in 1968 by the Royal Institution of Engineers in the Netherlands. The Centre has two main aims:

- to evaluate technological trends from the viewpoint of the engineering sciences and to assess their interaction with other social developments;
- to bring its findings to the widest possible notice in order to help build up a more integral picture of the future fabric of Dutch society.

The Center's studies are a source of information for industry government authorities, educational bodies and, of course, the interested layman.

The offices of the Netherlands Study Centre for Technology Trends are at Prinsessegracht 23, The Hague; tel. no. (0)70 - 64 68 00. Postal adress: Stichting Toekomstbeeld der Techniek, P.O. Box 30424, 2500 GK The Hague.

Published by
Delft University Press
Mijnbouwplein 11
2628 RT Delft
tel. no. (0)15 - 78 32 54

Copyright © 1981 by Stichting Toekomstbeeld der Techniek

No part of this book may be reproduced in any form by print, photoprint, microfilm or any other means without written permission from Stichting Toekomstbeeld der Techniek.

ISBN 90 6275 051 6

Contents

Foreword

In 1979, when the Netherlands Study Centre for Technology Trends began examining the field of biotechnology, there was already a growing body of literature on the subject from other countries. At that time, though, no systematic examination had yet been made of the potential importance of biotechnology for the Netherlands, nor of the obstacles in the way of its development. Such a study was obviously needed. A thorough and coordinated analysis of the possibilities of biotechnology, covering the entire field of possible applications, is an invaluable tool for industry in assessing the prospects of innovation. In addition, it can assist the academic world to tailor research to future developments, and it is absolutely essential to the government in deciding on measures designed to stimulate developments.
Advances in the basic sciences transcend national boundaries, so any study which was of purely Dutch interest would be invalidated on the grounds of parochialism.
However, the combination of a thorough knowledge of the basic sciences and considerable industrial expertise in various biotechnological applications places the Netherlands in a special position.
The Netherlands Study Centre for Technology Trends realises that biotechnology is poised on the threshold of a period of rapid development and expansion, but at this stage it is difficult to foresee how the various sectors of society will be affected by these developments over the coming decades.
Recent Dutch publications on the subject have concentrated on government policy, or on one particular aspect of biotechnology. The aim of the present study is to present an integral picture of the prospects for biotechnology in all sectors, and of the social factors which will interact with this new field to determine the future course of applied biotechnology.
The study is published in English because the Centre believes that the Dutch case can hold valuable lessons and pointers for other industrialised countries. The report will also serve as the keynote paper for the symposium on biotechnology on May 20, 1981, which is being organised by the Centre.
We are confident that the report and the symposium will contribute to a wider appreciation of the potential of biotechnology and of the challenge it poses our society.
The Netherlands Study Centre for Technology Trends is greatly indebted to all who have worked on this study.

Dr A.E. Pannenborg

Chairman of the Board
Netherlands Study Centre
for Technology Trends

I Introduction

1. What is biotechnology?

The definition

One definition of biotechnology is that it is the science of applied biological processes.
There is, however, a certain consensus for a narrower definition, with biotechnology being regarded strictly as the science of production processes based on the action of micro-organisms and their active components, and of production processes involving the use of cells and tissues from higher organisms.
Medical technology, agriculture and traditional crop breeding are not generally regarded as biotechnology.

The earliest applications of biological processes involved food and stimulants. Wine is probably the oldest biotechnological product, followed by beer and bread.
Other important products of traditional biotechnology are bakers' yeast, cheese and yoghurt.
Tobacco, tea and cocoa are fermentation products, as are a large number of soya derivatives.

Around 1920, fermentation processes began to be used for the production of such substances as acetone, butanol, ethanol and glycerine. Fermentation was also applied to the manufacture of lactic acid and acetic acid.
Penicillin production developed rapidly after the Second World War, and other antibiotics are the subject of increasing research and production efforts. The biotechnological production of certain vitamins, steroids and enzymes is of more recent date.

The rapid development of molecular biology and cellular biology in the last few decades has laid the scientific basis for entirely new technologies.
Enzyme technology and the technique of genetic engineering have brought a new dimension to biotechnology, and have greatly expanded the range of options.
The field which is now being opened up is defined as 'the science of the integrated application of microbiology, biochemistry and process technology'. Generally speaking, this only covers application for industrial processes and environmental control.

It is this new form of biotechnology which has aroused the greatest hopes for the future.

The study area

The present study concentrates primarily on biotechnology which reflects the integration of microbiology, biochemistry and process technology. The emphasis is on manufacture. Biotechnology is seen not so much as a scientific discipline as a range of allied activities aimed at the manufacture or preparation of a product.

The report therefore focusses on the application of biotechnology in industry and for environmental control.
In these areas use is made of bacteria, yeasts, moulds, algae, plant and animal cell cultures, viruses for the preparation of vaccines, enzyme systems and organelles.
There is also an important parallel with the development of biotechnological applications in agriculture, where the main emphasis is on the *in vitro* culture of higher plants and genetic engineering in plant cells and soil organisms.

2. The study

The object

The aim of the study is to examine the development prospects and possible future applications of biotechnology.
The emphasis is on the potential of biotechnology in the Netherlands, the obstacles to its development, and its consequences for Dutch society. The period considered is of the order of at least 10 years.
The study is based on a scientific and technical evaluation of the field. The examination of the realistic prospects for biotechnology in the Netherlands also involves the consideration of financial, economic, organisational and other social factors.

Developments in biotechnology have generated a growing body of literature, both in the Netherlands and elsewhere [1, 2, 3, 4, 5, 6]. The accent in these reports is on the prospects of industrial innovation offered by these developments, and on the stimuli to be provided by national governments.
The title of the present study, *Biotechnology: a Dutch Perspective*, has a dual significance. Not only does it present a Dutch view of the possible development of biotechnology, but it also examines the challenge which biotechnology offers the Netherlands. The contributors believe that the Dutch case could also be of value to other industrialised countries.

The report

Broadly speaking, the report falls into three parts.
Those who are pressed for time will find the essence of the report in the summaries and conclusions to Chapters IV, V, VI, VII and IX, and in the general study findings in Chapter X.
The first part of the report consists of Chapters II and III. Chapter II gives a brief historical outline of the subject, followed by a concise introduction to a number of basic sciences and techniques which play a major role in biotechnology. This chapter is aimed primarily at the non-specialist. It does not pretend to give a complete picture, nor to discuss the latest developments in the various fields. Chapter III lists the academic courses and research activities in biotechnology and subordinate disciplines.

The second part of the report, Chapters IV-IX, explores the possible applications of biotechnology in five sectors: food and allied products, pharmaceuticals, chemistry, waste treatment, and agriculture.
The potential contribution of biotechnology is evaluated for each sector, as are the bottlenecks and the consequences of application.
Chapter VIII contains a very brief introduction to the subject of microbial leaching.

Chapter X forms the third part of the report, and contains a discussion of the study findings, general conclusions and recommendations. This chapter applies almost entirely to the situation in the Netherlands. It provides a synthesis involving social, organisational and economic aspects. It ends with conclusions and recommendations addressed to industry, the academic world, and government.

The contributors

The Netherlands Study Centre for Technology Trends is greatly indebted to all who have worked on this study. Its thanks are due in the first place to the Steering Group and to the authors, who made their contributions either individually or in the working groups.
The Centre is also grateful to all those who took part in the many discussions, either during the preparatory phase of the study or afterwards.
The Steering Group for the study consisted of the following members:

Dr J.A.M. van Boxsel	Ministry of Science Policy
Dr J. de Flines	Gist-Brocades
R. Keuning	Unilever Research Laboratories
Prof. N.W.F. Kossen	Delft University of Technology
Prof. A. Rörsch	Central Organisation for Applied Scientific Research in the Netherlands
Prof. A.H. Stouthamer	Free University, Amsterdam

Dr H.J. Vink	formerly of Philips Research Laboratories
J.H.F. van Apeldoorn (secretary)	Netherlands Study Centre for Technology Trends

The members of the seven working groups and individual authors are credited in the appropriate chapters of the report.

The report was translated by Michael Hoyle, Louise Boer-Hoff, Elizabeth Crocker and J. Kearey.
Translation editor: Michael Hoyle
Editorial assistant: Mrs A.G. van der Kamp-Thomasson
The entire study was prepared and supervised by J.H.F. van Apeldoorn, project leader at the Netherlands Study Centre for Technology Trends.

References

1. Dechema, Biotechnologie, Frankfurt/Main, March (1976) 3rd Edition
2. F. Gros, F. Jacob, P. Royer, Sciences de la vie et société, La Documentation Française (1979)
3. J. de Rosnay, Biotechnologies et bio-industrie. Annexe au rapport Sciences de la vie et société, La Documentation Française (1979)
4. A. Spinks, Biotechnology, Report of a Joint Working Party, ACARD/ABRC/ The Royal Society, London, March (1980)
5. R.R. van der Meer, Biotechnologie en innovatie, Publikatie van de Voorlichtingsdienst Wetenschapsbeleid, Staatsuitgeverij, The Hague (1980)
6. J.C. Pelissolo, La biotechnologie demain? La Documentation Française (1980)

II Science and technology

1. Historical background

1.1 Introduction

The last few decades have seen major developments in the biosciences. It goes without saying that developments in this area have not taken place independently of those in the other natural sciences; it is a process of interaction, with advances in one area stimulating discoveries in another.
Research in the life sciences is prompted by various motives. In the first place there is man's constant quest for scientific knowledge. In the life sciences this is particularly marked, partly because of the often mysterious nature of life, due to its complexity, but also because of man's awareness that he is inextricably bound up with the natural life of the planet. This gives a perspective to the life sciences which differs from that of the technical sciences, which are concerned with man's own creations.
Then there is the desire to put discoveries to practical use. In the life sciences this means acquiring an understanding of physical and mental processes. This knowledge can be used to fight disease (medical applications), to enhance food supplies (agricultural and livestock applications), and to stimulate the development of industrial applications (food preparation, and latterly the production of hormones, antibiotics and enzymes). Although the application of bioprocesses for the benefit of mankind is presumably as old as history, the really important breakthroughs in the biological sciences only occurred after those in physics and chemistry. It was the knowledge and skills acquired in the latter two areas that laid the basis for a fundamental understanding of life processes.
The greatest advances were made in microbiology, genetics and biochemistry, and after 1950 these separate fields merged to form the discipline of molecular biology. This combination of disciplines speeded up developments in the field of genetic information, leading to discoveries about the composition and structure of that information, the way it is stored, and its manifestation (expression).
The discoveries and developments described in the following sections have made a major contribution to the overall potential of biotechnology.

1.2 Microbiology

The earliest developments in the field of microbiology illustrate the strong attraction which biotechnology has had for Dutch scientists. The first visual

examinations of microscopically small plants and animals were made in the 17th century. It is generally accepted that the science of microbiology dates from a letter written to the Royal Society of London on October 9th, 1676, by Anthony van Leeuwenhoek at Delft. In that letter van Leeuwenhoek gave a detailed description of infusoria in rainwater, and of spirilla and bacteria in pepper ectracts. The anaerobic spore-forming bacteria were discovered in 1680, a bare four years later. It was to take another 200 years before these bacteria again attracted attention, from Pasteur and especially from the famous Dutch microbiologist, Martinus Willem Beyerink (1852-1931), who was professor of microbiology at Delft University of Technology from 1895-1921.
Between 1900 and 1910 Beyerink developed the principle of selective culturing methods, also known as accumulating methods. This fact was stressed when Beyerink was awarded the Emil Christian Hansen Medal in 1922. Until then every microbiologist who wanted to study a particular micro-organism had to rely on his experience to tell him that certain micro-organisms were present at specific locations. Often, though, it was pure coincidence that the investigator actually found the micro-organism he was looking for. As a rule it is impossible to recognise bacteria in a natural environment (substrate) or to isolate them. This is mainly because, within certain limits, the form which bacteria assume can change, and this cannot therefore be used as a criterion of identity.
Beyerink was the first to apply the logical principle that when a natural substrate containing all sorts of micro-organisms is introduced into a medium which is especially adapted to the growth requirements of only one, it is this micro-organism which is seen to develop into the one that outnumbers all the others.
Useful microbiological processes have been known since the dawn of time. Their action was not understood, but they were used extensively in the preparation and preservation of food. The work of the three pioneers of microbiology: Anthony van Leeuwenhoek, Louis Pasteur and Martinus Willem Beyerink, concentrated on the variety of species and their metabolism. From around 1875 industrial microbiology was responsible for the large-scale production of alcohol, for technical and other uses. Other microbiological products were lactic acid (1881), mould amylase (1894),* glycerol (1914), citric acid (1923), gluconic acid (1928), vitamin C, acetone and butanol, and vitamin B_2.
Fleming's discovery of penicillin signalled the start of the large-scale pharmaceutical use of the products of micro-organisms. It was followed by the discovery of an almost inconceivable number of substances with a wide range of properties-antibiotic, pharmacological and hormonal.

1.3 Biochemistry

The chemical analysis of the composition and metabolism of organisms has made an immeasurable contribution to our understanding of biological structures and functions.
In 1783 Spallanzi had demonstrated that meat was dissolved by gastric juices,

*Amylase is an enzyme which breaks down starch.

and in 1814 Kirchhoff discovered that a protein fraction from wheat (amylase) converted starch into sugars (maltose, glucose). In 1883 Payen and Person isolated this amylase from malt.
Between 1830 and 1930 a large number of enzymatic (biocatalytic) activities were demonstrated, and the existence of co-enzymes and enzyme specificity were discovered.
In 1927 von Embden and Zimmermann isolated adenylic acid from muscle tissue. Lohman later demonstrated that there is a pyrophosphate compound of this acid, adenosine triphosphate (ATP), in other cells as well. It soon became clear that this compound, which is a carrier of energy, plays a vital role in the energy management of living organisms, and so bioenergetics was born [1].
The following discoveries and advances have been made since 1931.

1. The mechanism of action of some vitamins can be explained by their function as co-enzymes (i.e. they assist in catalysis) [2].
2. The dynamics of metabolism (i.e. the interplay of the various metabolic cycles, whereby the products of one cycle form the substrates for another).
3. The development of the concept of group transfer (the transfer of large molecule fractions).
4. Enzymatic CO_2 fixation (carbon bonding).
5. The localisation of enzymes in the cell.
6. Protein synthesis.

Biochemistry has also made an important contribution to molecular biology, and the clarification of the amino acid sequence and the three-dimensional structure of a number of enzymes has greatly increased our understanding of the way enzymes operate.

1.4 Immunology

In 1797 Edward Jenner (1749-1823) observed that milkmaids remained unaffected during a smallpox epidemic due to their having previously contracted cowpox infections on their hands. This observation eventually saved the lives of millions of people and was a discovery of the first importance. It was only after many years that this discovery led to the understanding that antibodies are formed in warm-blooded animals upon the invasion of foreign substances (antigens), such as pathogenic micro-organisms and their products. The antigens combine with their specific antibodies to form complexes, whereby they are neutralised.
Jenner's discovery resulted in the introduction of cowpox inoculation, also known as vaccination (from the Latin *vacca*, cow). Vaccination is the deliberate introduction into the body of a milder, closely allied infective agent (antigen), which stimulates the production of antibodies. Those antibodies can later combat a more acute form of the disease.
The resulting resistance to that disease is known as immunity, and the inoculation as vaccination or immunisation. This breakthrough was followed by the development of vaccines for various other diseases, in the course of which it was discovered that devitalised (deactivated) pathogens can also

provide a considerable degree of immunity, provided they are introduced into the body in sufficient quantity.
Even more spectacular was the work of Louis Pasteur (1822-1895), who succeeded in developing a vaccination against rabies. He extracted nerve tissue from rabid animals which he then administered to the patient in a series of graduated doses. Each successive inoculation contained an extract of infected nerve tissue which had been dried for a shorter time and was thus more virulent. Eventually a point was reached where the patient had built up an immunity sufficient to withstand a serious infection with fully virulent rabies virus.
In those days it was thought that immunity was due solely to certain white blood cells engulfing and devouring the pathogen. They were called phagocytes (cell-eating cells). This theory of cellular immunity was developed by Mechnikov (1845-1916), and it was not until 10 years after his death that it was combined with the theory of the free antibodies (antibodies in the body fluids). This was largely due to the work of Emil von Behring (1845-1917), who discovered that a major cause of immunity against diphtheria and tetanus lay in the ability of the blood to deactivate the harmful substances (toxins) formed by the bacteria found in those diseases.
Von Behring used that discovery to develop another method of combatting infectious diseases. This involved administering an antiserum (blood containing antibodies but no blood cells) prepared from the blood of immune organisms. In 1901 von Behring was awarded the first Nobel prize for Medicine and Physiology for his antiserum therapy, the best known of which is horse serum for use against tetanus.
These discoveries led to the establishment of the scientific discipline of immunology. The most important advance of the past 25 years has been our understanding of the structure of the basic substances involved: the antigens (vaccines) and antibodies (sera). The subdiscipline of immunochemistry was created, with applications not only in the direct treatment of infectious diseases, but also in diagnostics and in scientific research in other branches of the life sciences.
The recombinant DNA technique (which involves insterting alien genetic information which is stored in DNA and bringing it to expression) will make it possible to use micro-organisms for the mass production of vaccines and sera (see further section II.5, Genetic engineering).
Another major advance was the discovery of a class of proteins known collectively as interferon. Isaacs and Lindeman, working around 1960, found that the fluid of a cell culture infected with a virus contained a substance (protein fraction) which rendered other cells resistant to infection by a wide range of viruses. Interferon also has other properties; it inhibits the division of certain types of cell, it affects the immune response, and it affects the body's reaction to ionising radiation.
If it turns out that interferon does in fact inhibit the growth of certain tumours, then the production of interferon on an industrial scale seems fully justified.
Interferon genes are being cloned in many parts of the world. In addition to this clinical work, research remains to be done on the binding of carbohydrates to proteins and on specificity.

1.5 Genetics

Heredity is a fact confirmed by experience. All of us constantly experience phenomena which indicate the existence of external correspondence between organisms and their descendants.
One striking example of this is the famous 'Habsburg lip', which was possessed by so many members of that royal house. Studies by Galippe (1905), Haeker (1912) and Strohmayer (1937) showed that this jutting lower jaw had been in the family for centuries, and certainly since Ernest, duke of Austria (1377-1424).
For a long time heredity was regarded simply as a tendency for like to generate like. The realisation that it must have an underlying cause brought it into the realm of scientific investigation.
The first step was to separate characteristics into those that were inherited and those that were due to other factors. It was on the basis of this work that Johanssen (1909) defined the sum of observable characteristics as the phenotype, which was due to both genetic composition (the genotype) and environmental influences. The genotype governs the ability to react to environmental conditions.
The father of modern genetics was Gregor Mendel (1822-1884), who established the laws governing heredity on the basis of his own scientific investigations. Since 1900 the study of heredity (genetics) has developed into a major discipline.
A physical basis was provided by the description of the nuclear division of cells, of reduction division in the formation of reproductive cells, and of the associated behaviour of the chromosomes (which carry the genes). Hugo de Vries discovered that changes occurred with a very low frequency, and that they in turn became hereditary. These changes are called mutations.
Mutations, whether natural or artificially induced, still play a key role in research and development in genetics and molecular biology, and are of fundamental importance for biotechnology.

Molecular biology

Since 1950 there has been an integration of microbiology, virology, genetics and biochemistry. Work in these areas has revealed so much about life processes that we now have an understanding of the very basis of life, namely the information (DNA, RNA), the operative mechanisms (proteins) and the principles governing regulation. Using protoplast fusion (the fusion of two protoplasts), and above all with the recombinant DNA technique, man has been able to bring alien genetic information to expression in host cells.
In 1940 Beadle and Tatum demonstrated that changes in various genes of the mould *Neurospora crassa* resulted in the loss of a particular enzyme. This and similar experiments led to a better understanding of certain metabolic processes.
Experiments by Avery, MacLeod and McCarthy showed that a hereditary characteristic can be transferred by DNA alone [3]. They conducted their experiments with *Pneumococcus*, the bacteria responsible for pneumonia. The

results of their experiments led to molecular genetics.
The transfer of genetic material (DNA) from one micro-organism to another is called transformation. Within four years of this discovery Ledenburg had discovered two further mechnisms of DNA transfer, namely conjugation (transfer across a bridge linking two bacteria) and transduction (the transfer of bacterial DNA by means of a virus (bacteriophage)) [4].
The study of bacteriophages, whose structure and function is simpler than that of bacteria (limited information, DNA or RNA, and thus a limited number of proteins), has made a major contribution towards an understanding of genetic information and the regulation of expression.
In 1953 Watson and Crick postulated the double helix structure of DNA [5]. Since in the structure the two strands of DNA were complementary this answered the question of how hereditary characteristics were themselves able to contain the information necessary for their own reproduction.
The studies of Jacob and Monod on the control of genetic expression put researchers on the trail of messenger RNA [6]. The success of Nirenberg and Matthaei in synthesising protein outside a cell from known synthetic fragments of RNA and then analysing the protein (a polypeptide) led to the unravelling of the entire code [7] Mention should also be made of the brilliant synthesis work done by Khorana.
Further regulation research by Jacob, Monod and Pardee revealed the operation of the regulation signals (operator and promoter), while studies on protein synthesis explained the complexity of the mechanism involved [8]. This work gradually provided an understanding of genetic information and some insight into the way in which it is expressed. It also led to the discovery of mechanisms governing the selective expression of information, which is of great importance in biotechnology.
However, it was above all the discovery in 1973 by Paul Berg and his colleagues of techniques for dissecting the genetic information of a cell reproducibly and then recombining the fragments after inserting a piece from an alien organism which made it possible to incorporate new combinations of genetic properties in an organism [9].
Since the discovery by Kao and Michayluk in 1974 that polyethylene glycol considerably improves the fusion of the protoplasts of plant cells, the technique has also been used on the protoplasts of moulds, bacteria and yeasts. The technique was not only suitable for studying the genetics within a species, but it made it possible to breach the species barrier and produce hybrids (bastards) which could not be obtained in any other way.
One important application is the fusion of a human lymphocyte (white blood cell), selected for its ability to produce a particular antibody, with a cell from a human tissue culture. The combination is then cultured further in order to form this one antibody (monoclonal antibody).

2. Fermentation and tissue culture

2.1 Introduction

Since time immemorial man has employed mico-organisms in the preparation of such foods as cheese, yoghurt and beer. The production of gas and formation of alcohol which takes place in the brewing of beer was known as fermentation. In the course of time this term took on a wider meaning, and today it covers all conversions in bioreactors which are due to the action of micro-organisms. The micro-organisms convert a substrate, generally of carbohydrates but sometimes of hydrocarbons, into cell mass (biomass) and a number of secondary reaction products.

In recent years bioreactors have also been used for growing cells and tissues from plants and animals. These techniques are not yet classified as fermentation processes but are generally referred to as tissue culture. Compared to micro-organisms, plant and animal cells play only a very limited role in industrial production.

For many centuries fermentation was a process of trial and error in which a number of operations were brought to a certain pitch of efficiency. Only after 1870 was there a systematic attempt to master fermentation processes in order to manufacture products of a standard quality on a large scale and at a low cost. In the first industrial fermentation period, from 1880 to the First World War, products such as bakers' yeast and lactic acid were made for the food industry, ethanol (ethyl alcohol) for the chemical industry, and amylase, the first enzyme, for the textile industry. Research was still in its infancy, and process control was fairly primitive. The master distiller worked with an expertise based on tradition, with just a pinch of science.

The First World War brought a massive demand for acetone. Acetone can be produced by fermentation, and its large-scale manufacture was facilitated by a rapid growth of the research effort, which even then was multidisciplinary. Bacteriologists, biochemists and engineers developed methods for the preparation of pure cultures (in which the cell mass consists of a single type of cell) and sterile media, and they succeeded in raising the yields of many processes. This development continued in the inter-war years. New fermentation products appeared, among them riboflavin (vitamin B_2), glycerol, sorbose (an intermediate for vitamin C production), and citric acid.

One of the effects of the Second World War was to accelerate penicillin production. The pharmaceutical industry also became interested in the fermentative preparation of vitamins and antibiotics. The research effort expanded rapidly, and many new antibiotics were discoverd. Enzymes were added to the production range, as were steroids like hydrocortisone. The growing body of biochemical knowledge led to the preparation of amino acids, such as the flavouring agent monosodium glutamate (MSG) and polysaccharides for the food industry.

One dramatic development was the attempt to produce SCP (Single Cell Protein), a microbial biomass with a high protein content. As an animal feedstuff and as a food for human consumption, it was felt that SCP could

alleviate the world shortage of protein. Huge production plants were built in the belief that there was a large market to be won. Hopes were dashed when it was discovered that SCP could not compete with traditional sources of animal feed, and the first preparations also proved unsuitable for human consumption. Production in a number of countries was discontinued for toxicological and economic reasons. Only ICI remains optimistic, and it has developed a method for producing a bacterial biomass on the basis of methanol. ICI now has the world's largest bioreactor, with a capacity of 1,500 m^3. Today there are more than 145 fermentation plants producing pharmaceuticals and high-grade chemicals, as well as some 85 yeast factories. Between them they produce more than 250,000 tons of MSG and 10,000 tons of penicillin a year. In the 1960s and 1970s producers began adopting a quantitative approach as they learned more about the metabolism of industrially attractive micro-organisms. Mathematical models were introduced, raising the efficiency of many processes. The 1980s will probably see the first applications of recombinant DNA techniques.

2.2 The fermentation process [10, 11, 12]

Fermentation, which involves the proliferation of cellular matter and, in many cases, the production of substances from those cells, can in principle be carried out using micro-organisms and cells from higher plants and animals. The micro-organisms may be yeasts, fungi, streptomycetes, bacteria or algae.
The fermentation equipment has to be adapted to meet the widely differing requirements of these substances. Some cells need oxygen, while others do not. Algae need light, but most other cells grow in the dark. Some fungi can be grown on the surface of a medium, which is usually solid, but most organisms are grown as a suspension in a fluid medium.
The vast majority of fermentations are batch processes. The main variant is the fed batch, where the substrate is gradually pumped into the fermenter. This offers major advantages in certain cases. Continuous fermentation, in which there is addition of the medium with constant tapping of the product, has little commercial significance at the moment. In the first place there is the risk of contamination, degeneration and growth on the walls, any of which would necessitate halting production. Nevertheless, the system does have major advantages. Production per unit volume is greater, and the process can take place under controlled optimum conditions. ICI's SCP production is a continuous fermentation process. Some breweries are producing beer in this way, and in future it will probably be adopted for other products as well.
The fermentation process starts with the selection of a suitable micro-organism, which has to meet a number of criteria. It should preferably be
— genetically stable,
— have a long storage life, and
— be free of other micro-organisms.
The culture of the selected organism (seed culture) is grown on a suitable medium before being transferred to a fresh medium (inoculation). Since this regular transfer carries the risk of genetic alteration, storage methods are used to halt cell growth. The culture can be preserved in dry form (possibly on a

carrier), or on agar-agar (generally shaped into tubes) and stored at a low temperature (5° C). Occasionally the culture is perserved in oil. A common form of storage is in a freezer or even above liquid nitrogen. This has the advantage of halting metabolism, but there is the drawback that some of the cells may be killed.

The fermentation cycle begins with the transfer of the (micro-)organism from the inactive stage to a medium. The cells begin to multiply, consuming the nutrients forming the substrate. Once sufficient cells have formed and are actively dividing the culture is transferred once again to a fresh medium. The organism is grown step by step in increasingly larger volumes, rising by a factor of 10-50 each time. Finally, an inoculation of approximately 10% by volume is introduced into the production medium. Production is carefully monitored by measuring a number of parameters, such as pH, optical density, amount of oxygen dissolved, and product concentration.

The pH is generally maintained at a constant level by the addition of acid or base. Air is dispersed in the medium in order to maintain a particular concentration of oxygen. An agent is added to prevent excess foaming. Carbon dioxide, oxygen, alcohol and other compounds in the exhaust gases are generally measured and used as regulatory signals for process control.

The growth medium (substrate) is selected by the fermentation industry on the basis of yield, cost and availability. The main component of the medium is generally a substance rich in carbohydrates (molasses or glucose syrup made from potato flour, maize, etc.) or a hydrocarbon. The remainder of the medium is often a complex mixture of nitrogenous substances, vitamins and minerals. The choice of a good medium is one of the key factors in optimising production. Although waste products are often used as a substrate their application in the fermentation industry immediately gives them a value. There is no such thing as a free raw material. Changes in the composition of the medium are unattractive, since this can create problems with recovery. Nevertheless, change has been forced on the industry by large price fluctuations. Added to this, the industry prefers not to be dependent on a single supplier or a single feedstock.

In the early days of industrial fermentation the only processes which could be used were those in which the fermentation environment was so selective that only the desired micro-organisms could survive (protected fermentation). In vinegar preparation this protection was provided by the pH, and in alcohol production by the alcohol itself, which excluded insects and foreign micro-organisms from the fermenter. As described in section II.3, it was only after the discovery of the sterilisation process and the introduction of engineering improvements that fermentation could take place under aseptic conditions.

The fermentation cycle lasts anything from a few hours to many days. The aim is to maximise production per unit of time. As soon as the optimum product concentration is reached the fermentation process is halted by refrigeration, or (preferably) by extremely rapid recovery. That, though, is not as simple as it sounds. In some processes it is very difficult to remove the cell mass. This is generally done by centrifuging or by filtration. The centrifugate or filtrate generally contains the desired product in dilute form, so the next step is to separate it from the solvent (usually water).

Fermentation and product recovery have to be geared to each other. The usual configuration is to have several bioreactors connected to a single recovery system.

2.3 Applications [13]

Fermentation processes are carried out for a variety of reasons. The object may be to obtain the cell mass or biomass itstelf, or to produce metabolites (metabolism products), which are separated either during or after growth in the medium. Occasionally a substance is added to the medium so that it can be converted by the cells into a chemically related product, in which case the cell acts as a biocatalyst. Fermentation can also be used to remove waste products, which are broken down by (micro-)organisms and used for the growth of cell material, with methane sometimes forming as a by-product. These four categories are discussed and illustrated in the following paragraphs.

Biomass

The preparation of yeast is a typical example of biomass production. Yeast grows prolifically on a sugary medium like molasses, under conditions hostile to most bacteria. If yeast cells are introduced into this liquid there is not only formation of cell material but also of alcohol, particularly if oxygen is witheld. This was acceptable as long as the alcohol could compete with the synthetic product, but when that was no longer the case producers switched to aeration and later to fed-batch fermentation, in which the medium is gradually added to the growing cell mass. With optimum aeration it is possible to calculate the amount of nutrient that has to be added per unit of time in order to leave the cells with just enough oxygen to survive. The added nutrients are then converted almost entirely into cell mass. So by regulating aeration and nutrient supply it is possible to produce yeast and alcohol in predetermined proportions.
At first these developments were purely empirical; the mechanism governing the formation of cell material or alcohol was only discovered later.
The process is as follows. Glucose is rapidly absorbed by the yeast cell and is converted into pyruvic acid in a series of enzymatically catalysed reactions. Here the metabolic pathways diverge. A certain amount of pyruvic acid can be converted per unit of time into cell material and energy via the Krebs cycle. That cycle operates at its optimum if there is a certain degree of oxygenation. If the conversion from glucose produces more pyruvic acid than can be absorbed by the cycle, the increased concentration of pyruvic acid in the cell leads to alcohol formation. Adequate aeration and the sparing addition of sugar can suppress alcohol production almost entirely. Increased knowledge about cell metabolism and fermentation technology made it possible to draw up mathematical models of the process. This led to improved yields and standardised product quality (providing the baker with a reproducible volume of bread).

Metabolite preparation

The use of micro-organisms to prepare metabolites by fermentation is applied in the production of antibiotics, vitamins, enzymes and amino acids.
The majority of the products which have an important pharmaceutical application were found more or less by accident by screening a large number of micro-organisms. Ingenious selection methods have now been developed by microbiologists, and if a product appears interesting there is a lengthy refinement of the medium and the culture conditions, and often a further selection process after mutagenic treatment of the micro-organisms. The selection criteria also cover toxicity, environmental impact and admissibility norms. This often takes more time and money than all the preceding research.
There are also examples of controlled intervention in the metabolic process, with certain enzyme reactions being blocked and intermediates being released into the medium. Kinoshita, a Japanese scientist, was one of the first to use these techniques. The result was the production of amino acids such as MSG (monosodium glutamate) and lysine. There have been major advances in this area in the past 20 years, leading to the production of a growing range of microbially-prepared chemicals, antibiotics and enzymes.
Enzymes are an important fermentation product. Since they occur in every living cell they are produced by all organisms and micro-organisms. There are lengthy selection procedures for finding the micro-organisms which produce the desired enzyme in sufficient quantity. Fungi of the *Aspergillus* species are particularly good producers, as are some bacteria strains, such as *Bacillus subtilis*, and a limited number of yeasts. So far industrial production has been concentrated on enzymes which are secreted by the micro-organisms in the medium. The cell secretes those enzymes in order to decompose smaller molecules—a sort of digestive process outside the body. In recent years there has also been an increase in the applications of intracellular enzymes.
The production and application of enzymes is discussed further in section II.4.

Biocatalysis [14]

In metabolite preparation part of the raw material has to be converted into cell material before metabolite production can start. Each system has to be examined on its merits to find the optimum between biocatalyst production and product preparation. The main criteria are maximum product/substrate ratio and maximum output per unit of time.
Biocatalysis is generally carried out using micro-organisms. The cell material is first grown in the fermenter, and then a substance is introduced into the same environment to be biologically transformed into a marketable product. The steroid transformations are particularly important commercially, since it is often far simpler to introduce an OH group into a steroid molecule microbiologically than chemically. Certain anti-inflammatory steroids such as hydrocortisone, and derivatives and sex hormones for contraception purposes are produced in this way. The process generally occupies several stages, the

majority involving organic synthesis and only a few taking place biocatalytically.
If the preparation of the biocatalyst and the conversion of the substrate are separated in space and time it is sometimes possible to increase the yield even further. One method is to immobilise the micro-organisms after they have been grown (see para. II.4.4) and then to lead the substrate past the biocatalytic cells. This restricts fermentation to the preparation of the micro-organisms. This technology will probably replace a proportion of the classical fermentative preparation of metabolites. At the moment the industrial use of immobilised cells is still very limited, and there is a great potential here for Dutch industry (see section II.4).

Effluent treatment

In the not so distant past dissolved waste from industry and towns was generally pumped into open waterways, where it was broken down by micro-organisms. However, as towns and industry grew, the amount of waste generated was often so great that it imposed too heavy a burden on the oxygen in the water. Plants, animals and a proportion of the purifying micro-organisms were killed and started decomposing. The limits to growth could be smelt for miles. The answer was to build effluent purification plants. The majortity of these systems are a combination of mechanical, biological and physico-chemical purification methods. The main variant was the aerobic fermentation of biologically decomposable effluent in large open tanks, in which a large proportion of the dissolved organic components was converted into cellular matter. This biomass (active sludge) was then dumped in the countryside. Chemical and physico-chemical process stages removed the dissolved chemicals.
The rapid rise in energy prices stimulated the development of anaerobic purification plants, which not only consume less energy but actually produce methane in return. Another attraction of the anaerobic method is that it reduces the amount of biomass by 90%. Modern anaerobic plants are now being built to process effluent from the sugar, potato flour and intensive farming industries. The Netherlands has contributed to the development of a new type of reactor with more than 10 times the processing capacity of the classical systems and a far shorter processing time.
The increase in the number of anaerobic plants belonging to local authorities and industrial concerns is being matched by the development of small decentralised plants on farms. Manure converted into biogas could make the farmer virtually independent of outside energy supplies.

2.4 Tissue culture

Plant tissue culture [15, 16]

For many years now it has been possible to grow plant fragments on a sterile nutrient surface. The gardener can grow new plants from cuttings, and the scientist can do so from plant tissue. Under certain conditions the reverse can

be achieved in liquid nutrient solutions, by growing individual cells which divide and separate just like micro-organisms. Those cells are no longer differentiated according to root, stem or flower tissue, but have become growth cells. This was the beginning of a new and fascinating development which branched out in two directions. The most obvious path follows the model of fermentation mediated by micro-organisms, and in principle it makes use of the same systems: agar petri dishes on which the separate cells from a suspension can grow into colonies, shake cultures in liquid media, and bioreactors into which oxygen in pumped and finely dispersed by means of agitators. Continuous fermentation is also possible, with the nutrient medium being added continuously and the suspension containing the plant cells being tapped off. There are, however, some important differences. Some cells, for example, need light if they are to grow - the plant cells sometimes contain chloroplasts and are therefore green. Another difference is in the growth rate, which is a factor of 20 lower with plant cells than with micro-organisms. The formation of cell material or cell products is therefore far slower, and consequently far more expensive. This is complicated by the fact that the longer the culture time the greater the chance of contamination, and since the invading micro-organisms grow far faster than the plant cells they starve the latter of food. This is the main reason why plant tissue culture will never lead to an economically viable source of biomass. Moreover, there are generally cheaper alternatives, such as growing plants in the traditional way.

A far more interesting option is the production of high-grade substances which can be synthesised by plant cells, such as alkaloids.

Fermentation has made it possible to achieve higher concentrations of some secondary metabolites than occur naturally in plants. The Japanese have already succeeded in producing plant cells in large fermenters, and the first commercial fermentation products are on their way. The slow rate of production means that this technique will be restricted to very expensive substances. It will be possible to raise efficiency by using the methods which proved so successful in the fermentation industry: selection, mutation and optimising the culture environment. New techniques (see section 2.5 below and Chapter IX), such as the growth of haploid cells, protoplast fusion and recombinant DNA, will produce further improvements, but may lead to competition with the plant fermentation technique. It will soon be possible to produce potentially interesting plant enzymes far more quickly using micro-organisms after the genetic material of the plant has been transferred to a suitable bacterium. The anticipated problems with recombinant DNA rule this out for non-protein compounds for the next few years. Another variant of fermentative preparation is biotransformation, in which the culture of the plant cells is separated from the production of the desired substance. The cells are immobilised and then brought into production, which can continue for a long time provided sufficient raw materials are added. Certain plant steroids are already being produced in this way, but production on an industrial scale still lies some distance in the future.

A completely different application is the production of plants by cell culture. In the case of many plants it has proved possible to grow a large number of

individual cells with the same genetic composition which go on to form embryos in a second stage. These are then sown out on a solid nutrient medium, where they grow into small plants. This would have enormous practical value when applied to plants which produce only a few offspring by sexual reproduction (offspring which are moreover not genetically identical). This technique is being applied on a large scale for the first time at a number of oil palm plantations. Only time will tell whether it will also be suitable for other types of tree, and even for faster-growing plants. The two potential advantages of the technique, genetically homogeneous material and a faster rotation period on the land, will have to prove superior to traditional sowing methods.

Animal cell culture [17, 18].

The culture of animal tissues (tissue culture) and cells (cell culture) *in vitro* is used in scientific research and in the manufacture of cell and virus products. The latter application is the most interesting from the technological point of view, notably the preparation of virus vaccines and reagents for virus diagnosis and, more recently, for the production of cellular products like interferon, monoclonal antibodies and, to a limited extent, hormones. Tissue culture as such no longer plays an important role here.
The major breakthrough in animal cell culture came in the early 1950s with the development of the *in vitro* culture of separate cells on glass, known as monolayer culture. It was soon discovered that certain animal cells multiply in suspension, just as micro-organisms do. In the main, though, these were cancerous cells which had either come directly from tumours or which had been taken from healthy tissue and had become cancerous during *in vitro* culture. These so-called continuous cell lines can be cultured limitlessly *in vitro*. They generally have an abnormal number of chromosomes and are prone to chromosome exchange. The normal human diploid cell has 46 chromosomes, while cancerous cells have between 60 and 80. These cells cannot be used for the prepartion of virus vaccines or cell products for human applications, since it is feared that they might pass on their cancerous properties, which may or may not be built into the virus genome. They are in widespread use for the preparation of veterinary vaccines and reagents for virus diagnosis. In addition to being cultured *in vitro* in flat flasks these cells can be grown in suspension for large-scale production, using the same type of fermenter as for other micro-organisms.
At the moment only normal diploid cells can be used for the preparation of virus vaccines or cell products for human applications.
These include the primary cells and human diploid cell strains. Primary cells are cells taken directly from healthy animal tissue. The tissue is split up into separate cells using trypsin, and the cells are then cultured once *in vitro*. Human diploid cell strains are cells from embryonic tissue, but they can also be isolated from the skin of adults. It has been demonstrated that they retain their normal diploid character after culture *in vitro*. Cell growth, however, stops after some 50 divisions, in contrast to continuous cell lines, which can be grown limitlessly *in vitro*.

Certain continuous cell lines of human or animal origin have proved not to be carcinogenic when injected into immune-deficient animals, and so must be regarded as a separate group.
The use of this type of cell for the preparation of purified deactivated or sub-unit virus vaccines is already under discussion. These cells and the normal diploid cells are only able to multiply *in vitro* if they can attach themselves to a suitable carrier material, such as glass and certain plastics (polystyrene). At first this severely limited the large-scale culture of these cells, since it meant using many more bottles. The situation was improved by the development of plate and column fermenters, but they in turn made it difficult to monitor culture conditions and cell growth. In order to correct this the Netherlands Public Health Institute (RIV) at Bilthoven has developed a method for growing the cells homogeneously in fermenters. In this micro-carrier culture the cells are grown on special minute globules of DEAE-Sephadex suspended in the medium. The cells anchor themselves to these micro-carriers and form a monolayer during their growth period. This enables them to be grown in standard fermenters under properly controlled conditions. Sampling provides a regular check on growth. This opens up the way to the large-scale culture of these attachment-dependent cells. The RIV is now using this method to produce deactivated polio and rabies vaccines on a scale of 40-350 litres.
This micro-carrier method has been adopted in other countries for a variety of purposes, including the preparation of interferon from human diploid cells.
One of the great disadvantages of cell culture is that good cell growth requires the use of complex media (for example 5-10% of calf or bovine serum is generally added). This makes the process far more expensive than bacteria culture. It is widely expected that recombinant DNA in bacteria will prove a cheaper method of manufacturing a number of products, such as virus antigens for vaccine preparation and cell products. However, there are indications that animal cells will make it possible to manufacture more complicated biological preparations than can be done with bacteria, so it is not inconceivable that animal cells will also be used in recombinant DNA experiments aimed at producing more complex preparations. Monoclonal antibodies are already being obtained in this way. The fusion of a non-growing cell which produces an antibody with a cell cultured *in vitro* yields a cell which produces specific antibodies on further culturing *in vitro*. It seems likely that the use of recombinant DNA techniques will stimulate rather than reduce interest in the culture of animal cells.

2.5 Future developments

The fermentation industry owed its growth to increased demand for its products. Much of this was due to the two world wars and to the development of antibiotics. Scientific knowledge grew at the same time, and this helped the industry to refine its processes and products.
We are now witnessing a scientific development which opens up new prospects for the fermentation industry: recombinant DNA. Formerly the microbiologist presented a micro-organism which could supply a certain product. It was the

technologist's task to suit the environment to the micro-organism. The dialogue between the two disciplines is about to enter a new stage. It will be the task of the microbiologist or genetic engineer to produce a micro-organism geared to the demands of good energy management and the easy recovery of the product. *E. coli*, for example, the intestinal bacillus which has been studied so extensively by geneticists, is not such an attractive producer. Not only does it evoke negative emotional reactions, but it is small and not easy to remove. The aim nowadays is to work at higher temperatures so as to reduce the need for cooling. In some cases yields will also be very much higher than in the past. Not only will current fermentation processes be improved, but new products and processes will be developed. Micro-organisms will be making products which are at present being synthesised by plants and animals, or by micro-organisms which do not lend themselves to fermentation processes. Interferon, which is produced in humans and animals to combat infection, and which may prove to be an effective agent against viruses and possibly even against certain types of cancer, can already be made by a micro-organism, at least in principle. Cetus, one of the new genetic engineering companies in the United States, joined forces with an oil company to develop a biotechnological method for producing ethyle and propylene oxide, which until now have been manufactured from crude oil fractions. Not only does this provide the chemical industry with an alternative raw material, but it is an entirely new microbiological process. Genetic engineering will undoubtedly provide the fermentation industry with an important new stimulus. The scientific community imposed numerous restrictions on itself until more was known about the possible hazards of recombinant DNA techniques to man and the environment. Society reacted by introducing restrictive legislation. Now that we are in a better position to assess the risks, the politicians have adapted the legislation (although rather hesitantly in some countries). That being said, it is perfectly understandable that the layman should regard the new developments with a certain amount of mistrust.

Advances taking place in another area could lead to improved yields. Our greater understanding of biochemical mechanisms in the living cell make it possible to compare energy requirements in theory and in practice. It turns out that some micro-organisms waste more energy than others, and that the composition of the medium and culture conditions have an effect on the energy budget. ICI has turned this to account by applying recombinant DNA to realise a more energy-efficient metabolism for the production of Single Cell Protein.

A better knowledge of metabolism and reactor kinetics can also be used by the process engineer to construct reactors designed to make the best use of specific micro-organisms and for the manufacture of specific products. Multidisciplinary teamwork is beginning to bear more and more fruit. Mathematical models are being developed, resulting in higher yields and lower costs. These developments will probably prove more valuable than further scaling-up and continuous fermentation.

2.6 The position in the Netherlands

The application of fermentation techniques requires interdisciplinary teamwork in which the leading roles are played by microbiology, process technology and biochemistry. These disciplines are found throughout the Netherlands, but the only relevant criterion for evaluating the current position in this country is their combination in fermentation science.
The Netherlands has a large fermentation industry, and in many respects it is of an international standard. There are also a number of companies, some of them large, which make one or more fermentation products but which cannot be regarded as belonging to the fermentation industry. Research facilities outside the industry are very limited indeed, with the result that the Netherlands cannot compete with countries like the United States and Japan, where there are large institutes engaged in fermentation research.
The food industry is a very different matter. The Netherlands has a whole series of breweries and dairy plants whose sophisticated preparation of numerous products makes an important contribution to Dutch export figures.

3. Bioreactors and downstream processing

3.1 Introduction

A bioreactor is a more or less enclosed space in which micro-organisms or enzymes are employed to manufacture products from raw materials (substrate) under controlled conditions. The formation of the product almost invariably involves the generation of by-products and heat. The term 'bioreactor' is not yet fully accepted. The vessel (which is the term in general use) is traditionally known as a fermenter, but strictly speaking a fermenter is a bioreactor in which the process is anaerobic, i.e. oxygen is excluded. Since there are numerous processes in which oxygen does play a role (aerobic processes) the blanket use of the term 'fermenter' is misleading.
In some bioreactors micro-organisms can pass in and out freely (effluent treatment plants and algal ponds), while in others they can leave but no foreign organisms are permitted to enter (antibiotic manufacture). Other bioreactors are hermetic, i.e. nothing can enter or leave (manufacture of some vaccines).
The raw materials (substrate) consist of sugars, methane or similar substances, from which products such as antibiotics or biomass (fodder yeast) are made. In effluent purification the feedstock is a pollutant (possibly toxic), and the products are biomass, carbon dioxide and water (non-toxic). The product formed in the bioreactor rarely leaves it in a form suitable for immediate use, and so it generally has to go through a number of processing stages (filtration, centrifugation, precipitation, drying, etc.). This series of operations is known collectively as downstream processing.
The investment in processing equipment can far exceed the sum invested in the bioreactor itself.

Figure 1. Bioreactor and downstream processing (from P.G. Malby, Process Biochem. 5, 8 (1970) 22)

Figure 1 shows a bioreactor and the associated processing stages.
The relationship between product formation in the bioreactor and product separation in the processing equipment is of fundamental importance. A high product concentration coupled with poor processing characteristics, which may be due to slime formation (secretion of polysaccharides), can be far less attractive than a lower product concentration with good processing characteristics.

3.2 Mode of operation

Reactor

The feedstock (substrate), and possibly oxygen as well, is introduced into the bioreactor, where it has to be brought into contact with the micro-organisms or enzymes. This requires some form of transport (mixing). The removal of the products, by-products and heat also entails transport (see figure 2). Mixing is done by agitation or gas entrainment.

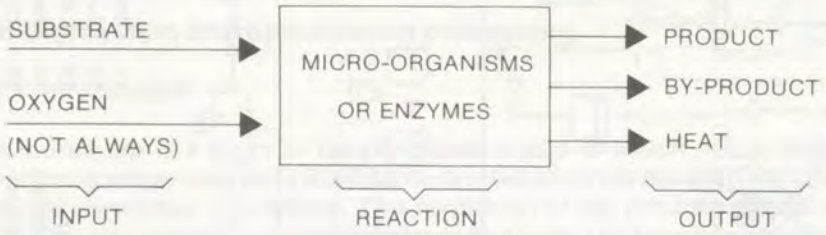


Figure 2. Bioreactor conversion process

Table 1 list the various ways in which fermentation can be carried out

Table 1. Fermentation methods

aseptic	non-aseptic
monoculture	mixed culture
aerobic	anaerobic
batchwise addition of the substrate	continuous supply of the substrate
dispersed organisms or enzymes	immobilised organisms or enzymes, attached to each other (micro-organisms only) or to a solid substance (carrier)
constant process conditions	variable process conditions
stirred vessel	non-stirred vessel

By combining these configuration in various ways it is possible, in principle, to obtain a large number of reactor types and modes of operation.

Downstream processing

The first stage involves separating the micro-organisms from the liquid (culture broth) in which they have been grown. If the products do not consist of the micro-organisms as such, they have to be concentrated, often after being released from the micro-organisms.

3.3 Historical background

An outline of the oldest known biotechnological processes was given in sections II.1 and II.2. The following account focusses on the equipment used in those processes.

Wine preparation dates from at least 10,000 years ago. It is an anaerobic process in which fermentation takes place spontaneously in fruit juice which is simply allowed to stand. The preparation of vinegar from wine (an aerobic process) must be about as old, and it too can take place spontaneously. Up until the 19th century the 'bioreactor' was nothing more than a simple earthenware or wooden vessel. Other long-established fermentation processes include the use of leaven (Matthew 13:33), and the manufacture of cheese, yoghurt, etc.

Bioreactor design began to evolve with the improvement of aerobic processes, particularly with the attempts to increase oxygen transfer. In the oldest European vinegar process (the Orleans process) the oxygen was taken up by a stationary liquid (wine) via the surface, on which the micro-organisms grew as a film (surface culture). This method was still being used in the Netherlands for citric acid manufacture until only a few years ago. Hermann Boerhaave (1668-1738) improved oxygen transfer by submerging wood shavings in a vessel with wine every 12-24 hours and then pumping the contents to an identical vessel. Bacteria adhered to the wood shavings and used the oxygen absorbed during the pumping process to oxidise the alcohol remaining in the film to acetic acid. This greatly increased the surface for oxygen transfer. J.S. Schützenbach modified the Boerhaave process by allowing the wine to flow continuously as a thin film over wood shavings (the quick vinegar process), which provided a relatively large surface for oxygen transfer. This reactor (figure 3) was the first application of a continuous process using immobilised micro-organisms. The control variables were the wine flow and the volume of air rising through the reactor, which was regulated by vents in the reactor wall. It was only after 1960 that vinegar was manufactured in stirred vessels, in which there is also a high oxygen transfer (the surface per volume for oxygen transfer is actually not much larger than in the quick vinegar process).

In the early bioreactors used for the preparation of bakers' yeast the yeast growth took place largely under anaerobic conditions (the Dutch and later the Vienna process). In 1879 Marquardt took out a patent for a method in which the yeast yield was greatly increased by aeration. In this process air was introduced into the fermenter (the bubble column) but there was no stirring.

A rotary aerator was being used for bakers' yeast manufacture by around 1933, although for a long time it was only used on a very small scale. In other

processes (the manufacture of gluconic acid, for example) oxygen transfer was improved by rotating a horizontal cylindrical bioreactor. Horizontal fermenters are still in use today, but oxygen transfer is now achieved by a rotating brush aerator. In the early days penicillin was manufactured in horizontal rotating bioreactors, but it was here that the breakthrough came, in the Second World War, with the development of a vertical vessel with a number of turbine agitators mounted on a single shaft. This is now the standard reactor for many processes, the bubble column being used mainly for yeast manufacture. Oxygen transfer has increased substantially from the days of the Orleans process, which had an oxygen transport of approx. 7×10^{-6} kg O_2 per m^3, to the present figur of approx. 2×10^{-3} kg O_2 per m^3, and there has been a corresponding improvement in the productivity of aerobic reactors.

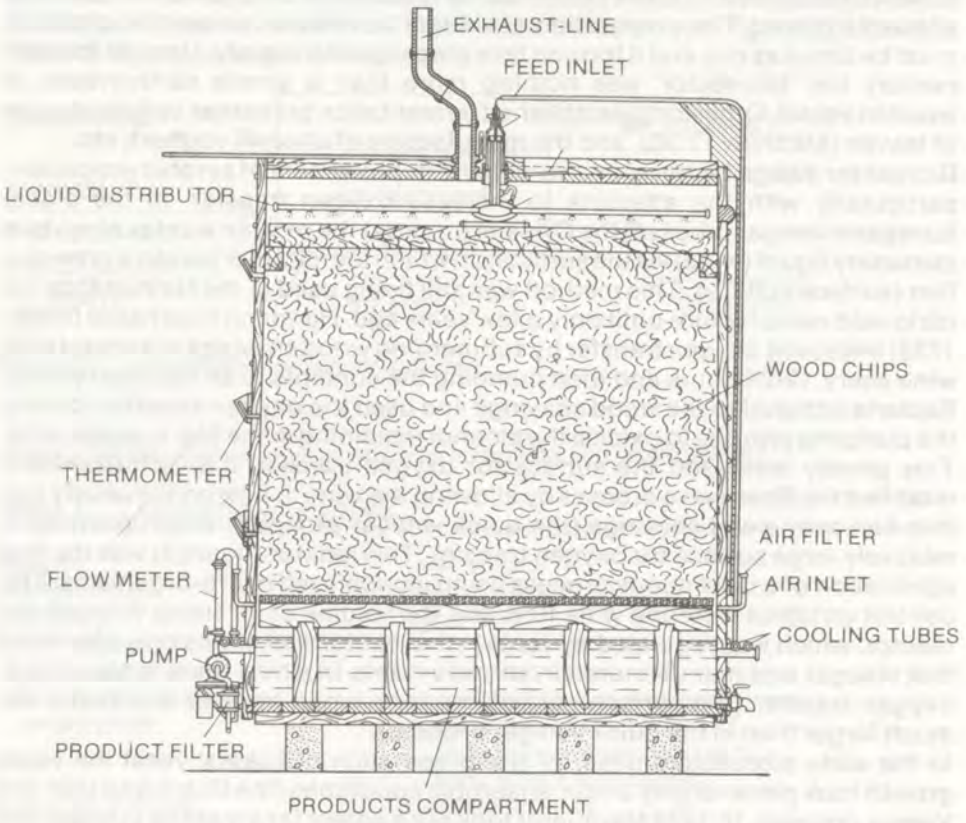


Figure 3. Quick vinegar reactor
(from R. Heiss, Lebensmitteltechnologie (1950) 297, J.F. Bergmann, Munich)

There have been a number of other important developments in bioreactor design and use.

1. *Inoculation*
   At first fermentation processes were due to natural contamination (spontaneous fermentation). At a later stage the micro-organisms were transplanted (repeated inoculation), and finally inoculation was made from a pure culture (end of the 19th century).
2. *Asepsis*
   See section II.2 for the main principles. In the past there was a natural selection of processes that led to the creation of fairly stable microbiological populations, allowing for the imperfect process conditions of the day. The switch to pure cultures opened the way to processes which could be duplicated more easily, although elaborate technical facilities were needed to maintain asepsis. There was a comparable development in agriculture.
3. *Substrate feed*
   The substrate was initially introduced in batches, but before long small amounts were being added semi-continuously while fermentation was going on (Orleans process, and in yeast manufacture). The first patent in this area only dates from 1915 (*Zulaufverfahren*). Some processes are fully continuous (mainly a post-war development).
4. *Cooling*
   The necessity for cooling grew as conversion rates increased. It was initially left to natural flow (free convection), but later the wall of the vessel was trickled with a film of water. Nowadays reactors are built with double walls or have cooling devices in the vessel itself. Another method is external cooling with recirculation.

Product recovery has also developed and grown in complexity. In the Vienna process the yeast was in the protein-rich foam layer (produced by carbon dioxide formation), and it was simply skimmed off. In the other processes the yeast was harvested by sedimentation and/or filtration. The use of the filter press for yeast separation dates from 1867, and the use of the centrifuge from 1906. The distillation process for concentrating the alcohol was developed by the Arabs in the 9th century (Arabic *al kul*, the finest). The bubble-cap plate was being used in alcohol distillation by the end of the 19th century.
Reviewing the overall development of biotechnological equipment it can be seen that most of the principles were known in the 19tn century: improved aeration due to the increase in surface area, semi-continuous feed (Orleans), continuous feed (quick vinegar), use of immobilised micro-organisms, process control by regulating the oxygen supply, the use of cooling coils, and pure culture. The use of agitators is of more recent date.

3.4 Current situation

Bioreactors

Figure 4 shows the type of bioreactor currently in use for most processes (with the exception of effluent treatment, alcohol production, many bakers' yeast processes and a few miscellaneous processes). It has an agitator assembly and equipment for heat removal (cooling coils), sterilisation, process control, and for the supply and removal of substances. The agitator serves a wide variety of purposes. It mixes the substances, breaks up air bubbles, exerts forces on the micro-organisms (to prevent flocculation, for example), and enhances heat transfer. Processes generally take place under aseptic conditions, with cultures consisting of organisms which can move independently of each other (free cells) and which belong to a single strain (monoculture). With the exception of alcohol preparation there is a marked preference for aerobic processes and for a batchwise addition of the substrate (batch process), or a gradual addition of the substrate without the simultaneous harvesting of the product (fed batch process). In recent years, though, a number of continuous fermentation processes have graduated from the laboratory stage, among them beer preparation and the production of xanthan gum (a thickener) and glucose-isomerase (an enzyme). In many cases the reactor design is not geared precisely to production requirements, partly because the necessary design data may be lacking, and partly because a vessel is often used for very different bioreactions over a period of time. A third reason is that the constant improvement of micro-organism strains alters the requirements made of the bioreactor. There is a constant adjustment of the cooling and aeration capacities. The biomass concentration varies from process to process, but can amount to 10-30 kg/m^3 (dry matter). Other types of bioreactor have recently come into use (including ICI's pressure cycle fermenter) in which the functions of mass transfer, flow and heat removal are rigorously separated.

Effluent purification in the Netherlands is generally an aerobic process which takes place in ditches. These are long, annular channels with a rectangular cross-section (figure 5). Air is introduced at a number of points in order to break down the waste. These plants are used for purifying domestic (dilute) waste and industrial (concentrated) effluent. The biomass concentration amounts to approx. 4 kg/m^3 (dry matter). The anaerobic purification of effluent, and particularly of concentrated streams, is gaining ground rapidly. This process takes place in vessels, generally of concrete, which are isolated from the outside air. The biomass concentration can easily amount to 60 kg/m^3 (dry matter). Both reactor types are used in a continuous process.

The present generation of enzyme reactors consists of oblong, cylindrical columns. The enzymes are immobilised by being contained in or attached to heaped particles. An aqueous solution of the substance to be converted flows past the particles. In some cases it is not the pure enzymes which are immobilised, but entire micro-organisms which have been treated in such a way as to inhibit further growth but leaving intact that part of the enzyme system required for the conversion process. See section II.4 for a description of immobilisation techniques.

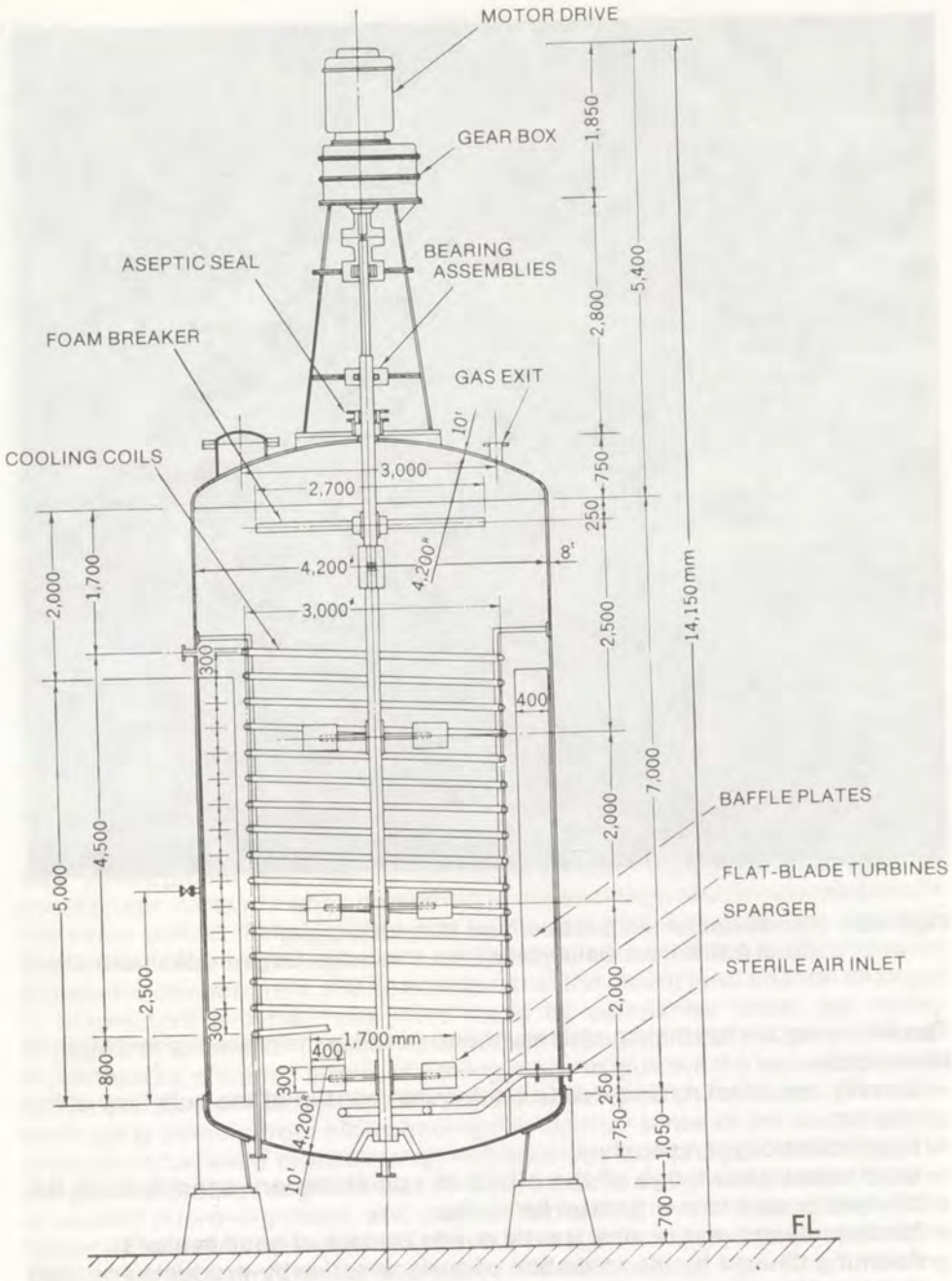


Figure 4. Bioreactor with agitator assembly
(from S. Aiba, A.E. Humphrey, N.F. Millis, Biochemical Engineering (1973) 304, Tokyo University Press)

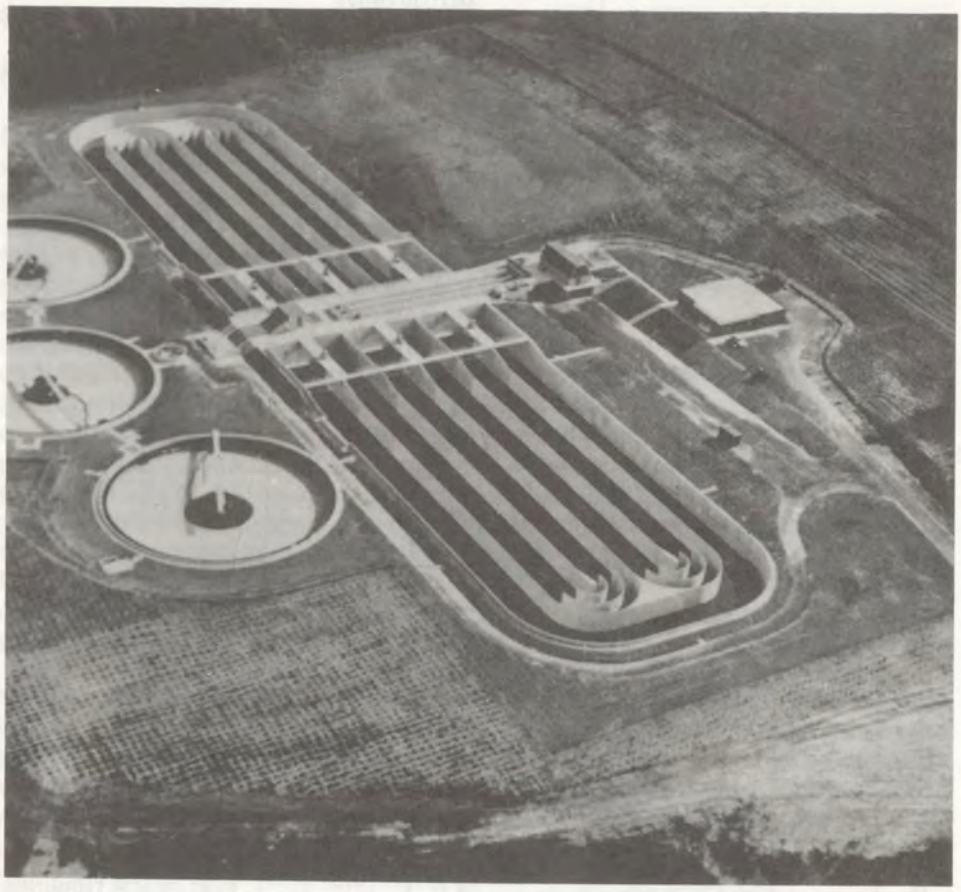


Figure 5. Bioreactor for effluent treatment (Caroussel type)
(from A.C.J. Koot, Behandeling van afvalwater, Uitgeverij Waltman, Delft)

The following are the main problems encountered in the present generation of bioreactors.

— Locally imperfect mixing due to inadequate control of the bulk flow of the reactor.
— Insufficient oxygen supply.
— Insufficient knowledge of the effect of non-stationary conditions in the bioreactor on micro-organism behaviour.
— Inadequate process control (partly due to the lack of good sensors).
— Foaming caused by the formation of surface-active by-products and high aeration intensity.

These problems make scaling-up an uncertain process and impede optimisation.

Downstream processing

The micro-organisms are separated from the culture broth by precipitation, filtration or centrifugation.
There are numerous ways of concentrating the dilute solution of the desired product. The usual method is to introduce a second, non-mixable phase, which may be a gas, a solid substance or an organic liquid, for which the desired product has an affinity differing from that of the by-products or the water (distillation, adsorption, extraction, etc.). The concentration can also be raised by introducing a medium (membrane, filter) whose permeability for the product is different to that for the other substances (membrane processes, microfilters).
In fermentation processes the product is always formed as a highly dilute aqueous solution. Distillation processing consumes a great deal of energy, particularly in the case of alcohol production.
There is an essential difference between the processing of robust molecules such as alcohol or acetone, and of sensitive molecules such as proteins (enzymes, for example). It is often difficult to process sensitive molecules without affecting their desired properties. A wide range of specific processing methods has been developed, a discussion of which will be found in Gestenberg *et al.* [19].
The separation of the micro-organisms from the culture broth frequently poses problems. Furthermore, in some cases downstream processing causes considerable product loss. Energy consumption can reach unacceptable levels, particularly with bulk products. In practice downstream processing always takes place after product formation, but harvesting during formation can offer major advantages, particularly in the case of growth inhibitors [20].

3.5 Future developments

The development and use of bioreactors could hardly be described as spectacular, certainly when compared to the evolution of chemical reactors in the same period. This is partly due to the fact that molucules are easier to manipulate than micro-organisms, and partly to conservative attitudes towards bioreactor development. It is no accident that the recent fundamental changes in bioreactors and their use were made by companies which are chiefly engaged in chemical process technology (ICI and Shell, among others). Nevertheless, attempts are clearly being made to improve the bioreactor, both within the biotechnology industry itself and at research institutes. The main stimulus is coming from biotechnology in the true sense of the word, i.e. the interaction between biochemistry, microbiology and process technology. In the past the process engineer was virtually asked to design a bioreactor around a specific micro-organism, and caution dictated that the design should be based on tried and trusted principles. Nowadays the micro-organisms and the reactor are so integrated as to produce the optiumum design. The following paragraphs highlight a number of trends which will have a major effect on future developments.

General

We still know very little about the 'limits to growth' as they affect micro-organisms. The theoretical limits of growth rate or the rate of product formation are uncertain, and our knowledge about product formation and growth under deliberately non-stationary conditions is scanty in the extreme. The sparse literature on these topics suggests that considerable improvement can be expected, and this will undoubtedly affect reactor design (higher rates of transport will be needed, together with more advanced forms of process control).
The vessel itself imposes limits on growth, partly due to the restrictions on transport rates (mixing, mass and heat transport). A large increase in agitation capacity involves unacceptably high energy consumption, and beyond a certain rate of rotation the increase in heat generation caused by agitation is greater than heat removal.
The biomass concentration in the vessel is often low as a result of poor sludge separation (in aerobic effluent plants, for example). There is considerable room for raising this concentration.
Vessel limits are not known, particularly in the case of novel designs. Recent reports, though, speak of spectacular improvements (one being in the power required per kg of oxygen transferred).

Reactor type

1. Reactors with better defined flow conditions.
2. Separation of functions: mixing, mass transport, heat transfer, and the forces acting on the micro-organisms.
3. Reactors which consume less energy. The energy fed to the vessel via the agitator is many times greater than the theoretical requirement. Considerable improvement could be made here.
4. Reactors designed more for a single product.
5. The increased use of immobilised systems (this also applies to effluent purification), and particularly of immobilised micro-organisms, which may be on or in a carrier, or attached to each other. This entails developing process conditions or micro-organisms such that no net growth occurs in the reactor, so that the biomass concentration remains constant. Immobilisation makes it far easier to separate the micro-organisms from the culture broth. Increasing the range of applications for immobilised enzymes will require a great deal of biochemical work on cofactor regeneration.
6. The use of fluidised beds (moving particles) instead of packed beds (stationary particles), in connection with gas formation and continuous operation.
7. A relative increase in the use of anaerobic reactors (for such processes as solvent and alcohol production, and in effluent treatment). The main improvement in anaerobic effluent treatment will be in sludge retention in the vessel (by immobilisation and other techniques).
8. Dry fermentation (solid bed fermentation, but also in a fluidised bed).

Reactor use

1. Increased use of semi-aseptic methods, for example by extreme temperature, acidity of concentration, closed niche (a community of organisms which is so balanced that there is no room for other organisms), micro pure culture (a fermentation method employing an immobilised pure culture in globules, the globules being in a non-aseptic environment), or by using very rapid growers. This simplifies the production process. To a certain extent there is nothing new in this; in the past the majority of these methods were the only way of working with micro-organisms. These old methods, possibly with further refinement, could prove to be extremely useful again today.
2. Mixed cultures. These are often referred to but rarely used.
3. Working with high temperatures (up to approx. 60°C) and product concentrations (alcohol, for example). This saves cooling water and energy for processing. Micro-organisms meeting these requirements might be developed by genetic engineering.
4. The deliberate use of non-stationary conditions. For example, it is possible to produce glycerol by transferring algae back and forth between sea water and fresh water.
5. Better process control.

Downstream processing

1. Processes requiring less energy (the wider use and improvement of membrane processes, for example).
2. Separation during fermentation. The removal of alcohol during fermentation means that a far heavier load can be placed on the reactor.
3. Larger organisms, improved flocculation or attachment (by genetic engineering).

Some of these options may appear speculative, but all have been investigated successfully at one time or another. Further study will have to show whether that was solely a scientific success, or whether it can be converted into an economic success as well.

3.6 The position in the Netherland

The Netherlands has an excellent tradition of microbiology, biochemistry, process technology and plant engineering. It also has a leading international position in the production of a number of biotechnological products and in effluent treatment. All the facilities are available for improving current plant and for developing new bioreactors and processing equipment.
The report *Chemie nu en straks* [21] identifies the weak areas in university research and suggests remedies. Research is insufficiently mission-oriented, and although it is thorough it tends to be unspectacular and inflexible, and there is a lack of teamwork.
Some sectors of industry have given only a lukewarm reaction to new developments. This seems to be a congenital Dutch failing. The overall picture,

though, does give grounds for hope. In the past few years there have been examples of creativity, mission orientation and teamwork in research which were thought impossible 10 years ago (anaerobic effluent treatment being a case in point). Industry, too, seems to be having a change of heart. These trends could be accelerated by the right stimuli (financial and structural). The numerous parliamentary memoranda show that the politicians are aware of this.

4. Enzyme technology

4.1 Introduction

This section considers certain aspects of enzyme technology, particularly as they affect industrial production and application. There are two aspects of fundamental importance. In the first place, there has to be the ability to produce enzymes relatively simply and economically. Secondly, it is essential to have a good understanding of the areas in which the enzymes will be used. This requires a multidisciplinary approach, with contributions from chemistry (knowledge of substrates and reaction mechanisms), biochemistry (enzyme kinetics, properties of the enzyme), microbiology (enzyme production) and engineering (large-scale aspects of preparation and application). This multi-disciplinary teamwork is essential if enzymes are to be used successfully, and moreover there has to be a constant process of feedback between preparation and application.
The possible applications of an enzyme are governed by the way in which it is prepared, and hence by its properties. Occasionally the application will result in a better formulation and even modification of the preparation process.
This section focusses on the preparation and application of enzymes. This is preceded by a discussion of some concepts and definitions, and the section ends with a survey of the current state of enzyme technology in the Netherlands.

4.2 Concepts and definitions

Enzymes are catalysts of biological origin. All enzymes are proteins whose highly specific structure enables them to speed up numerous chemical reactions in a living organism. The great value of enzymes is that their catalytic ability can be utilised under moderate conditions of temperature, pressure, etc. This makes them more attractive for industrial applications than other catalysts, which often require more extreme conditions. A second advantage is their high degree of specificity. One single enzyme catalyses only one or at most a few chemical reactions, with the result that there are few unwelcome side reactions.
Processes based on the catalytic action of enzymes have been known for thousands of years, the preparation of wine, cheese and beer being the most obvious examples. In these processes, though, catalysis is carried out not by a single enzyme but by a micro-organism in which a number of enzymes work together as a system.

In 1926 Summers obtained the first absolutely pure enzyme. This was urease, the enzyme that breaks down urea. Since then numerous enzymes have been discovered, isolated, purified and studied, and to date more than 2,000 enzymes have been described in greater or lesser detail.
Enzymes are found in every living cell. An enzyme generally occupies only a very small portion of the cell, and since tonnage amounts are required for large-scale applications, human beings, animals and plants are as yet inadequate sources of enzymes for industrial use.
Micro-organisms – yeasts, moulds and bacteria – are the most suitable source, since they multiply fairly rapidly and are easily grown in large quantities.
Enzymes are classified according to the type of reaction they catalyse. There are six classes: oxidoreductases, transferases, hydrolases, lyases, isomerases and lipases. Each class is subdivided, producing an internationally agreed enzyme nomenclature [22].
Another important factor from the industrial viewpoint is whether an enzyme is intracellular or extracellular. An intracellular enzyme is located within the cell of the micro-organism, so the cell wall has to be ruptured before the enzyme can be extracted.
An extracellular enzyme is excreted by the micro-organism into the culture medium, which greatly simplifies the purification process.
By far the majority of the enzymes in large-scale use at the moment are of the hydrolase class, together with a few oxidoreductases and isomerases [23]. Almost all of them catalyse fairly simple reactions.
A single enzyme can catalyse only one step in a reaction, so conversions consisting of successive steps require the use of several enzymes (multi-enzyme systems). Enzymes very often need the assistance of accessory substances (cofactors, simple chemical compounds) in order to carry out their task properly.
The use of cofactors, however, creates an added problem. In principle, enzymes have the same structure after the reaction as before it, and as long as they remain intact they can carry on catalysing the same reaction. The process is not endless, however, for sooner or later every enzyme loses its activity.
Cofactors, on the other hand, are changed by the reaction, and they have to be restored to their original form if they are to continue doing their work. This is known as regeneration.
In the 1960s there was an important breakthrough in the search for ways to use enzymes more widely and more efficiently. Enzymes normally do their work dissolved in water, but then methods were developed for anchoring them within or on an insoluble carrier (immobilisation). This offers a number of advantages. The technique of enzyme immobilisation developed rapidly, and today not only single enzymes, but multiple systems and even cofactors can be attached to carriers.
This development, from a simple enzyme system dissolved in water to a multi-enzyme system plus cofactors attached to a carrier, brings us to the threshold of achieving a number of complex reactions which can otherwise only be carried out by a living cell.
The next step is to immobilise entire micro-organisms, combining the catalytic

ability of a complete cell with the advantages of immobilisation.
There are so many parallels between immobilised enzyme systems and immobilised cells that the latter will also be dealt with in this section.

4.3 Possible applications of enzyme technology

In the following paragraphs we will examine the possibilities of the large-scale industrial application of enzymes.

Table 2. Breakdown of enzyme sales per industrial sector (x 10^6)

Starch	55 approx.
Detergents	65 "
Dairy	10 "
Distilling	10 "
Brewing	5 "
Fruit, wine	15 "
Milling, baking	10 "
Other	10 "
	approx.180

Table 3. Breakdown of sales by enzyme (x 10^6)

Bacillus protease	65 approx.
Glucoamylase	25 "
Bacillus amylase	20 "
Glucose isomerase	25 "
Microbial rennet	10 "
Fungal amylase	5 "
Pectinase	20 "
Fungal protease	5 "
Other	5 "
	approx.180

Approximately 65% of total sales is accounted for by the use of enzymes in the detergent sector (proteases) and in the starch industry (amylase, glucoamylase and glucose isomerase). The percentages in the other sectors are as follows: dairy (5%), distilling (5%), brewing (3%), fruit and wine (8%), milling and baking (5%) [24].
Apart from detergent enzymes, virtually all the applications lie in the food and beverage sector.
These figures apply to enzymes supplied by enzyme producers to outside customers. It is difficult to get an accurate idea of the quantities of enzymes used internally by large fermentation concerns. Examples are the enzyme-catalysed production of 6-amino-penicillanic acid for the production of semi-synthetic penicillins, and the production of various amino acids.

At the moment only 10-15 different enzymes have attained true industrial status. The greatest potential for the future appears to lie in the food and beverage industry [25, 26], but the conversion of waste materials into a usable raw material is another promising option. It is also expected that enzymes will penetrate increasingly into the world of chemistry as a biochemical alternative to processes which at present can only take place under extreme conditions. Finally, there is a major research effort into the use of enzymes in the development of new energy sources (fuel cells) and primary fuels.
With the exception of food and beverage processes the number of actual applications of enzyme technology is small, and a great deal of development work still remains to be done.
Table 3 gives an idea of enzyme costs for a number of products.

Table 4. Direct enzyme cost in enzyme processes, USA 1977 [24]

Application	Units	Enzyme costs (US c)
Washing	1 kg detergent	2-4
Starch liquefaction	1 kg starch	0.2-0.5
Starch saccharification	1 kg starch	0.4-0.8
Starch isomerisation	1 kg starch	1.0-1.5
Cheese manufacture	1 litre milk	0.1
Alcohol manufacture	1 litre alcohol	0.2-0.5
Alcohol manufacture	1 litre alcohol	0.7-1.4
Brewing	1 litre beer	0.1
Baking	1 kg flour	0.01
Juice	1 litre juice	0.1-0.2
Wine	1 litre wine	0.1-0.2

In general, it can be said that the enzyme cost is small in relation to the value of the product produced with the enzyme. The costs should rather be compared with the processing costs of the operation in which the enzyme is used. Unfortunately figures for these costs are usually not available.

It is expected that attention will focus in future on possible uses of enzymes under process conditions which are unusual for them. Biochemists will have to get used to the idea that enzymes do not only operate in aqueous environments. It has been shown that many enzymes retain their catalytic ability in mixtures of water and organic solvents. In addition, there may be surprising changes in the operating mechanism or specificity of the enzyme, which shows that they can do more than would have been expected on purely biochemical grounds.
Increased knowledge of these aspects of enzymes greatly expands the area of possible applications. One example is the use of reactors combining chemical catalysis and biocatalysis.

4.4 State of the art

Production method

Although enzymes are found in every living organism, micro-organisms (yeasts, moulds and bacteria) have so far proved to be virtually the only suitable source of enzymes which are to be used on a large scale.
The growing body of knowledge, particularly in microbiology, has led to a better understanding of how to stimulate a micro-organism's production of a particular enzyme, and methods have been developed for making the micro-organism produce far more of the desired enzyme than it does naturally. The problems are far more complex with multiple enzyme systems. As yet these systems have attracted relatively little attention, and much work needs to be done in this area. Feedback between the isolation and application phases is particularly important. In the case of the more complex enzyme systems, application research will have to identify the enzymes which should be made during the production phases.
One illustration of this is the use of the enzyme cellulase in the breakdown of cellulose to glucose. The complete breakdown of cellulose requires a cellulase complex consisting of at least three enzymes. These enzymes have to be in a particular proportion if the breakdown reaction is to be achieved under conditions which are acceptable in practice. It wil be up to microbiologists to supervise the production of the enzyme preparation in such a way that it meets as many of the application requirements as possible. If that proves impossible, various complementary enzyme prepations will have to be used in order to optimise the application.
In many cases the price of enzymes for large-scale application is still far too high. The main cost price components are fermentation costs and isolation and downstream processing costs. There is considerable room for reducing fermentation costs by raising enzyme production per cell. It is expected that genetic engineering could help to bring this about.

Isolation and purification

Isolation (possibly followed by purification) reduces the enzyme to a form suitable for distribution and use. Complete separation yielding a pure enzyme is very rare. The purification process can itself reduce an enzyme's activity. In general, pure enzymes are far less stable, due to the removal of stabilising substances during purification. Enzymes for technical applications are generally fairly impure preparations, which is partly due to efforts to keep the price down, and it is difficult to catalyse very advanced reactions using these impure preparations. The sole task of detergent enzymes, for example, is to remove protein stains from fabrics. There is a slow but definite shift towards processes in which an enzyme is used to carry out a highly specific task in which side effects, such as the formation of by-products, are undesirable. There will be an increased demand in future for enzymes which are more highly purified. The main problem in this area is the discrepancy between the wide range of

isolation and purification methods used by the biochemist in the laboratory and the techniques which have so far been applied in industry [27].
Biochemists have succeeded in isolating more than 2,000 enzymes on a laboratory scale and have achieved a certain measure of purification (up to 100% in some cases).
There are advanced mechanical, enzymatic and chemical techniques for breaking open micro-organisms so that a start can be made with purifying the enzymes.
The numerous techniques for improving purity include gel permeation chromatography, ion-exchange chromatography, affinity chromatography and ultrafiltration.
Only a few of these advanced techniques are used in a large scale. The first problem with intracellular enzymes is to break open the cell (autolysis). Chemical and thermal autolysis methods are the most widely used in industry. On the small scale the preference is for mechanical and enzymatic methods, which often give better resulsts. Enzymatic methods require the availability of large quantities of the desired enzymes.
Mechanical methods suitable for industrial use require equipment which can process large quantities, preferably continuously. A great deal of work still has to be done on large-scale rupturing techniques.
Geneticists might be able to help here by providing mutants which excrete the desired enzymes, or mutants whose cell wall is easily ruptured.
The further large-scale purification of enzymes generally involves fairly straightforward techniques like centrifugation, filtration, precipitation and drying, but the resulting enzyme preparations are generally fairly impure. The development of really large-scale purification techniques is another area where much work still has to be done.

Enzyme modification and synthetic enzymes

Enzymes have a number of attractive features, and some which are rather less attractive. They often turn to be less than ideal for the process envisaged. A simple solution would be to go back to the geneticist and ask him to provide a mutant which does make an enzyme with the desired properties. Sometimes, though, that is impractical, either because it takes too long, is simply impossible, or because the requirements change too frequently. Other ways then have to be found of modifying enzymes.
The following three examples illustrate ways of modifying the form and structure of enzymes.
The original protease preparations for use in detergents generated a considerable amount of enzyme dust. A modified version was developed, and the enzyme was marketed in a non-powdering granular form. During the search for the right formulation (form in which the enzyme is delivered) steps were taken to ensure that the enzyme was in a protected environment, so that it would not be deactivated by the actual detergent.
Another property of enzymes which is not always welcome is their solubility in water. They can be made water-insoluble in numerous ways (immobilisation).

A third modification route is to change the enzyme molecule chemically. By bringing about (minor) chemical changes one can try to enhance one or more of the enzyme's properties. The aim may be to improve the stability of the enzyme, to alter the optimum acidity, or the acidity at which the enzyme operates.
Considerable work is being done on the chemical modification of enzymes. The results have shown that there is little logical connection between the modification process and the effect it has on the working of the enzyme. It is above all a matter of trial and error.
A closely related field is the work being done on so-called synthetic enzymes, which follows two, fundamentally different approaches [28]. The first is the chemical modification of the enzyme. For example, the transfer of a major functional group from one enzyme to another, fully intact enzyme can yield enzymes whose action has been altered. A hydrolase, for example, might gain an oxidoreductase activity while retaining other attractive features of the hydrolase (such as stability). This in fact amounts to a new enzyme combining the attractive properties of two other enzymes. In this approach the basis of the catalyst is still an enzyme (protein chain), the modification being made solely to the active centre. A variant of this approach is to alter the specificity of an enzyme by modifying the cofactor, which may entail separating it from the enzyme for a while.
The second route approaches the problem from a totally different direction by trying to build a synthetic enzyme. Various attempts are being made to arrange active groups involved in the catalysis reaction in the correct spatial structure relative to each other. In the majority of cases no molecule of biological origin is used as a carrier for the functional groups.

Enzyme immobilisation

Immobilised enzymes are enzymes which are physically confined or localised in a certain defined region of space with retention of their catalytic activities, and which can be used repeatedly and continuously.
The advantage is that the catalyst can be separated after use and recycled. No enzyme remains behind in the product. It also makes continuous processes possible. The result is a more efficient use of the enzyme and a corresponding reduction in enzyme costs.
A considerable amount of work has been done on immobilised enzymes in the last 15 years, and dozens of different immobilisation methods have been described. There are already reports of the successful immobilisation of more than 70 enzymes. There are over 1,000 publications in this one area alone, and in view of the range of immobilisation methods and the fact that more than 2,000 enzymes are known, the number of publications will undoubtedly continue to grow [29,30].
Given the number of successful immobilisations already achieved, the potential in this area is virtually limitless. In the majority of cases, though, the publications deal with systems which have only been prepared and tested on an laboratory scale, and they tend to overlook the many problems involved in the large-scale preparation and use of immobilised enzymes.

These problems include the compressibility of the catalyst particles (which is of particular importance in column reactors), abrasion resistance and suitability for operations on an industrial scale. Closer examination reveals that since 1965, when the first industrial process was carried out using immobilised enzymes, only five systems have been developed to full application on a large scale. Weighing this against the more than 1,000 publications which have appeared in the past 15 years it is clear that there is still a great deal of work to be done, the more so in that all the processes so far applied on a large scale are biochemically straightforward, involving either hydrolysis or isomerisation.
The great value of enzyme technology, and certainly of immobilised enzymes, will be in the catalysis of more complex reactions, in which the enzymes, assisted by cofactors, will synthesise complex molecules.
The systems will become biochemically more intricate, and this in turn will complicate scaling up and application.

Multi-enzyme systems, immobilised cells

As already noted, future developments will be towards the catalysis of increasingly complex reactions, in which several enzymes and even cofactors will be required. Cofactors, unlike enzymes, are changed by the reaction. Since they are expensive they have to be recycled, so they first have to be restored to their original form, which also requires enzyme reactions. This leads to the use of complex multi-enzyme systems. These systems have been demonstrated on a laboratory scale, but the vital question concerns scaling up. The process involves isolating and then immobilising different enzymes and cofactors, which then have to brought together in the correct proportion. A classic example is the synthesis of the antibiotic gramicidin S using an immobilised multi-enzyme system. Synthesis has proved practicable on a laboratory scale, but it is evidently still far too complicated for large-scale application, since this has not yet been done.
An alternative would be to use a complete micro-organism, immobilised or otherwise. In principle micro-organisms are capable of catlysing extremely complex reactions. The problem is whether it will be possible to control the micro-organism so that it does exactly what is required of it without producing any undesirable reactions.
Micro-organism immobilisation has been an area of intense activity in the past few years. Many immobilisation techniques have been described, and they are often analogous to those for immobilised enzymes. Despite the many problems it has proved possible to immobilise a wide range of micro-organisms while preserving their catalytic ability [31]. Although excellent results have been achieved in the laboratory there is still a question mark around their industrial applicability. The techniques are complex and difficult to implement, and the main applications may well prove to be in the fermentation industry itself. The large-scale use of immobilised micro-organisms will depend on the advantages of immobilisation, some of which already suggest themselves. A simple procedure could lead to a cheaper product. The chemical reactions involved in immobilisation might result in a modified or improved specificity. It is possible

that cell growth could be slowed down or even halted by embedding the cells in the carrier so that as much of the nutrient as possible is used for manufacturing the desired product. Better protection against extreme conditions could be another advantage. Research will have to identify the realistic options, and this will determine the future for immobilised micro-organisms.

Large-scale use

The preparation and use of enzymes, immobilised enzymes and immobilised cells is an area in which biochemistry, microbiology and process technology meet. Process technology takes on an important role in various areas as soon as enzyme work begins to scale up to the industrial level.
The first area is large-scale preparation (fermentation), and process technology should also play a leading role in purification and downstream processing. This is a relatively underdeveloped area at the moment.
Process engineers will have a key part to play during the formulation phase, particularly if the enzymes have to be produced in particle form in large quantities. This will apply not only to the traditional soluble enzymes (detergents), but also to all forms of immobilised enzymes and cells. Considerable research is still required in this area.
The last stage covers the actual large-scale application of enzymes. This involves reactor technology, generally applied to immobilised systems. A proper understanding of immobilised enzymes and cells also requires study of certain aspects of process technology.
It is generally appreciated that the use of enzyme technology demands an interdisciplinary approach, with the 'bio' and the 'techno' disciplines working in harness. There have already been encouraging practical demonstrations of this teamwork, but a great deal still remains to be done before enzyme technology is introduced into all those areas where it is felt that there are good potential applications.

4.5 The position in the Netherlands

The Netherlands has one of the world's major producers of enzymes. This company, Gist-Brocades, is carrying out intensive study on the production, isolation and application of enzymes, on the large scale as well as at the laboratory level.
The marked interest of Dutch industry in enzyme technology has not (yet) been reflected in university involvement. The Netherlands does not have the institutes with years of experience in this area which are found in France, Germany, Britain and, above all, in Japan and the United States.
It is fair to say that there is a lot of leeway to be made up, particularly in the field of university research.
Fortunately it is now being recognised that the Netherlands is trailing in this area, and in the past few years various initiatives have been taken to remedy the situation.
Biotechnology research is now being carried out at Delft and Wageningen, and

a start has also been made with enzyme technology. The current restrictions on staff and funds for academic research do not make this a particularly opportune time to begin exploring a new research area.
The successful development of enzyme technology will require close cooperation between basic research institutes (universities and universities of technology) and those involved in the application side (industry and TNO).

5. Genetic engineering

5.1 Introduction

Genetic engineering can perhaps best be defined as the controlled modification of the hereditary factors (genes) of a living cell, or of their environment, in order to affect the function of those genes.
Genetic engineering can be used both to amplify specific genes in a cell, and to transfer genes from one organism to cells of another organism. The latter option is particularly interesting, since it opens up the possibility of producing valuable proteins (hormones, enzymes, etc.) which are normally produced by cells of higher organisms, such as plants and animals (and often only in very small quantities), from cells of micro-organisms, which are far more suitable for technological purposes.
Before going into genetic engineering in greater detail it is worth explaining briefly how the genetic material in a living cell is organised, and how it is brought to expression [32].
In every living cell there is genetic material containing the information necessary for all the processes taking place in that cell. The chemical basis of this genetic material is the compound deoxyribonucleic acid (DNA). A DNA molecule consists of two long strands (a single strand in the case of some viruses) coiled around each other in a particular way. The strands are composed of a large number of four building blocks: adenine, thymine, cytosine and guanine. The two DNA strands are coiled in such a way that a thymine in one strand is always opposite an adenine in the other strand, and a cytosine opposite a guanine.
The sequence of these four building blocks determines the hereditary factor in question. A DNA molecule can incorporate several thousand hereditary factors (genes), and a gene in its turn contains an average of 1,000 building blocks per strand. In higher organisms the DNA in the cells is organised in clearly recognisable structures known as chromosomes, which are located in the nucleus of a cell. Such cells are called eucaryotic. This clear structure is lacking in other organisms, such as bacteria, and here the cells are called procaryotic.
How is the information stored in the genes in the DNA molecule brought to expression?
The process takes place in two distinct stages. First a fragment of the DNA, consisting of one or more genes, is transcribed into ribonucleic acid (RNA). This consists of a single strand bearing the information of part of the DNA, and is known as messenger RNA. The process of information transfer is known as transcription.

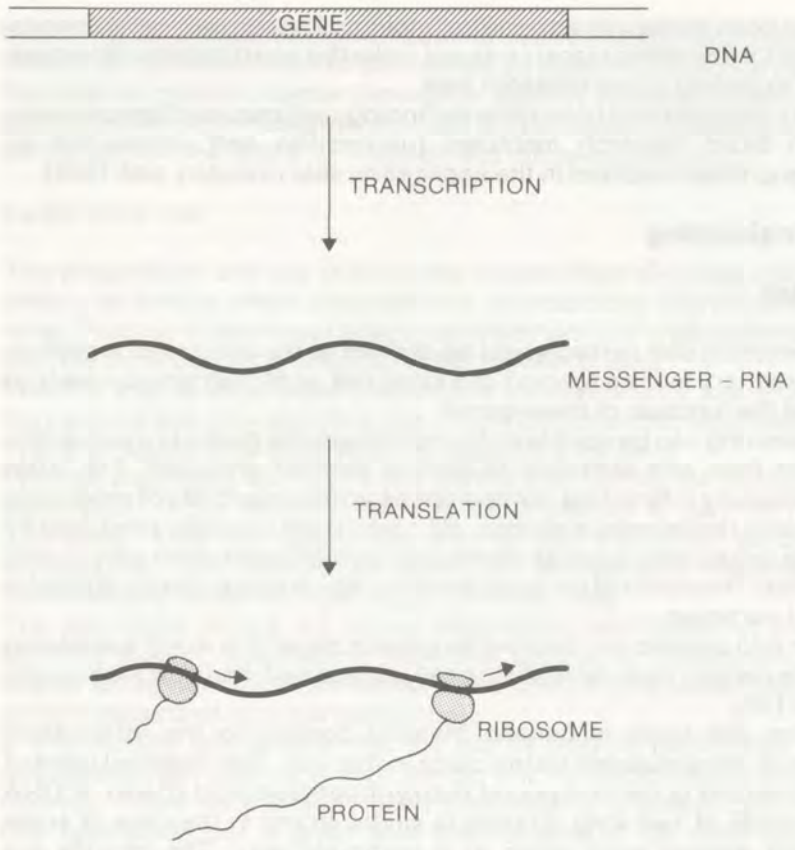


Figure 6. The formation of gene products in a living cell

The messenger RNA is then translated into a protein by ribosomes (see figure 6). This process is known as translation. The ultimate product of a gene is thus an extremely specific protein, which carries out a particular function within the cell. That function may be enzymatic (an enzyme), structural (a membrane component, the coat protein of a virus, etc.), or regulatory (a protein hormone, for example).

The amount of protein manufactured by one particular gene is a matter of regulation, which can take place at either the transcription or the translation level.

Genetic engineering can be used for various purposes. The object may be to obtain a particular gene or gene fragment in order to study it. Studies of this type are usually purely scientific, the purpose being to discover more about the relationship between the structure and function of the DNA. For example, what is the structure of the DNA at the sites where the replication of this DNA begins, or the structure of DNA or RNA where transcription or translation begins or ends? This sort of study may also be important from the technological point of

view if one intends to make deliberate alterations to the structure of the DNA in order to modify the gene product (protein), or to optimise the expression of a gene. Structural investigation of the DNA can also have a medical value in the study of hereditary diseases and other genetic anomalies in the living cell (such as cancer). That being said, the main industrial interest in genetic engineering is concentrated not on the possibility of investigating the DNA itself, but of obtaining the gene product. That product might be an enzyme, a protein hormone such as insulin, or a structural protein such as the coat protein of a virus which could be used for vaccination against that virus. The goal will generally be to extract the product, but it is also conceivable that the optimum formation of one or more gene products within the cell itself could yield an organism capable of carrying out a particular conversion more efficiently.

5.2 Brief history of genetic engineering

Genetic engineering was first practised on the bacterium *Escherichia coli*, which inhabits the intestines of all animals, where it serves a useful function. *E. coli* is still the most important organism for experimenting with genetic manipulation. But why this special interest in *E. coli*, and not in other micro-organisms, such as yeasts or bacillus species, which offer a number of distinct advantages from the point of view of process technology? The first reason is that the development of micro-organism genetics in the 1950s centred around *E. coli* and, to a lesser extent, *Salmonella*. This was occasioned by the discovery that these bacteria occur in both male and female forms. If a male bacterium comes into contact with a female the genetic material of the male cell can be transferred to the female cell, where new combinations take place with the genetic material from that female cell (recombination). A second reason is that the fundamental physical study of DNA and DNA-dependent processes (replication, transcription, etc.) was carried out using DNA from bacteriophages (bacteria viruses) which parisitise *E. coli*. Knowledge of the genetics of the *E. coli* chromosome and of the properties of one particular *E. coli* bacteriophage, lambda, made it possible, in the mid-1960s, to transfer one gene of the *E. coli* chromosome to the chromosome of the bacteriophage using natural recombination processes. Since a phage can replicate up to approximately 1,000 copies in each *E. coli* cell it is also possible to reinforce the gene in question (*lac*, which is involved in the fermentation of lactose). This ultimately led to the isolation of the *lac* gene [33]. The potential of this method was greatly increased when it was discovered that DNA can also be recombined outside a cell by using enzymes [34, 35]. This form of recombination employs so-called restriction enzymes which can split the DNA into extremely specific fragments. The enzyme ligase is then used to reunite those fragments in the desired sequence. These new DNA combinations can also be made with DNA fragments from other organisms, and they are brought to life again by allowing the DNA to be taken up by living cells. This latter process is known as transformation (see figure 7). Initially it was thought that only a few micro-organisms were transformable (*Haemophilus influenza, Bacillus subtilis, pneumococci*), but it is now possible to transform to a large number of different

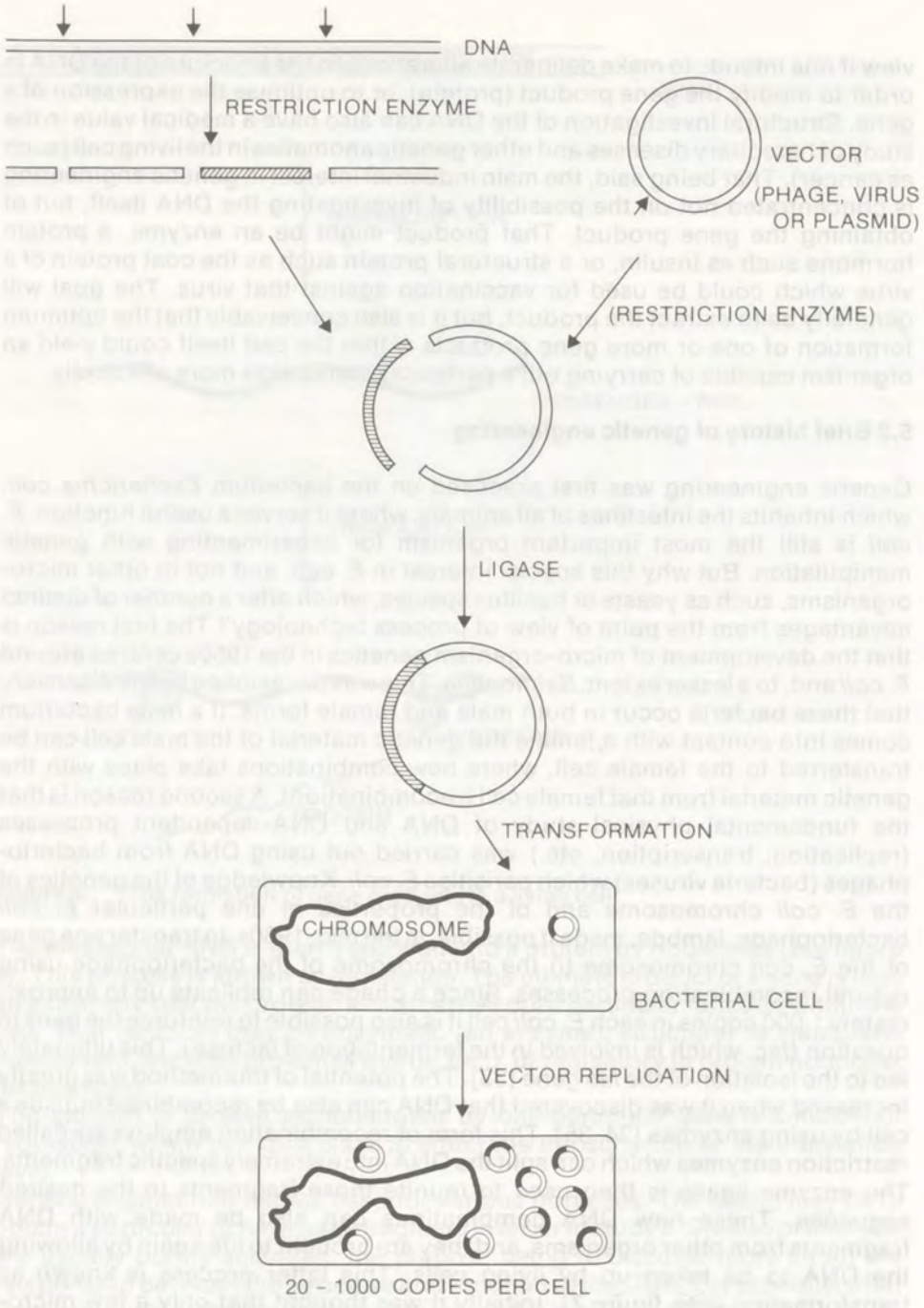


Figure 7. Gene amplification by means of molecular cloning on plasmids

cells, both procaryotic and eucaryotic (*E. coli*, yeast, human cells).
In principle this new technique makes it possible to reinforce any gene in the cell to which it normally belongs, or to transfer it to cells of other organisms. This has greatly enlarged the prospects for biotechnological applications.

5.3 Practical potential of genetic engineering

5.3.1 Homologous system

In order to obtain the maximum expression (and thus the greatest amount of product) from a gene it is essential to optimise both the process of transcribing the DNA sequence into an RNA sequence, and of translating the RNA into protein. The simplest way of doing this is in a homologous system. In other words, the optimum expression of a yeast gene is best achieved in yeast cells, of an *E. coli* gene in *E. coli* cells, and so on.
There are certain requirements for the use of homologous systems. In the first place it must be possible to culture the organism rapidly and relatively inexpensively. This generally limits the field to micro-organisms. In addition the genetics of the organism have to be well developed and, most important of all, it has to have suitable vectors for transferring the genes. In general these vectors are phages (or viruses), or certain types of plasmid. Plasmids are autonomously replicating DNA molecules which, like bacteriophages, can occur in very large numbers of copies per cell. The organism which best meets all these requirements at the moment is *E. coli*.
Occasionally the disadvantages inherent in other organisms can be overcome, partly or entirely, by isolating hybrid vectors. These are vectors which are capable of replicating in two organisms. This means that certain experiments can be carried out in *E. coli*, with others taking place in the organism to be investigated. This is particularly valuable in acquiring information on the genetics of the organism in question, which is essential if one wants to submit that organism to genetic manipulation. Hybrid vectors have been isolated for *E. coli* and yeast [36], for *E. coli* and blue-green algae (in order to study the genetics of photosynthesis), and for *E. coli* and human cells [37]. For example, if a human gene were to be transferred on a hybrid vector that vector could be replicated in *E. coli* cells. Although the human gene would not come to expression of its own accord it would be easy to isolate it in fairly large quantities in order to study its structure, and for other purposes. If the same hybrid vector was transformed to the human cell then it could also be replicated there. For the transferred human gene this would be a homologous system, and so it could easily be brought to expression.

5.3.2 Heterologous system

If an organism is ill-suited or even totally unamenable to genetic engineering it is possible to transfer the gene whose product is required from that organism to another, suitable organism (generally *E. coli*, at the moment). The problems this raises are discussed briefly below.

Expression

If a gene is transferred from one organism to another it will generally not come to expression. There are various reasons for this. The first is that the regulatory elements which are necessary for expression differ from one organism to another. As a result correct transcription will either not take place at all, or will be very poor. In principle, though, this problem can be overcome. The stripped gene, with its natural regulatory elements removed, is implanted (using genetic engineering) behind the regulatory elements of a gene from the organism in which it is to be brought to expression. The regulatory elements most in use at the moment are from the *lac* genes of *E. coli* (for example, in the synthesis of the hormone somatostatin [38] and the human growth hormone) [39]. In other cases use is made of a very strong promoter (point at which transcription begins) from the *E. coli* phage, lambda.

Gene structure

Another problem is that structure of the gene itself can prevent its proper expression in a heterologous system. Many eucaryotic genes, for example, contain segments of DNA sequences (introns) which do not properly belong to the functional gene. After transcription of the gene these introns are removed from the RNA in a process known as splicing, leaving just the functional messenger RNA to be translated into protein [40].
Since splicing cannot take place in *E. coli* it is necessary to present this micro-organism with the eucaryotic gene stripped of the introns if the gene is to come to expression. This can be done if the messenger RNA, which no longer has any introns, is enzymatically converted into DNA. The trick is to extract the desired messenger RNA from the cells, and highly ingenious methods have been developed for doing this. One method is based on the use of a small piece of synthetic DNA which has been designed so that it is homologous to the desired messenger RNA. The target messenger RNA can be extracted from a mixture of a large number of messenger RNAs using DNA-RNA hybridisation. The base sequence of this fragment of synthetic DNA can be deduced from the amino acid sequence of the protein associated with the messenger RNA [41]. Needless to say, it must be possible to isolate that protein.
Another method is to synthesise the entire gene (which is done in the case of the gene for somatostatin) [38]. These two methods can also be combined. These methods enable us to bring more and more genes from higher organisms to expression in micro-organisms.

5.3.3 Cell fusion

Cell fusion offers a totally different way of isolating gene products.
In the case of cells cultured *in vitro*, the cells of the same or different species are fused. The result is a hybrid cell which, in most cases, is capable of cell division (proliferation). Cell fusion is achieved either with a deactivated virus (generally the Sendai virus) or with polyethylene glycol. The genes of both parent cells

come to expression in the hybrid cell. This is used for isolating antisera. The fusion of tumour cells from mice with antibody-forming plasma cells from mice results in the limitless production of antibodies. This is because the cell hybrids resulting from the fusion have two important properties:
— antibody formation continues in the hybrid cell;
— the tumour cell retains its property of continuous proliferation.

5.4 The safety of recombinant DNA research

In 1972 Paul Berg and his colleagues published an article in which they described how a gene from *E. coli* could be linked in the test tube with the DNA of the animal virus SV40 [42]. The authors stated that their goal was 'to develop a method by which new, functionally defined segments of genetic information can be introduced into mammalian cells'. Numerous researchers recognised the implications of this new technique within a very short time. These recombinant DNA molecules, which are combinations of DNA segments from different organisms, can also be inserted in bacteria. The bacteria could thus acquire additional genetic properties which might make a normally harmless organism pathogenic. That risk would be even greater if undefined DNA segments from an organism, which might contain unknown viruses, were to be inserted in bacteria. The assumption was that the insertion of foreign DNA of this kind might initiate the production of a foreign protein in the bacteria. Various experiments were carried out, and a few years later it was shown that this assumption was false [43, 44, 45]. Foreign DNA does not come to expression in bacteria without special experimental intervention [46]. In other words, it does not initiate the production of a protein which might be pathogenic.
In 1974 a conference on the possible dangers of recombinant DNA research was held at Asilomar (California) [47]. With the approval of many prominent scientists it was agreed to call a voluntary halt to recombinant DNA experiments until such time as there was adequate scientific data which could give an idea of the possible dangers of bacteria carrying recombinant DNA. Consultations with epidemiologists and population geneticists led to a fundamental change in attitudes, and the consensus was that recombinant DNA research should be allowed to continue, but under stringent safeguards. The period 1975-1979 was marked by a number of important developments. Committees were set up in the United States and Britain, under the auspices of the National Institution of Health (NIH) and the British government, to regulate recombinant DNA research and to lay down guidelines for experimentation. At the same time studies were carried out in these two countries to fill in the blank areas in our knowledge of the possible pathogenicity of bacteria incorporating recombinant DNA. The general conclusion to emerge from these studies was that it is extremely difficult, if not impossible, to make the non-pathogenic *E. coli* bacterium pathogenic by means of recombinant DNA. It was also found that recombinant DNA in no way enhances the bacterium's ability to survive or to replicate, either inside or outside the human body [48]. These conclusions were regarded as sufficient grounds for easing the safety regulations for recombi-

nant DNA work, but without doing away with safeguards altogether.
The safeguards fall into two categories: physical security (containment) and biological security. Physical security, which is subdivided into classes P1-P4 (American system) or CI-CIV (British system), covers the standards to be met by the laboratory, as well as the way in which the manipulations are carried out. Few special safeguards are required of a P1 (or CI) laboratory, but a P4 (or CIV) laboratory has to be under partial vacuum and has to have facilities for decontaminating personnel and equipment. The European Molecular Biology Laboratory (EMBL) at Heidelberg (West Germany) is the only facility in Europe where recombinant DNA research can be carried out under CIV conditions, and as such it acts as a host laboratory for visiting scientists. In the Netherlands there are plans to install a CIII facility at the TNO Medical Biological Laboratory at Rijswijk. Most Dutch universities and a few industrial concerns have CI or CII laboratories.
The second category of safeguards covers biological security (classes EK1 and EK2), which is prescribed for experiments with *E. coli*. EK1 strains of this bacterium are strains which are totally incapable of transferring genetic material (DNA) to other bacteria, such as bacteria which are found in the human intestine. It should be noted that the survival and replication of EK1 strains is extremely low compared to natural *E. coli* strains either inside or outside the human body. EK2 strains of *E. coli* are EK1 strains which, due to the introduction of mutations at certain sites in the genetic material (DNA), have an even lower survival capability, mainly because their growth depends on constituents which are not available either in the intestine or in nature.
An *ad hoc* committee on guidelines for genetic engineering was set up in the Netherlands under the auspices of the Royal Dutch Academy of Arts and Sciences, to which all recombinant DNA projects have to be reported. On September 18, 1980 the committee submitted an advisory report to the Dutch government. Its two main recommendations were as follows [49].

1. Grading of recombinant DNA experiments (a combination of physical and biological security) according to the guidelines drawn up by the NIH in the United States.
2. Observance of the regulations drawn up by the committee for the equipping and use of CI, CII, CIII (and CIV) laboratories, which are even stricter than the American P1-P4 standards.

These recommendations mean that recombinant DNA research using *E. coli* bacteria will generally be classified as CI and EK1. Experiments involving other organisms (such as *Bacillus subtilis* or yeast) fall under a more stringent physical security (CII or CIII). The main reason for this distinction is that *E. coli* has been studied fairly extensively as regards both its genetic properties and its epidemiological behaviour. The second consideration is that foreign DNA does not come to expression in *E. coli* unless it is actively encouraged. This has been noted independently by many scientists. In other words, the bacterium itself provides the best biological security. If a property does not come to expression then one is merely introducing a dead fragment of DNA which does not give rise to the synthesis of any (harmful) product.

The Dutch committee's report also outlines the many experiments which have been carried out in order to evaluate the possible risks of recombinant DNA work. The conclusion of the Dutch committee, and of other committees in Europe and the United States, is that the fears expressed in the period 1972-1974 have turned out to be unfounded.
Attitudes in the Netherlands towards recombinant DNA work developed more slowly than in many other countries, and public debate was set in motion several years later than in the United States, Britain, West Germany and France. Moreover, for a long time the Dutch government reserved its position on the issue, and that attitude is still common on the part of other bodies involved, such as the local health inspectorates and city councils. University and industrial research groups have consequently been at a disadvantage compared to scientists in the countries mentioned above. The delay in the regulation of recombinant DNA work has meant that in recent years Dutch scientists have been carrying out their experiments in neighbouring countries, such as Belgium, Switzerland and West Germany, where adequate regulations are in operation and where there are properly equipped laboratories.

5.5 Conclusions

The time is approaching when it will be possible to bring a large number of genes from higher organisms to expression in micro-organisms. Some of these gene products are medically valuable (vaccines, hormones, etc.), while others are of more interest to the biotechnology industry (enzymes).
There are also a large number of micro-organisms which can perform processes which are extremely interesting from the technological standpoint. The problem is that those micro-organisms are still relatively inefficient. The technique of genetic engineering makes it possible to analyse a number of these processes genetically, and this could open up the way to reinforcing those processes in the organism.
Processes such as photosynthesis and nitrogen fixation in plants are already being studied in this way. Genetic engineering techniques are also being employed to study processes in yeast and in human cells, and for examining the process of oncogenic transformation. This will undoubtedly lead to new medical and technological applications.
A high priority should be given to the framing of a national code of regulations for recombinant DNA research, and that code should have the backing of parliament.

Members of the working group

The working group which produced Chapter II consisted of the following members:

Dr G.M.A. van Beynum	Gist-Brocades, Delft
E.H.M. Greuell	Unilever Research Laboratories, Vlaardingen
Prof. N.W.F. Kossen	Delft University of Technology
Dr P. van de Putte	Leiden State University
Dr P.J. Strijkert	Gist Brocades, Delft
J.H.F. van Apeldoorn (secretary)	Netherlands Study Centre for Technology Trends, The Hague

The following provided additional information or made valuable contributions to discussions:

Dr H. Pannekoek	Leiden State University
A.L. van Wezel	Netherlands Public Health Institute, Bilthoven

References

1. K.Lohmann, Darstellung der Adenyl pyzophosphorsaüre aus Muskulatur. Biochem. Zeitschrift *232* (1931) 460 - 469
2. D.W. Hutchinson, Nucleotides and Coenzymes. London, Methuen & Comp. Historical Introduction (1964) 1 - 5
3. O.T. Avery, C.M. MacLeod, M. McCarty, Studies on the chemical nature of the substance inducing transformation of pneumococcal types; Induction of transformation by a desoxyribonuleic acid fraction isolated from pneumococeus Type III. J. Experimental Medicine *79* (1944) 137 - 158
4. E.L. Tatum, J. Lederberg, Gene recombination in the bacterium *Escherichia coli*, J. Bact. *53* (1947) 673 - 684
5. J.D. Watson, F.H.C. Crick, Molecular structure of nucleic acids. Nature *171* (1953) 737 - 738
6. F. Jacob, J. Monod, On the regulation of gene activity. Cold Spring Harbor Symposia on Quantitive Biology Vol XXVI (1961)
7. M.W. Nirenberg, J.H. Matthaei, The dependence of cell-free protein synthesis in *E. coli* upon naturally occuring or synthetic polyribonucleotides. Proc.Nat.Ac.Sci USA *47* (1961) 1588 - 1602
8. A.B. Pardee, F. Jacob, J. Monod, The genetic control and cytoplasmic expression of 'Inducibility' in the synthesis of beta-gelactosidase by *E. coli*. J. Mol.Biol. *1* (1959) 165 - 178
9. M.F. Singer, Genetic Engineering, Principals and Methods. Vol I Plenum Press, N.York (1979) Introduction and historical background 1 - 13
10. H.J. Peppler, D. Perlman, Microbial Technology. New York, Academic Press (1979).

11. J.E. Bailey, D.F. Ollis, Biochemical Engineering Fundamentals. New York, McGraw Hill Kogakusha Ltd. (1977).
12. A.T. Bull et al, Microbial Technology Current State, Future Prospects. Cambridge, Cambridge University Press (1979).
13. B.Dixon, Invisible Allies, Microbes and Man's Future. London, Temple Smith, (1976).
14. D.I.C. Wong et al, Fermentation and Enzyme Technology. New York, Wiley Interscience (1979).
15. J. Reinert, Y.P.S. Bajay, Tissue and Organ Culture. Berlin, Springer Verlag, (1977).
16. W. Barz, E. Reinhard, M.H. Zenk, Plant Tissue Culture and its biotechnological applications. Proceedings in Life Sciences. Springer (1977).
17. J. Paul, Cell and Tissue Culture. E & S. Livingstone (1979).
18. P.F. Kruse, M.K. Patterson, Tissue Culture, Methods and Applications. New York, Academic Press (1973).
19. H. Gerstenberg, W. Sitting, K. Zepf, Aufarbeitung von Fermentationsprodukten. Chemie Ingenieur Technik, *52* (1980) 9 - 13
20. G.R. Cysewski, C.R. Wilke, Process Design and Economic Studies of Alternative Fermentation Methods for the Production of Ethanol. Biot. & Bioeng. XX (1978) 1421 - 1444
21. Chemie nu en straks. Publication of the Netherlands Ministry for Science Policy, The Hague 1980
22. Enzyme nomenclature: Recommandations of the International Union of Pure and Applied Chemistry and the International Union of Biochemistry BBA (1976) 429 1
23. R.D. Schmid, Process Biochemistry (1979) May 2.
24. K. Aunstrup, Trend in Enzymology. Vol *61* (1979) 3
25. R.L. Ory, A.J.St. Angels, Enzymes in Food and Beverage processing. ACS Symposium series *47* (1977)
26. N.D. Pintauro, Food processing enzymes. Food Technology Reviews *52*. Noyes Date Corporation.
27. J. Melling, B.W. Philips in: A. Wiseman, Handbook of enzyme technology. E. Horwood Ltd. 58
28. In: Chemical & Engineering News, April 9 (1979) 26
29. I. Chibata, Immobilized enzymes. Halsted Press
30. Z. Bohak, N. Sharon, Biotechnical applications of proteins and enzymes. Academic Press.
31. K. Venkatasubramanian, W.R. Vieth, Progress in Industrial Microbiology Vol *15* (1979)
32. G.S. Stent, R. Calendar, Molecular Genetics. W.H. Freeman and Comp. San Francisco (1978)
33. J.R. Beckwith, D. Dipser, The Lactose Operon. Cold Spring Harbor Laboratory, New York (1970)
34. R.F. Beers, E.G. Bassett, Recombinant Molecules: Impact on science and society. 10th Miles International Symposium, Raven Press (1977)
35. J.K. Setlow, A. Hollaender, Genetic Engineering: Principles and Methods. Plenum Press, New York and London *2* (1980)

36. J.D. Beggs, Nature *275*, (1978) 104
37. R.C. Mulligan, P. Berg, Science *209* (1980) 1422
38. K. Itakura, T. Hirose, R. Crea, A.D. Riggs, H.L. Heyneker, F. Bolivar, H.W. Boyer, Science *198* (1977) 1055
39. P.H. Seeburg, J. Shine, J.A. Matial, R.D. Ivarie, J.A. Morris, A. Ullrich, J.D. Baxter, H.M. Goodman, Nature *276* (1978) 795
40. R. Breathnach, J.L. Mandel, P. Chambon, Nature *270* (1977) 314
41. D.V. Goeddel, E. Yelverton, A. Ullrich, H.L. Heyneker, G. Miozarri, W. Holmes, P.H. Seeburg, T. Dull, L. May, N. Stebbing, R. Crea, S. Maeda, R. McCandliss, A. Sloma, J.M. Tabor, M. Gross, P.C. Familette and S. Pestka, Nature *287* (1980) 411
42. D.A. Jackson, R.M. Symons, P. Berg, Proc.Natl. Acad.Sci USA. *69* (1972) 2904
43. L. Maturin, Curtiss III, R. Science *196* (1977) 216 (see also R. Curtiss III (1977) Letter dated April 12th to D. Fredrickson, Director NIH)
44. B.R. Levin, F.M. Stewart, Science *196* (1977) 218
45. M.A. Israel, H.W. Chan, M.A. Martin, W.P. Rowe, Science *203* (1979) 883 and 887
46. K. Itakura, T. Hirose, R. Crea, A.D. Riggs, M.I. Heyneker, F. Bolivar, H.W. Boyer Science *198* (1977) 1056
47. P. Berg, D. Baltimore, H.W. Boyer, S.N. Cohen, R.W. Davis, D.S. Hogness, D. Nathans, R.Roblin, J.D. Watson, S. Weissman, N.D. Zinder, Science *185* (1974) 303
48. E.S. Anderson, Nature *255* (1975) 502
49. Commissie ad hoc Recombinant DNA werkzaamheden: concept richtlijnen voor Recombinant DNA bewerkingen. Brief 18 september 1980 en Jaarverslagen onder auspiciën van Koninklijke Nederlandse Akademie van Wetenschappen van voorgaande jaren.

III Research and teaching

Inventory of university teaching and research in biotechnology and subordinate disciplines[1]

It must first be emphasised that the purpose of this inventory is not to provide a 100%, comprehensive survey, but rather to gain an all-round picture of teaching and reseach in biotechnology and subordinate disciplines. It has been compiled on the basis of:

— a number of personal interviews;
— a questionnaire to which a number of responses were obtained in writing.

By these means, about 40 representatives of various university departments – mostly at Delft University of Technology, Wageningen University of Agriculture and the State University of Groningen – were contacted in some form or other.

I. Multidisciplinary biotechnological research and teaching

1.1 Introduction

Although biotechnology, according to the definition adopted, embraces microbiology, biochemistry and process technology, it should not be assumed that each and every biotechnological research or education project must include aspects of all these disciplines. It is quite feasible, for example, for the purely biological or chemical aspects of a biotechnological research project to be of minor importance.

1.2 Delft University of Technology

If a choice had to be made on historical grounds as to which Dutch university would be the centre best suited for biotechnological education and research, Delft would stand a very good chance indeed. The school of Beyerink and Kluyver has a high international standing, and it can even be argued that it was the excellent (micro)biological /(bio)chemical/technological background

[1] This section has been taken in slightly modified form from section 3.3. of 'Biotechnologie en Innovatie', compiled by Dr R.R. van der Meer of the Central Institute for Industrial Development (CIVI) and published for the Voorlichtingsdienst Wetenschapsbeleid (Scientific Policy Advisory Service) by the Staatsuitgeverij, The Hague, 1980.

supplied by Delft University of Technology which has enabled a company such as Gist-Brocades to develop into one of the world's largest fermentation companies. It therefore stands to reason that biotechnological research has already been conducted here for several years (Biochemical Reactors Section). A few years ago, the first multidisciplinary university course in biotechnology in the Netherlands was started at Delft. The first biotechnologists have now graduated.

The following summary outlines the most important biotechnological and biotechnology-related activities.

Microbiology (Prof. Kuenen, Prof. Roels, Dr van Dijken, Dr Bonnet) Work is being or will be (Prof. Kuenen has just been appointed) carried out on the energy balance, maintenance energy and physiology of yeasts (e.g. *Saccharomyces cerevisiae*) and on fungi, mixed cultures and mixed substrates (for both productive fermentation and water purification).

Biochemistry (Duine, Frank) Research is being conducted on enzymatic conversions in methanol oxidation for purposes of Single Cell Protein production.

Biology (Prof. Fuchs, Prof. Roels) Work is proceeding on microbiological and other projects, in particular for the elimination of sulphides and heavy metals from the environment. Other projects include the kinetics of anaerobic water purification, macrobalances and other model descriptions.

Organic chemistry (Prof. van Bekkum) Work is being carried out, with the collaboration of Prof. Roels, on hybrid catalysis (combined inorganic and biological catalysis) for use in carbohydrate chemistry.

Biochemical Reactors Section (Prof. Kossen) Projects include the cultivation of fungi in pellet form, immobilised micro-organisms, the design of anaerobic digestion plants for water purification, the modelling of biological systems and the energy balance of micro-organisms.

Chemical technology (Prof. Wesselingh) Work is being carried out, in collaboration with Prof. Roels and others, on the use of molecular sieves for the separation of microbial process products.

Nearly all of the above projects are being carried out on a collaborative basis. The most important link-ups are between the Biochemical Reactors Section, the General and Technical Microbiology Department and the General and Technical Biology Department (under Profs. Kossen, Kuenen and Roels respectively). Delft has no molecular biology and genetics; moreover, there is not enough collaboration with biochemistry. The fundamental and applied technical disciplines of particular interest for biotechnology are well represented. Further dialogues and other forms of collaboration are currently being

established (with process dynamics, chemical and physical unit operations, operations research, and other disciplines).

1.3 Wageningen University of Agriculture

There is naturally a close correlation between agricultural and biotechnological research, so it is not surprising that research of great relevance for biotechnology has been carried out for years at Wageningen University of Agriculture. This has led to the setting up of a multidisciplinary course in biotechnology on the two-phase model: in the first phase (four years), basic knowledge is acquired in a number of fields such as biology, microbiology, biochemistry, genetics, biokinetics, physical transport phenomena, separation methods and general reactor studies. In the second phase, this knowledge has to be applied in multidisciplinary projects. This biotechnology course was started in September 1980.

The various biotechnology-related research projects under way at Wageningen are outlined below.

Microbiology (Profs. Mulder and Bulder) Work is being carried out on the epoxidation of alkenes with biocatalysis, on the isolation of xanthine oxydase and on anaerobic processes. Prof. Mulder has recently retired. At present it is argued that more attention must be devoted to technical microbiology at Wageningen for it to form a better basis for biotechnology.

Genetics and molecular biology (Profs. Sybenga and van Kammen and others) Projects include the genetic manipulation of fungus protoplasts, the carbohydrate metabolism of *Aspergillus nidulans*, the production of extracellular enzymes by bacilli and aspergilli and the application of recombinant DNA techniques for plant genes (nitrogen fixation).

Biochemistry (Profs. Veeger and Müller) Research is being conducted on the photochemical/photocatalytic/photovoltaic production of hydrogen with the enzyme hydrogenase (in both free and immobilised form) and on the application of xanthine oxydase (in collaboration with Prof. v.d. Plas, Organic Chemistry) in organic synthesis (immobilised).

Biophysics (Prof. Schaafsma) Research projects include the development of NMR (nuclear-magnetic resonance) and solids NMR techniques for the study of biological systems (including membranes).

Foodstuffs technology (Prof. Pilnik, Dr Kleter) Work is being carried out on enzymatic saccharification, pectolytic enzymes, cheese-maturing processes and protein isolates.

Process technology (Dr Tramper) Projects include the continuous production of gluconic acid with immobilised *Gluconobacter oxydans*, epoxidation

processes with *Mycobacteria* and decaffeination with the aid of micro-organisms.

Water purification (Dr Lettinga, Rensink) Projects include the anaerobic purification of waste water, manure processing, nitrification/denitrification and phosphate elimination.

Apart from the projects outlined above, most of the other applied research projects within the various departments at Wageningen are oriented towards biotechnology, and are often carried out on a collaborative basis. All this, in conjunction with the fundamental research work, means that a Biotechnology Sector within the University of Agriculture could well make a significant contribution towards the development of this field.

1.4 State University of Groningen

Groningen, like Wageningen, has in principle all the subordinate disciplines needed for a multidisciplinary course in biotechnology and for research in this field. For some years now, a chemical/physical technology course has been offered; organic chemistry has a strong biological bias (there is a chair for bio-organic chemistry); in biochemistry, work is proceeding on biotechnological processes and recombinant DNA research; biophysics is also represented. All these sections, each of which could play a part in multidisciplinary research and higher education in biotechnology, are located in the same building, which also houses the structural chemistry group under Prof. Drenth and Dr Hol. One small problem is that genetics and microbiology are housed in the University Biology Centre in Haren, some distance away from the other groups.

Several years ago, the fact that an important starch-processing industry was located in Groningen led the technology section to concentrate on biotechnological matters. This in turn led to a project for the study of the isomerisation of glucose to fructose with the aid of immobilised glucose isomerase.

Activities in the biotechnological field are currently concentrating on the study of reactors for multiphase systems.

Personal interests, and the fact that more and more emphasis is now being laid on the applications of biochemical and organic chemical research, have encouraged the departments concerned to embark on biotechnological research. This has naturally led to a link-up with the technology section (Prof. Wijnberg (organic chemistry), Prof. Witholt (biochemistry) and Prof. Joosten (chemical technology)).

At present, microbiology and genetics do not participate in this collaboration, although these departments do take part in multidisciplinary biotechnology teaching in that microbiology and genetics courses, seminars and practicals are being integrated and extended to cover more (bio)chemical topics.

Whether this will work out remains to be seen.

As has already been argued, and on the basis of the above, Groningen is in a good position for further development into a focal point for biotransformations and (fine) chemical syntheses. The initiatives taken so far have been promising but have almost all originated from the subordinate disciplines; there is at present no real centre for biotechnology at Groningen. However, this should not be seen as a major problem or obstacle, as such a centre would need time to develop.

2. Teaching and research in the subordinate disciplines

2.1 Microbiology

In the Netherlands, microbiology teaching and research are highly advanced. A great deal of attention is paid to the microbial physiology of bacteria. As priority is given to fundamental, physiological matters, there is not so much scope for collaboration on biotechnological projects.

However, there are various groups engaged on projects that could yield biotechnologically useful results. These include:

— a group working under Prof. Stouthamer at the Free University of Amsterdam on the mathematical description of micro-organism growth and production processes, on regulation processes and on improving the efficiency of nitrogen fixation in *Rhizobium* bacteria by increasing the hydrogenase activity (via genetic manipulation);

— a group working under Prof. Tempest at the University of Amsterdam on continuous cultures of *Klebsiella aerogenes*, *Bacillus subtilis* and *E. coli*, two-stage systems of acid-forming and methane-forming anaerobic bacteria, bio-energetics and the overflow mechanism;

— a group working under Prof. Vogels at the Catholic University of Nijmegen on anaerobic micro-organisms;

— a group working under Profs. Veldkamp, Harder and Konings at the State University of Groningen on interactions between micro-organisms in continuous cultures, sulphate-reducing bacteria, lactic acid bacteria, the bio-energetics of C_1 compound conversions (e.g. oxidation of methanol with yeasts) and the active transport of matter through microbial membranes.

The Wageningen and Delft groups (under Profs. Bulder and Kuenen respectively) have already been mentioned. From the information acquired on microbiology in the Netherlands, it can be concluded that little work is being done on micro-organisms other than bacteria. It has been remarked from many sides that hardly any physiological research is being conducted at present on

fungi/yeasts, streptococci and actinomycetes. More attention should also, it is thought, be paid to (products of) anaerobic micro-organisms.

The following reasons have been cited for this rather one-sided development of microbiology.

— The fact that microbiology has always been classified under biology. The relatively low teaching loads in this field have adversely affected the funds made available for research; this has also had repercussions on microbiology.

— The over-emphasis on bacteria (*E. coli*) is partly due to the fact that working with fungi and other micro-organisms entails greater technical problems, so that concrete scientific results are not so readily achieved. But it is in view of the likelihood of results that research grants tend to be awarded by SON and BION. For this reason, many departments are reluctant to embark on laborious research projects in which results cannot be so readily guaranteed.

Genetics in the Netherlands is also of a very high quality. As in the case of microbiology, development has always been rather biased towards bacteria (*E. coli*). Relatively little is known of the genetics of micro-organisms of commercial importance.

In molecular genetics, a distinction is made between two subordinate fields. The first of these fields – the molecular biology of micro-organisms – is studied at various universities. For example:

— the group working under Prof. Borst at the University of Amsterdam on yeast mitochondria DNA, kinetoplast DNA and nuclear DNA for *Trypanosoma brucei brucei* and kinetoplast DNA of *Crithidia fasciculata* and *Crithidia luciline*;

— the groups working under Profs. Planta, Groot and Nijkamp at the Free University of Amsterdam on ribosomal DNA, t-RNA from yeast (*saccharomyces carlsbergensis*), nuclear genes from yeast and ohizobia plasmids;

— Prof. Venema's group at the State University of Groningen working on *Bacillus subtilis* plasmids, etc.;

— Prof. Schilperoort's group at the State University of Leiden working on t-DNA of *Agrobacterium tumefaciens*, the Ti-plasmid of rhizobia etc.;

— Prof. van Arkel's group at the State University of Utrecht working on cyanobacteria, etc.;

— Prof. Schoenmakers' group at the Catholic University of Nijmegen working on the regulation of expression of bacterial virus genes, etc.;

— Prof. van Kammen's group at Wageningen University of Agriculture working on nitrogen-fixing genes in *Rhizobium leguminosarium*, etc.

It can be argued that all these molecular biology activities are being conducted on a relatively modest scale.

The second group of molecular genetics activities concentrates on applications in plants. Such activities form a more consistent whole than the first group, although no major inter-university collaboration has yet been initiated.
In early 1979, a preliminary dicussion was held under the auspices of the NRLO to try to formulate important subjects for research. At present, fundamental research in molecular genetics that might assist agriculture is being conducted by the following groups.

— Prof. Schilperoort's group at the State University of Leiden (molecular basis differentiation and oncogeny in plants / MOLBAS). Work is being carried out on *A. tumefaciens* and *Rhizobiaceae* plasmids, somatic cell genetics with plant cells, expression of Ti-plasmid DNA fragments *in vivo* and *in vitro* and the isolation and characterisation of the tumour-specific enzyme nopaline dehydrogenase. These research projects are starting to yield results which could lead to practical applications.

— Prof. Nijkamp's group at the Free University of Amsterdam. Work is being carried out on the molecular genetics of rhizobia plasmids in order to improve the efficiency of nitrogen fixation by increasing hydrogenase activity. Research is also being conducted on the somatic cell genetics of plants (chloroplast biogenesis) to gain greater insight into the molecular basis and the transfer of male sterility in *Petunia hybrida*.

— Profs. van Kammen and Sybenga's groups at Wageningen University of Agriculture. Work is proceeding on cowpea mosaic virus, chromosome transfer, etc.

— Prof. de Groot's group at the State University of Leiden. Research projects include the genetics of radiation damage repair in higher plants (*Nicotiana spp.*).

The Minister of Agriculture and Fisheries has recently appointed Professor de Groot to supervise the new programmes entitled 'Advanced methods for the genetic improvement of crops' and 'Biotechnology' at ITAL in Wageningen. These ITAL programmes are intended to concentrate on practical applications and so could tie in well with the results and work of the groups in Leiden, Amsterdam and the University of Agriculture.

Apart from the above groups, mention should also be made of the groups working under Profs. Gruber and Ab at the State University of Groningen. This

group is working on the recombinant DNA research of eucaryotic DNA from chickens. Eucaryotic DNA from calves is being studied at Nijmegen.

At the State University of Utrecht, a group under Prof. Jansz is working on the cloning of mammalian DNA which codes for peptide hormones such as cattle growth hormone, calcitonin, endorphin and ACTH.

2.2 Biochemistry and biophysics

In the Netherlands, biochemical research is highly sophisticated. Research in this field at Wageningen and Groningen ties in well with the biotechnological activities at those universities. Elsewhere, biochemical research is very fundamental in character and does not relate so well to present biotechnological developments.

In early 1980 the Minister for Science Policy set up the Biochemistry Reconnaissance Committee, with the intention that it should prepare proposals for the synchronisation and coordination of biochemical research in the Netherlands. This committee will undoubtedly cover the relationships between biochemistry and biotechnology in its terms of reference.

At present, the structural chemistry group in Groningen under Prof. Drenth and Dr Hol stands outside the biotechnological initiative at that university. Work is proceeding on the study of the three-dimensional structure of proteins by X-ray diffraction, etc. The activities of this group could be of great significance for biotechnology. It might be a good idea if the choice of enzymes to be studied could be coordinated, at the request of the biotechnologists, with the biotechnological processes that they are already studying.

Prof. van Boom's group at the State University of Leiden is working on the chemical synthesis of DNA fragments and the glycosylation of proteins. The first of these fields is becoming more and more important because of the rapid advance of recombinant DNA methodologies. This is an excellent technique for producing tailor-made DNA fragments that can be very profitably used in a wide range of applications in DNA research. The most striking example has been the cloning of somatostatin by Heyneker *et al.* at Genentech in California. This was done with synthetic DNA. The second example is insulin: a major part of the recombinant DNA was synthetic.
This technique can also be of great assistance in the modification of enzymes.

Since the cloning of interferon (which cannot be provided with the natural sugar and carbohydrate fragments by recombinant DNA techniques), the glycosylation of proteins has attracted more and more attention as well.

The Netherlands cannot complain as regards the availability of sophisticated measuring equipment. Although little attention has been paid to this question in this study, it can be pointed out that, on the average, good facilities are on hand

in the Netherlands for electron microscopy, ESR (electron spin resonance), NMR (nuclear-magnetic resonance), X-ray diffraction and mass spectrometry. The NMR of solids may become a major tool in biophysical research. It appears that this technique is also well suited for biological systems. The Catholic University of Nijmegen has this apparatus at its disposal.

2.3 Process technology

Process technology is, in fact, a back-up discipline rather than a subordinate discipline such as microbiology or biochemistry. This means that developments in this field have not so much provided new stimuli for biotechnology but, when judiciously applied in biotechnological processes, have helped such processes to develop fruitfully. On the international scale, a sequence can be perceived whereby developments in microbiology, molecular biology, and biochemistry stimulate new activities in biotechnology which in turn often lead to the corresponding development work in process technology. The fact that process technology is a very important sub-discipline is starting to be appreciated in a wide range of countries.

In most countries, however, the various initiatives taken to adapt process technology for biotechnological purposes have so far yielded hardly any concrete results. Dutch activities in the process technology field are concentrated at the Universities of Technology in so far as physical and chemical technologies are concerned, and at the University of Agriculture for foodstuffs technology. In Groningen (Profs. Stemerding, Drinkenburg and Joosten), work is mainly proceeding on physical technology; in Amsterdam (Prof. Boelhouwer) on chemical technology.

Teaching and research in chemical, physical, foodstuffs and dairy produce technologies, physical transport phenomena, reactor studies/vessel construction, physical separation methods, catalysis, measurement and control engineering, etc., have attained a high level in the Netherlands in the past 40 years, and they have made a significant contribution towards industrial development in that period. The fact that industrial expansion has slackened off over the past 10 years in the Netherlands, together with the fall-off in the number of chemistry students recently - which has tended to make the chemistry departments contract - has led to a certain standstill and perhaps even, here and there, to a decline in process technology. It has been argued that, although the quality is still very high and even unexcelled at some places, the all-round quality is not what it once was.

The same trend seems to be occurring in other countries. One advantage which the Netherlands has is that a great deal of the industrial research of Shell, Unilever and Philips is carried out here at the Royal Dutch/Shell Laboratory, Amsterdam, the Unilever Research Laboratories, Vlaardingen, and the Philips Research Laboratories. Technology has always played an important part in these companies' research. There are also the technological activities of

companies such as AKZO, DSM and Gist-Brocades. All in all, it can be said that as far as process technology is concerned the Netherlands is well placed for the development of biotechnology.

As this favourable position rests on two pillars – one academic, the other industrial – careful preparation is essential before concrete stimulation and initiatives can be implemented.

As Delft, Wageningen and Groningen have already been discussed, we will restrict ourselves to outlining some process technology projects under way at Eindhoven, Twente and the University of Amsterdam.

At Eindhoven University of Technology (Physical Technology Department under Prof. Thijssen and Ottengraf), work is being carried out on freeze-concentration processes; the improvement of preservation processes; aerobic water purification (kinetics, mixing, reactor models); and the elimination of pollutants from spentgases by means of biological filters.

At Twente University of Technology (Chemical Technology Department under Profs. Smolders and Bantjes), work is being carried out on membrane technology (in collaboration with Wafilin and the TNO Research Institute), and biomedical materials and medical technology. Points of interest in the latter field include:

— specific adsorbents and membranes on polymer basis for adsorbents or enzyme systems for medical applications;
— development of artificial organs;
— coupling of medicines to biodegradable polymers for slow dosing in the body;
— biocompatible surfaces with respect to blood and tissue.

At the University of Amsterdam (Chemical Technology Department under Prof. Boelhouwer), work is being undertaken on the acid-forming stage in anaerobic water purification, and on the anaerobic purification of dilute waste water flows (liquid domestic waste) after concentration of the contaminants by ultra and hyper filtration. There is collaboration with Prof. Tempest's group (microbiology, Amsterdam).

IV Food and allied products

1. The food industry

Introduction

The food industry can be regarded as one of the oldest forms of industry. The grinding of grain and the baking of bread in Roman times can be considered to be technology. The large scale on which these operations were carried out was the result of technical developments. In the same period an olive oil industry came into being. The preservation of meat, fish, fruit and vegetables involve even older technologies but there is no certainty as to whether it was done on a commercial scale.
A food industry based on scientific research, however, is only 200 years or so old. It was Lavoisier who established the fact that food was the body's fuel and it was Liebig who introduced the chemical analysis of food. In 1850 a few sections on food technology appeared in Wagner's *Manual of chemical technology*.
In this period growth was of an explosive nature — take the production and industrial-scale processing of beet sugar. In 1811 the quantity involved was six million kg; by 1870 it had grown to 7,000 million kg. Another rapid development was the food canning industry. Although Appert had already experimented with this technique in 1809, it took until 1870-1880 (Pasteur) before the industry really got going. This development ran parallel with that of the process plant industry and the improvement of transport systems.
In the Netherlands there was already a starch industry by 1840. The oldest beet sugar factory dates from 1858, butter and cheese factories from 1870, and the margarine industry from 1871.

The history of biotechnology in the food industry

Long before the existence of micro-organisms was discovered (A. van Leeuwenhoek, 1676) they were being used to make certain foods. By keen observation our forefathers learnt that during the storage of certain fruit juices and milk enjoyable keepable products were formed.
The first product to have been produced intentionally by biotechnological means was probably a fermented fruit juice in the hollowed out trunk of a tree. In any event it is known that around 3000 BC the Chinese were already producing a fermented rice and that in 200 BC the Assyrians had planted out vineyards and were making wine on a commercial scale. About 300 BC, Babylon had some twenty different kinds of beer and there is evidence that at the commencement of our era the steppe dwellers were using a distilling process to make a strong

alcoholic beverage from fermented milk. Other fermented milk products are thousands of years old. Homer described various kinds of cheese in his *Odyssey*.
In the making of these products things will often have gone wrong because people did not know how these mysterious changes took place. It was not until 1818 that Erxleben discovered that yeasts had to be the living organism that brought about fermentation. However, it is Louis Pasteur who is regarded as the founder of the modern fermentation industry as we know it today. Not only did he describe in 1857 the acidulation of milk and contend that an extract from yeasts — alcoholase — brought about the conversion of glucose to alcohol, but by working out pasteurisation and sterilisation methods he more particularly laid the foundations for the present-day fermentation industry.

Tables 1 and 2 show the importance of the food industry in the Netherlands and the significance of biotechnology.

Although products manufactured by biotechnological means account for 15% of overall sales, the influence of biotechnology is not much greater than it was 25 years ago and no striking progress has (yet) been made.

Table 1. Food and allied products industry in 1978 (excluding tobacco industry) [1]

		Production (Dfl. x million)
Slaughterhouses		2,480
Meat products		3,720
Poultry packers		1,170
Dairy industry (incl. ice cream)		9,460
Flour milling		1,150
Sugar		1,140
Margarine and oil		3,220
Fruit and vegetables processing		1,530
Bread and flour confectionery		2,030
Cocoa, chocolate, sugar confectionery		2,580
Alcohol and distilling		450
Brewing		1,580
Soft drinks		670
Animal feedstuffs		6,910
Total		38,090
Total industrial production (excluding public utilities and mining)in 1978	approx.	165,000
Export of food and allied products	approx.	19,000
Total exports	approx.	108,000

Table 2. Major fermentation products in 1976 [2]

	Dfl. x million
Cheese	1,900
Beer	1,700
Butter	1,460
Yoghurt	190
Fermented sausage	150
Sauerkraut	12
Export of fermentation products	1,400

2. Biotechnology and the food industry today

Most traditional foods date from before Appert's discovery of preservation by heat. They are based on fermentation (wine, sauerkraut, meat products, vinegar, beer, yoghurt, spirits) sometimes combined with the action of native enzymes (yoghurt, cheese, bread, beer). They are all due to chance discoveries made during the course of time and have progressed from the home via the artisan to the factory. We can only talk of biotechnology when basic knowledge is employed to introduce food engineering processes for improving quality and keeping properties, for scaling up and for continuous and automated production with a view to increasing profits. Vinegar manufacture is a very obvious example.
The transition from artisan to industrial production is not always complete. Next to the industrial bakery we still find the baker who bakes his own bread, and alongside the large industrial wine producers with their continuous fermentation processes there is still the small wine cellar. In some cases a product is unsuitable for manufacture by food engineering processes. Scaling up is then more a matter of increasing efficiency, e.g. by improved internal transport and by parallel production lines (sauerkraut, sausage, pickles). It is interesting to realise that biotechnology is involved in traditional products for which there is a tradition of advanced training, e.g. dairy, brewery, wine and distilled products.

The genetic engineering aspect would appear to be a less attractive proposition where new organisms forming other secondary fermentation products would jeopardise the sensory quality.

There are very few non-traditional biotechnology products: (bakers') yeast (1870), citric acid (1920), monosodium glutamate (1957), nucleotides as flavour enhancers (1960), lysine (1960), starch derivatives, among them isoglucose (1975 Institute of Food Technologists Industrial Achievement Award), xanthan gum (1974 Institute of Food Technologists Industrial Achievement Award) and Single Cell Protein (1970). These are not actual foods but additives or processing aids, which include enzymes. The impact of biotechnology on the food industry (the dairy industry excepted) is neverthe-

Table 3. Matrix showing relevance of process systems and techniques to production processes and additives

Product category	Fermentation			Enzyme technology	
	liquid	solid	gen. eng. potential	single use	immob. (incl. cells)
1. Meat		x		x	
2. Dairy	x	x	0	x	x
3. Fish		x		x	
4. Flour, hulled barley, rice				x	
5. Sugar	x			x	
6. Oils and fats	0		0	x	0
7. Fruit and vegetables					
8. Bakery products		x		x	
9. Cocoa, chocolate, sugar confectionery				x	
10. Starch, starch derivatives				x	x
11. Animal feed		x		x	
12. Alcohol, yeast	x	x	0	x	
13. Beer, wine	x		0	x	x
14. Vinegar	x	x			
15. Soft drinks, fruit juices				x	
16. Edible fungi		x		x	
17. Proteins, peptides, amino acids	x		0	x	0
18. Coffee		x			
19. Tea		x		0	
20. Tobacco		x			
Food additives					
21. Colours	x		0	x	
22. Flavours and aromas	x	x	0		
23. Stabilisers, thickeners	x		0	x	
24. Antimicrobial substances	x		0		
25. Vitamins and antioxidants	x		0	x	

x already applied
0 possible future application

less low. Of the industrial achievement awards made by the Institute of Food Technologists only two have been given for biotechnology processes. True, food processing is more concerned with the prevention of microbial and enzymatic changes than with promoting them. This will be obvious if we try to make a list of major product developments.

pre - 1870	preservation (by heat)
1870-1900	margarine, yoghurt, bakers' yeast, baby cereal, evaporated milk, saccharin, artificial varillin, frozen meat
1900-1920	hardened fats, dried foods, enzymes
1920-1940	citric acid made by fermentation, instant coffee, processed cheese, quick-frozen products, enzyme-treated fruit juices
1940-1960	pasteurised liquid milk, monosodium glutamate
1960-1980	freeze-dried products, xanthan gum, microbial rennets (dairy), concentrated microbial starters for dairy products, isoglucose, nucleotides as flavour boosters, amino acids.

The most important development affecting the future of the food industry is the change from selling food to selling nutrition. This has come about directly via the catering services of food companies or via the marketing of complete meals. Indirectly, nutrition is being stressed via nutrition labelling and by particular attention to avoid nutrient losses in heat treatment and in peeling, washing, blanching and similar operations. Nutrition is involved in the development of non-meat non-dairy proteins but Single Cell Protein appears to be less promising than plant sources. In general, the agricultural side (varieties, mechanical harvesting of plants, selective breeding and mechanical boning out) will be decisive factors in the profit structure of the food industry. Another important field is convenience; and this concerns the packaging industry to a large extent. The place of biotechnology in these developments would appear to be limited. Improved enzyme and fermentation technolgy certainly has a future but not on any spectacular scale. The application of genetic engineering for improving crops is dealt with in Chapter IX.

3. The potential for biotechnology in the food and allied products industry

A matrix showing the relevance of biotechnology developments for certain selected products has been compiled (see Table 3). It is elucidated in the following sections. The notes do not necessarily only apply to the situation in the Netherlands.

3.1 Meat and meat products [3]

Proteolytic enzymes are used as meat tenderisers. The ripening (acidulation) of meat products such as salami-type sausage can be accelarated by the inoculation of cultures with bacteria such as lactic acid bacteria. Both methods are old ones. No marked increase in applications need be expected in the future.

3.2 Dairy products

During the past decades scaling up has been the major characteristic of dairy farming and dairy industry developments in the Netherlands. This can best be decuced from the extent to which the number of dairy plants has decreased against the increase in milk production. The overall volume of milk delivered to creameries rose from 6,000 million kg in 1960 to 11,000 million kg in 1978 but in the same period the number of dairy farms dropped from 183,000 to 75,000, and the number of dairy plants from 499 to 173. Dairy industry sales in 1978 amounted to Dfl. 8,600 million. To this should be added the proceeds from ice cream manufacture, which were Dfl. 72 million in 1978.
The major fermented dairy products are: yoghurt, cheese, butter and butter milk. In 1978, 213, 412, 211 and 142 million kg respectively were produced. Of the cheese 249 million kg were exported and of the butter 145 million kg as butter and 40 million kg as butter oil. These volumes are equivalent to Dfl. 1,300, 840 and 157 million respectively. Overall dairy exports in 1978 amounted to Dfl. 4,250 million (information from Productschap voor Zuivel).
Biotechnological developments in the dairy industry are outlined below.

The fermentation process and bioreactors

Trends in all but a few dairy processing methods are marked by scaling up, mechanisation and automation. Research on fermented dairy products expecially is geared to maintaining and improving quality.

For yoghurt a fully continuous method has been perfected. It consists of a stirred reactor in which the milk ripens and a coagulator (plug flow fermenter) in which the yoghurt milk coagulates as ripening progresses [4, 5]. Improved control of the properties of the finished product (particularly acidity and viscosity) is expected to result.

In mechanised cheese manufacture the biggest change, which was intitiated several decades ago, is the separation of the curd-making and drainage (removal of whey) processes in cheese vats and drainage tanks respectively [6]. Although a fully continuous manufacturing process, this takes place in a moulding cylinder. Some dairy plants combine batch cheese vats (or curding tanks) with a buffer tank and several continuous moulding cylinders, the latter taking the place of the drainage tank. This development is expected to continue.

The CIP (cleaning in place) equipment widely in use and the utilisation of closed equipment makes it possible to design a process which need not be interrupted so often, because contamination by undesirable micro-organisms (bacteria and phages) can more easily be prevented.
In the Netherlands the process by which butter is made from ripened cream has been largely replaced by a process whereby special starters are worked into the mixture along with the starter permeate which regulates acidity [7]. This method has made for better control of butter properties without there being any deterioration in flavour. This method is also being employed in Germany, France and other European countries.

The equipment used in making cultured butter milk from skim milk will soon be subject to marked changes. The flavour is stabilised by a special method of aeration and de-aeration.

An important feature in the manufacture of fermented dairy products is the use of starter bacteria in concentrated form. They are being used for the daily inoculation of the bulk starter milk. There are instances of the milk being inoculated with concentrate direct, and despite higher costs this technique will spread. Improved control of acid production and of the properties of the finished product are among the great advantages of starter concentrates.

Transfer of genetic material [8].

In dairy research, efforts have been made to obtain suitable lactic acid bacteria cultures by selection and mixing. It has been found that many properties of importance to the dairy industry are due to plasmids (formation of proteases, fermentation of lactose, citric acid, resistance to bacteriophages, etc.). The transfer of genetic material by transduction and conjugation is possible in the case of lactic acid bacteria. The transfer of pure DNA has been demonstrated in these bacteria (transformation) as has the fusing of protoplasts. The transfer of genes in vector DNA (plasmid or bacteriophage) with the aid of enzymes and the subsequent transfer to lactic acid bacteria so that the new DNA is also replicated is something to speculate on. The lack of sufficient knowledge of the genetic pattern of these bacteria consitutes an obstacle. The introduction of genes for the production of rennin in lactic acid bacteria would be a great step forward for the dairy industry; and there are probably many more examples.

Enzyme technology [9, 10, 11].

Rennet obtained from the stomachs of calves is used in cheese manufacture. This is certainly the most important application for enzymes in the dairy industry. Owing to the scarcity of calves' stomachs in the world market, rennets of microbial origin have been produced in the past 20 years. In some countries (e.g. USA and France) these cheaper rennets have partially or wholly taken the place of calf rennet. As Dutch cheese of comparable quality cannot be manufactured with microbial rennets they are not permitted in the Netherlands

[12, 13, 14]. The use of equal parts of animal rennet and pepsin from pigs, and bovine pepsin [15, 16] present better prospects. There has been extensive research to see whether rennet can be immobilised and used in that way. Only partial success has been achieved.

Beta-galactosidase has great potential. The sweetening capacity of the mixture of glucose obtained from lactose with galactose is greater than that of lactose alone. The enzyme can be immobilised in many ways and is already being used in this form. However, large-scale commercialisation is a slow process.

Lipases and proteases other than rennet are only used on a small scale in cheese manufacture. Certain enzymes can be used to accelerate the ripening process. The modification of proteins with the aid of proteases is also of importance. The use of superoxide dismutase to prevent oxidation defects and of sulphydriloxidase to eliminate the boiled flavour of milk is still at the research stage. This off-flavour occurs in ultra-high temperature short-time sterilisation of milk, the UHT process. Aseptically packed UHT milk has become an important consumer good in some European countries (Germany, Italy). Although the use of glucose oxidase combined with peroxidase to improve the bacterial keeping properties has been patented, the method has not been applied.

Immobilisation of biosystems

The immobilisation of lactic acid bacteria to enable flavour products (e.g. diacetyl) to form in milk has already been described in the literature. The manufacturers of dairy products are certain to attempt to use these immobilised multi-enzymes in sub-processes in the near future.

3.3 Fish products [3].

In the past efforts have been made to convert fish waste to protein for use in foodstuffs. Proteolytic enzymes were used. A drawback was that bitter peptides were formed. Moreover, the process was so costly that it was not possible to compete with soya or milk protein. The decreased availability of fish has meant that interest in the process has ceased.

3.4 Flour, hulled barley, rice

The rising capacity of flour can be improved by the use of different enzymes during production. No drastic developments are expected [17].

3.5 Sugar

In the sugar industry invertase is used for making invert sugar. There is interest in improving the sweetening capacity by converting glucose to fructose with the aid of the enzyme glucose-isomerase.

3.6 Oils and fats

Edible oils are of vegetable and animal origin (35.1 and 8.8 million tonnes respectively were produced in 1976). The fatty acids composition of some of these products in 1976 was as follows [18].

	10^6 tonnes/ yr in 1976	palmitic acid	stearic acid	linoleic acid	linolenic acid
soya oil	10.0	11.0	4.5	22.0	53.0
sunflower oil	4.1	10.5	3.0	50.0	30.0
groundnut oil	4.5	6.5	3.5	23.0	64.5
palm oil	3.3	43.0	5.0	40.0	10.0
animal fats	8.8	28.5	20.0	39.0	3.0

Since the First World War it has been known that certain micro-organisms are able to make triglyceride as a reserve material and to store it intracellularly. Some of them (e.g. *Lipomyces*, *Rhodoturula*, *Aspergillus*, *Penicillium* and *Pythium*) have a useful fatty acid composition. Although it is not yet economically viable to produce fatty acids in this way from waste products like vinasse and whey, the position may change in the future if the demand for fatty acids in the food industry increases owing to the higher cost or even exhaustion of certain petrochemical compounds. Such a development could be greatly expedited if the composition and yield of fatty acids could be further improved by better fermentation methods, conventional methods or genetic engineering.

It has been calculated that the present cost of microbial oils would be between Dfl. 2,900 and 3,600 per tonne. Not until methods of growth and/or strains are improved and more particularly the carbohydrate substrate (e.g. enzymatic or acid hydrolysed cellulose) becomes cheaper could the cost drop to Dfl. 2,000. This figure is an interesting economic proposition.

The use of enzymes (lipases and phospholipases) for modifying lipid-like substrates will also gain significance in the future [17], especially once attempts to produce such reactions in non-aqueous environments succeed. Phospholipids will also play a more important part in the modification of lipoproteins to improve their emulsifying capacity.

3.7 Fruit and vegetables

No developments are expected in this sector for the time being.

3.8 Bakery products and pasta

Oxidants, emulsifiers and enzymes are used in the production of pasta, bread and other bakery products. The generally make for a better, more constant

finished product. Most additives are used to influence the two major reactions which take place during processing: the conversion of high molecular proteins to smaller units by reducing the disulphide bonds, and the degrading of high molecular polymers containing pentoses.
There is great interest in boosting the action of the customary additives or even completely replacing them with specific enzymes such as reductases, glucanases and xylanases.

3.9 Cocoa, chocolate and sugar confectionery

Cocoa fermentation. When the cocoa fruit has been opened the beans and the pulp are thrown on a pile or into crates. Fermentation means microbial decomposition of the pulp, a fairly well-understood process. The heat, the changing anaerobic and aerobic conditions, and the destruction of the bean at 50° C initiate chemical and enzymatic processes which form flavour precursors, which on roasting of the bean are converted to flavour compounds. Although fermentation is extremely important to the quality of the cocoa no new developments are expected.
Invertase. Invertase is used in the manufacture of chocolates to prevent the formation of bloom.

3.10 Starch and starch derivatives [19, 20, 21, 22].

Some 70% of the world's starch is employed in products that are used in the food industry. Three categories of product are involved: glucose products (glucose syrups, dextrose), isoglucose (= isomerose = fructose syrups = high-fructose glucose syrup) and modified starches (which have retained their starch properties). Most glucose products and isoglucose are manufactured with the aid of biotechnological processes. Modified starch products are made in physical and/or chemical processes.
Table 4 gives an idea of the great and growing significance of the manufacture of starch derivatives with the aid of biotechnology.

Table 4. Starch and starch derivatives manufactured with the aid of biotechnology (as dry matter, estimated figures).

World production	1970 t x million	1980 t x million	1985 t x million
Starch	10	14	17
Glucose syrup+dextrose	6	7	8
Isoglucose	0	2.6	4
Modified starch for food manufacture	1	1.5	1.7
West European production			
Starch	2.0	2.7	3.2
Glucose syrup+dextrose	0.8	1.2	1.3

Isoglucose	0.0	0.3	0.4
Modified starch for food manufacture	0.3	0.35	0.4

Glucose products (glucose syrups; dextrose)

Until 1960 most glucose products were made by hydrolysing starch with the aid of acids. Since then this method has largely been replaced by enzymatic methods (using amylases).
In addition to their sweetening properties glucose syrups have a number of functional characteristics such as binding capacity, viscosity, moisture control, softening, hygroscopicity, gloss-giving, browning, colour stabilising, flavour boosting, crystallisation control of saccharose, and preserving. These properties can be varied by modification of the composition (glucose, maltose, maltotriose, maltotetraose, etc.) of the glucose syrup. The principal applications for these syrups are in sugar confectionery, bakery products, dairy products, soft drinks, meat products, canned fruit, sauces, desserts, baby foods and ice cream.

The consumption of glucose products may be expected to increase. The following are some of the determing factors.
— Glucose products (glucose syrups in particular) are cheaper than saccharose.
— Obstacles to the utilisation of glucose products currently presented by food regulations will disappear.
— The composition of glucose syrups can be widely varied. The functional properties can therefore be adapted to the required application.
— In some countries a trend towards less sweet foods is detectable. If the sweetening capacity of saccharose is rated at 100, that of dextrose is 70 and that of glucose syrup 30-60 (measured as a 1 : 10 aqueous solution).

Isoglucose

If the sweetening capacity of saccharose is indexed at 100 that of glucose is 72 and that of fructose 114 (all in 1 : 10 aqueous solutions). The isomerisation of glucose to fructose will thus make for a great increase in the sweetening capacity. The sweetening power of equal parts of glucose and fructose is almost the same as that of saccharose.
Since 1940 the starch industry has given much thought to the chemical (alkaline) isomerisation of glucose to fructose. No successful commercial process has hitherto resulted, largely due to the low isomerisation yield and the occurrence of a large quantity of undesirable colouring matter, minerals and by-products.
The enzyme glucose-isomerase, which acts as a catalyst in the partial isomerisation of glucose into fructose, was discovered in 1957. The isomerisation balance is attained at 55% fructose. In practice the process is arrested at 42% fructose because the reaction then slows down too much. The cost of

manufacturing glucose-isomerase is high so it will have to be used in an immobilised form if commercial use is to be worthwhile.

The basic material for the incorporation of glucose-isomerase is a starch hydrolysate with a very high glucose content, e.g. 95% dry matter (the remainder consists of maltose and higher sugars). This starch hydrolysate is obtained by using the enzyme alpha-amylase to convert the starch to a low viscosity syrup with a low glucose content. The resulting hydrolysate is treated with the enzyme amyloglucosidase until the desired glucose content has been attained (enzymatic juice).
The enzymatic juice can be isomerised by a continuous process in a reaction column. The isomerised product is then concentrated to a syrup containing 70% dry matter. This syrup is called isoglucose, isomerose, fructose syrup or high-fructose glucose syrup (high-fructose corn syrup (=HFCS)). Isoglucose contains about 42% fructose, 52% glucose and 6% higher sugars (all calculated on dry matter).
In the meantime a second generation of fructose syrups is being produced. They have a much higher fructose content (55-95%). This increase is brought about by passing isoglucose over ion exchangers, enabling a 90% fructose content to be attained. By mixing syrup containing 90% fructose with syrup containing 42% fructose one obtains syrups containing 55% and 70% fructose. The main applications for fructose syrups are in soft drinks, jams and canned foods.

Since 1970 isoglucose has undergone tremendous development in several countries, particularly the United States and Japan. Estimated consumption in these two countries and in the EEC is given in the table below.

Table 5. Carbohydrate sweetener consumption in the United States, the EEC and Japan (in millions of tonnes; all figures relate to dry matter)

	1970	1975	1980 (estimates)	1985 (estimates)
United States				
saccharose	10.1	9.3	10.0	10.4
isoglucose (HFCS)	0.0	0.5	1.8	2.4
glucose syrup+dextrose	1.7	2.3	2.1	2.2
EEC				
saccharose	10.5	10.0	11.0	11.5
isoglucose (HFCS)	0.0	0.0	0.3	0.4
glucose syrup+dextrose	0.8	1.1	1.1	1.2
Japan				
saccharose	3.0	2.8	3.0	3.3
isoglucose (HFCS)	0.0	0.1	0.3	0.5
glucose syrup+dextrose	0.8	1.1	1.1	1.2

The use of isoglucose instead of saccharose (in soft drinks, jam, bakery products, etc.) will increase as one or more of the following conditions obtain:
— expensive saccharose;
— cheap starch;
— the need to import saccharose.

The country coming closest to these conditions is the United States, so this is where the greatest increase in isoglucose consumption is being experienced, as shown by Table 6.

Table 6. Per capita consumption (as kg dry matter) of carbohydrate sweeteners in the United States (estimated for 1980 and 1985)

Year	Saccharose	Isoglucose	Glucose syrup	Dextrose	Honey	Total
1960	44.3	0.0	3.7	1.5	0.9	50.0
1972	46.3	0.4	7.0	2.0	0.7	56.3
1975	40.6	2.1	8.0	2.3	0.6	53.6
1980	41.6	7.7	8.3	2.0	0.5	60.2
1985	39.7	11.6	8.5	1.9	0.5	62.2

The manufacture of isoglucose in the EEC has been restricted as the result of a quota system and levies. Even discounting these considerations, conditions for isoglucose production in the EEC are less favourable than in the USA due to a surplus of beet sugar and the higher cost of starch. Isoglucose cannot be fully substituted for saccharose because the latter has properties that isoglucose lacks. Saccharose is a non-hygroscopic solid that does not contain any reducing groups (it is alkali-stable and thermostable), whereas isoglucose is a hygroscopic syrup and does contain reducing groups.

Fructose can be used as a sweetener in low calorie foods.

Modified starch

Modified starch (cold-soluble starch, oxidised starch, starch esters, starch ethers, cross-linked starches) are made by physical and/or chemical processes. The chemical reactions can occur in aqueous suspensions at a low temperature (below 60°C). Chemical methods are relatively cheap and produce pure end products.
Despite trends under the influence of food regulations to replace chemical reactions by physical and biotechnological ones, it does not look as though the latter will play any significant part in the near future.

3.11 Animal feedstuffs

Efforts are being made to raise the digestion value of dry feed by the addition of enzymes.

In recent years there has been an increase in the use of ensilage methods. This is due to higher energy costs, which have made the drying of grass, lucerne, etc. a costly business. Grass, maize and other green feed have joined spent grains in the ensilage process, the lactic acid formed having a preserving effect.
Although biotechnology as such is not expected to have a great influence on ensilage methods, there is still a need for continuing study of the micro-biological processes that take place. It is desirable for a potentially defective silage process to be recognised at an earlier stage.

3.12 Alcohol and yeast [23, 24, 25, 26, 27]

In alcohol manufacture a distinction can be made between industrial alcohol and alcohol for human consumption. The latter necessitates suitable agricultural products as the raw material is specified in the EEC customs tariffs (e.g. grain and molasses). The micro-organism is not specified. Dutch spirits production in 1979 was 5×10^5 hectolitres (calculated as 100%). The following are ways of applying and improving biotechnological methods.

a. Improving the liquefaction and processing of raw materials with the aid of enzyme preparations.
b. More efficient conversion of sugars to alcohol. Besides the conventional micro-organism yeast, the anaerobic bacterium *Zymomonas mobilis* would seem to offer potential. This bacterium can convert glucose to alcohol. To convert other sugars to ethanol at the desired speed the genetic structure will have to be modified.
c. Selection of mutants with high sugar concentration tolerance and increased alcohol tolerance. This will reduce the energy costs involved in distillation.
d. Accelerated fermentation. This can be attained by the transfer to the organism of additional genetic information for amylolytic enzymes.
e. Continuous fermentation systems, where necessary utilising immobilised micro-organisms.

Yeast

Bread bakeries have always been the principal sector of use for bulk yeast. In recent years there has been increasing use in wine making of pure culture yeasts, which are grown in large quantities. Bulk yeast is also used as a base for certain flavour compounds. Of the yeast produced in the Benelux in 1979 34,500 tonnes were sold there.
In yeast production it is essential for as much of the substrate as possible to be converted to biomass. For optimum production glucose repression must be eliminated. In the conventional batch fermentation system the sugar content of the substrate is so high that respiration is almost completely repressed. In contrast, in the fed batch system the sugar content of the bioreactor is kept constant and low, enabling respiration to take place. In this system therefore the yeast yield is appreciably higher than in the conventional system. Research will thus have to be conducted to find ideal initial conditions. Parameters must also be given (aeration, substrate feed, alcohol concentration) for optimum

processing conditions. Research will primarily be geared to process automation.
Even where it is a matter of finding cheaper or better quality raw materials than the currently used molasses, more traditional biotechnological methods are involved. The transfer of genetic information to yeast for the efficient conversion of substrate to biomass without the properties of the yeast being affected would seem to be potentially viable in the longer term.

3.13 Beer and wine [23, 24, 25, 26, 27]

Beer production in the Netherlands in 1979 was 15.4 million hectolitres; wine production was negligible. Most of the technologies referred to below are therefore geared to brewing. Since the flavour and aroma patterns of both beer and wine are set during the fermentation process, most of the points relating to that stage of the process can be applied accordingly to wine making.
The following are potential applications for enzymes.

a. The use of immobilised or non-immobilised enzymes in the preparation of syrups which are fermented with the usual wort or separately. This will have the effect of reducing costs. Depending on the percentage of fermentable sugars in the syrup it will also enable products with differing alcohol content to be brewed.
b. The use of immobilised enzymes for quality control, eliminating undesirable flavour components (diacetyl, sulphurous compounds)
c. The use of immobilised enzymes for specific parts of the process (e.g. stabilisation).

There is potential for genetic engineering in fermentation. The modification of brewers' yeasts and wine yeasts is only acceptable if the specific aroma and flavour patterns remain unaltered. The ultimate pattern is determined by genes, so it will be a lengthy process and it is doubtful whether it will be successful. The possibilities for applying genetic engineering are:

a. Manipulation of yeast for the manufacture of new products (e.g. with high or low alcohol contents)
b. Manipulation of yeast for quality control purposes. For instance, the transfer of genetic information for the control of diacetyl, esters and hydrogen sulphide. If it proves possible to control these features, the very distant future will see a very great reduction in production times with unchanged quality.
c. Continuous fermentation will again become possible if methods are developed for the constant monitoring of substrate and fermentation product. Genetically manipulated yeasts could also play a role here.

Generally speaking, it will be possible to put much of the waste from all product groups to good use within the plant or elsewhere.

3.14 Vinegar

No new processing developments are anticipated.

3.15 Fruit juices

Modern fruit processing is inconceivable without the use of enzymes. In fact, enzyme manufacture and fruit juice (products) have developed hand in hand, and until recently several large fruit processing companies were also producing their own enzymes [28]. The main task of the enzymes is to depolymerise pectic substances and they are known commercially as pectinases [29, 30]. They are all derived from *Asperigillus* species, mainly from *Aspergillus niger*. They are manufactured by various companies in Europe, USA and Japan and are used industrially in the processes described below [30, 31, 32].

Fruit juice clarification

This is the oldest and still the principal use for pectinases. The juices in which they are mainly used are apple juice followed by pear and grape juice. Freshly pressed juices are viscous and are persistently turbid. The addition of pectinase rapidly reduces the viscosity and breaks the turbidity: cloudy particles agglomerate forming flocs which settle out. Flocculating agents are added to achieve complete flocculation. A clear juice is then obtained either by filtration or by centrifugation.

Fruit juice extraction

Fruit juices are generally extracted by pressing, but this is by no means easy with soft fruits such as black currants, strawberries and raspberries. The partial destruction of pectin by enzymatic treatment of the pulp facilitates processing and ensures high yields of juice and anthocyanin pigments. The same is true for the Concord variety of *Vitis labrusca*, which is used in the USA for the extraction of red grape juice. In wine making the fermentation of red grape juice is an attractive alternative to the traditional fermentation of the skins. In the European apple juice industry the decline in the pressing quality of apples due to variety (Golden Delicious) on storage has made the enzymatic treatment of pulp a necessity.

Maceration

In enzymatic maceration, suspensions of loose cells are produced from fruit and vegetable tissues. The process may be applied in the manufacture of fruit nectars. These are pulpy drinks prepared from a variety of fruits by milling the fruit, adding water, acids and sugar, homogenisation and pasteurisation. Enzymes reduce the viscosity, making it possible to make concentrated nectar bases. The process is also recommended for the production of finely dispersed constituents for baby foods made with carrots and other vegetables.

Application in citrus technology

In the citrus industry pectinases are mainly used to upgrade by-products. The coarse pulp which is screened from freshly pressed citrus juice contains

considerable amounts of juice and soluble solids. These are recovered in a counter-current washing operation in which pectinases are used to obtain 'pulp wash' with a maximum of soluble solids and lowest possible viscosity so that it may be concentrated. The peel and rag left behind after juice extraction contain large amounts of substances which can give beverages the desirable cloudiness. There is a great demand in the soft drink industry for cloudy products. Highly turbid, low viscosity preparations suitable for concentration can be obtained by thermomechanical comminution, followed by pectinase treatment. Pectinases are also useful in the isolation of essential oils and carotenoid pigments from citrus peel.
For these industrial applications a pectinase production worth $2.7 million (US only, 1980) is estimated.
Future developments on the brink of industrial utilisation are the liquefaction of fruit and vegetables and the enzymatic debittering of citrus products.

Liquefaction of fruit and vegetables

The previously mentioned maceration process can be carried through to almost complete liquefaction of fruit and vegetables if cellulase is used in conjunction with pectic enzymes. In this process the cell walls are largely digested so that 90-98% juice is obtained. The process is suitable for products for which there is no juice extraction equipment (tropical fruits) and considerably increases yields in the manufacture of traditional fruit juices.

Enzymatic debittering of citrus products [32].

Products from grapefruit and navel oranges may have an unpleasantly bitter taste due to two chemically distinct bitter compounds: flavonoids and limonoids. The main bitter citrus flavonoid is naringin, which may occur in large amounts in grapefruit, especially in the peel. The enzymatic hydrolysis of naringin as a means of debittering grapefruit products is possible. Limonoid bitterness is associated with navel oranges and with grapefruit. Suitable enzymes for its elimination are still being sought [32].
So far pectinases are not being sold as immobilised products but performance studies have been published [33, 34].

3.16 Edible fungi

Mushrooms are grown and marketed on a large scale in the Netherlands. There is a lot of waste, consisting mainly of cellulose. When mushrooms are picked part is thrown away. Research here has proved that an edible product can be made from this mushroom waste. Besides mushrooms there are other edible fungi on the market. In Europe most grow wild. Japan is one of the few countries where numerous varieties of edible fungi are grown commercially. In the early 1970s the Dutch TNO's Domestication of Edible Fungus Department attempted to grow new varieties of fungus. The result of their work were presented in a paper entitled 'Possibilities for the cultivation of the *Boletus* species *in vitro*' at

the Fourth International Fermentation Symposium held in Japan in 1972.
Research into the use and enriching of cellulose waste with edible fungus has also been conducted. Several species that would grow on chaff have been found. Production on a larger scale is being considered.

3.17 Proteins, peptides and amino acids [35, 36, 37,38, 39, 40].

Proteins

The production of Single Cell Protein (SCP) was studied widely in the 1970s and in some cases put into practice. SCP can be made from petrochemical materials (methanol, paraffins, etc.) agricultural products (sugar, glucose, etc.) or from waste materials (straw, cellulose, effluent from paper mills, etc.).
The quality of SCP is usually adequate for animal feed and possibly for human consumption, although there have been doubts about the latter in Italy and Japan. In Italy this has caused a factory to be closed down.
The cost of SCP is made up of the cost of the raw material and the cost of processing. The former is variable and amounts to over Dfl. 0.50 per kg. Processing costs (some of which are fixed and some variable) are between Dfl. 0.50 and 2.00 per kg, depending on the process, the scale of production, location, etc. Taking yield into account the cost of SCP is between Dfl. 1.50 and 4.00 per kg. SCP has to compete with soya protein and other sources of protein used in animal feed. Since these other sources cost under Dfl. 1.00, SCP is still too expensive.
The future does not look particularly rosy, owing to the high rise in the cost of energy. Although ICI have succeeded in influencing the metabolism of ammonia by genetic means, the result has only been a 5% increase in the yield on raw materials. SCP is therefore not expected to make a breakthrough. The production of SCP protein from waste may become worthwhile when the alternative waste treatment process becomes more expensive.

Protein hydrolysates and peptides

The use of protein hydrolysate in food is fairly important because of its flavour. The hydrolysis process is a special one in which minor proteins (peptides) can be formed from vegetable material or yeast in combination with other components. Certain peptides and derivatives have a specific flavour; aspartame, for instance, is developed as a sweetener.
It may be expected that, in addition to the chemical methods of synthesising these peptides, enzymatic means will also be found.

Amino acids

Traditionally, the manufacture of amino acids is dependent on the production of hydrolysed protein, from which they are obtained. For some time now methods entailing fermentation processes have been employed to produce a large number of amino acids, largely from sugars. There are also chemical

synthesis methods for certain amino acids and combinations of chemical and enzymatic synthesis steps.

On the whole, it may be said that for very small quantities and at high cost per kg extraction is still used, but the major amino acids are made by fermentation (L-glutamic acid, L-lysine). The production of glutamic acid and lysine making use of *Corynebacterium glutamicum* has been going on for years and amounts to 270,000 and approximately 30,000 tonnes annually for the respective products. Synthetic methionine is produced on a scale of approximately 100,000 tonnes a year. Some amino acids can be made via combined synthetic and enzymatic reactions (among them L-phenylalanine, D-phenylglycine, L-lysine and L-tryptophan).
Improvement is expected in the economics of production methods involving fermentation with the aid of recombinant DNA techniques, as recently evidenced by the 500% increase in prolase production.

3.18 Coffee

Fermentation is a part of the wet or West Indian method of processing the green fruit. The washed fruit is pulped. This operation removes some of the jelly-like material surrounding the bean. The remainder decomposes due to bacteria (i.e. fermentation) within one or two days and can then be washed away.
The fermentation process has been well studied from both the microbiological and the enzymological angles. As the process has very little influence on the quality of the bean, no major developments are expected.

3.19 Tea

When the leaves of the tea bush have been picked they are first allowed to wither. Mechanical operations (rolling, crushing) break up most of the cell structure. For several hours ambient oxygen and the enzymatic systems in the cell moisture (e.g. polyphenoloxidase) act on the crushed leaf. The reactions occurring during this fermentation have been studied fairly widely (in the USSR and elsewhere). They make an important contribution to the formation of the flavour and flavour precursors which are converted to the actual tea flavour during the ultimate firing process.
Although improvements to quality by better control of the enzymatic reactions and the use of exogenic enzymes cannot be ruled out in theory, no major developments are expected in the foreseeable future owing to the complexity of the processes, the structure of the market and the infrastructure of the producing countries.

3.20 Tobacco [41, 42].

As a result of the action of tobacco enzymes in the conventional tobacco fermentation process, components are formed which largely contribute to the quality, taste and aroma of the product. As a complex of reactions involving

different enzyme systems are concerned, no major developments are expected in the near future.
In some modern processes the tobacco is fermented in suspension directly after harvesting and then, part of the protein having been separated off (for use as animal feed), processed as homogenised tobacco leaf. More technological processing and utilisation of specific enzymatic processes, e.g. to reduce the physiologically active or harmful components, are among potential developments.

3.21 Colouring matter

The production of natural colours with the aid of micro-organisms has been widely studied but is not an attractive proposition at the moment. This is largely due to the fact that the yield is low. It takes six days to produce 1.4 grams of beta-carotene per litre using *Mucor hiemalis*. If it were to be possible to increase the yield of natural colours produced by micro-organisms by mutation or recombinant DNA methods, it should be possible to produce them by fermentation.

3.22 Flavours and aromas

Amino acids and proteins can be used to flavour foods (section 3.17). There are several other materials that are important for flavour and aroma. Lactones, methylketones, aldehydes, alcohols and fatty acids are important for giving dairy products this aroma. In flavours such as meat, chicken and fish these compounds are also important, although their ratios vary greatly.

Hardly anything is known about the biosynthesis of lactones in plants and animals, although certain materials, such as coconut oil, have a high lactone content. Genetic changes in the plants and/or the tissue cultures from which they are derived may lead to increased production.
Methyl ketones are derivatives of fatty acids and are produced with the aid of certain fungi. Conversion takes place during the ripening of cheese and of some varieties of sausage. The conversion of fatty acids with the aid of immobilised fungi spores may result in ideal conversion.
Long-chain aldehydes and alcohols are also products of fatty acid metabolism. These materials can be formed by enzymes (alhydrogenase and alcohol dehydrogenase in the presence of cofactors).
Immobilised enzyme systems will have to be developed and the problem of protecting the cofactor against undesirable oxidation will have to be solved if these kinds of flavour and aroma are to be made by biotechnological means.
The oxidation products of amino acids are also important aroma materials. A method for converting amino acids using immobilised amino acid oxidase has already been described.

3.23 Thickeners and stabilisers

At present there are about thirty hydrocolloids being used as stabilisers and/or thickeners and/or gelling agents in the food industry. Of these the only one derived from an animal source is gelatine. Others are derived from higher land plants (tree exudates, vegetable gums, pectins, starch and cellulose) or from seaweeds. They may also be of microbial origin. A special case is the manufacture of cultured dairy products, e.g. stirred yoghurt by means of selected cultures of lactic acid bacteria, which produce capsular polysaccharides. Together with the coagulated milk proteins these bacteria form a structure in milk which gives the desired viscosity after stirring.

Another product of microbial origin is xanthan gum, produced by the bacterium *Xanthomonas campestrix*, which was admitted for food uses by the US Food and Drugs Administration in 1969 and has since also found its way into the legislation of most European countries. Production for food uses does not amount to more than a few thousand tonnes and is confined to one US company. The production facilities of one French and one English company are expected to be on stream in the early 1980s. Xanthan posesses a unique combination of rheological, synergistic and stabilising properties which make it eminently suitable as a thickening, binding and suspending agent in all kinds of foods. It is produced by submerged aerobic batch fermentation in a medium of carbohydrates and certain other nutrients. The gum was discovered in a screening programme of the microbial culture collection of the Northern Utilization Research and Development Division of the United States, which was carried out in the 1950s to detect useful biopolymers. Its use in a crude form in oil drilling allowed Delco to develop a food grade product and to carry out the necessary safety evaluation programme which has made xanthan gum one of the most extensively investigated polysaccharides.
Although the production of microbial gums would seem ideal for gum manufacturers in view of the possibility of controlling impurities and of being independent of crop and trade conditions in other parts of the world, the high cost of obtaining international food use approval makes it unlikely that new microbial gums will appear in the food industry.

3.24 Antimicrobial materials

Many natural systems protect themselves against micro-organisms. Micro-organisms themselves use specific substances such as bacteriocines, killing factors and antibiotics. Higher organisms also have such protective systems. The best known are the lysozyme/avidine system in egg and the lactoperoxidase/thiocyanate/H_2O_2 system in milk. In the sperm of mammals, too, there are enzymes which effectively kill off micro-organisms. Little use is as yet being made of enzymes of this type in the preservation of food. However, in view of the trend towards natural products which do not contain preservatives and have not been sterilised to death, it would seem desirable to examine natural antimicrobial substances more closely. Studies will have to be based on the

specific structures in micro-organisms which do not occur in higher organisms. Both the cell walls of bacteria and the skin of bacterial spores contain these unique structures. Combinations of D-aminopeptidase, keratinase and lysozyme-like enzymes would appear to offer good prospects. Although initially thought should be given to using this type of hydrolytic enzyme in end products, there is a possibility of using them for the partial decontamination of additives in the production of foodstuffs. Partial decontamination could be followed by milder physical decontamination (which consumes less energy).

3.25 Vitamins and antioxidants

Vitamins are added to foods to provide consumers with an adequate quantity in their diet. Some vitamins act as antioxidants.
In the past vitamins have always been produced by extraction from natural materials. Nowadays most vitamins are obtained by fermentation and/or synthesis. For instance, multi-stage syntheses are used for vitamins A and E on the basis of chemicals such as acetylene. Vitamin C is synthesised from glucose by a combination of fermentation and chemical synthesis processes. In some of these relatively big products new syntheses in combination with enzymatic processes may be expected. Existing antioxidants based on chemical compounds with an aromatic structure are under some discussion due to investigations into their toxicological properties.

4. Aspects affecting the development of biotechnology in the food industry

The success of new biotechnological processes partly depends on the utilisation of a suitable biological system and the equipment used. It is of great importance for process plant manufacturers to meet the requirements of industry. Major factors include reactor design, heat transfer, mixing, sealing and ease of cleaning and disinfection. They must enable users to have the greatest possible control over processes. It is necessary to develop control systems with newly designed sensors enabling deviations to be recognised and rectified at an early stage. If biotechnology is to be raised to higher levels, attention will have to be paid to the industries supplying materials and equipment. The Netherlands has several medium sized firms qualifying for further development.

When a food manufacturing process is modified there is a risk that the flavour will change. If a product is already widely accepted, the manufacturer will have to be very wary of making drastic changes in the production process. Changes in existing products can only be introduced in gradual stages. It is a different matter with newly developed products. Here the economies of the process can be weighed against the cost of marketing. However, in both cases extensive sensory testing will be necessary if acceptance among a wide spectrum of consumers is to be investigated.

Generally speaking, changes to food manufacturing methods can only be made slowly. If basic materials and additives produced by biotechnological means are used (e.g. microbially produced colouring matter), compliance with official regulations will have to be borne in mind. In most cases lengthy toxicity tests will have to be conducted before a particular product is cleared. This may mean that five years are lost before a product can be put to use. The high cost of toxicity testing may be an obstacle to early commercial production. Although there is a possibility of shorter toxicity tests in the near future, manufacturers may still expect to spend a long time on toxicity research in order to clear substances for use in foodstuffs.
Social and economic aspects can have a great influence on the application of biotechnology in the food industry. Consumers may be put off by the knowledge that a certain food has been produced with genetically manipulated micro-organisms. The wish for more natural products can be met by replacing chemical preservatives with natural preserving systems. Such systems are also potential energy savers in both the factory and the home. Less energy may be necessary for sterilisation or pasteurisation and for quick freezing. If new continuous production methods are introduced, considerable opposition on the part of operatives to consequent round-the-clock working may be expected.
In view of all these factors:
— the need to assist the supplying industries in raising the standard of the products they supply,
— the risk of changing sensory characteristics,
— the possible need for lengthy toxicity testing to meet official requirements,
— various social and economic factors which may hinder the application of certain biotechnological processes,
the development of new technologies in the food industry will be slow. There will be heavy demands on risk-bearing capital, so a government which aims to promote biotechnology may have to make financing easier for industry.

Finally, there is the problem of education. Biotechnology covers a very wide area, which in fact consists of several sub-areas that are related to a greater or lesser degree. Consequently, the knowledge that has to be conveyed can never be provided by one person. Biotechnology can therefore best be developed if it is possible to combine several biotechnology subject areas. At a gathering of Dutch university professors these areas were defined as being covered by a minimum package consisting of:
— separation methods, processing methods, chemical reactors, chemical kinetics, enzyme kinetics, cell physiology, general microbiology and bacterial genetics.
According to the report compiled by these professors, this minimum package can be provided at Wageningen University of Agriculture, Delft University of Technology, and the University of Groningen. In the new two-phase structure of Scientific Education this course would cover the first four years. The University of Agriculture and Delft University of Technology have applied to continue biotechnology in the second phase. That is where the *centres*

d'excellence for biotechnology, with a high degree of interchangeability of professors and students, will have to develop.

5. Summary and conclusions

Man has been making foods and similar products for 5,000 years. It will probably be necessary to go back 2,000 years to find the first production on a commercial scale, while technology was only introduced 200 years ago. For 100 years we have been able, thanks to Louis Pasteur, to talk of biotechnology and its applications in the food industry. The food and allied products industry's current contribution to industry's overall sales in the Netherlands is about 25%. The share of biotechnological processes is quite high (15-20%).
Development of biotechnology in the food industry will probably be important in meeting the needs of an ever-growing world population.

There are basically four groups interested in the development and applications of biotechnological processes: the manufacturer, the supplier of equipment and raw materials, the consumer and the government.
Processes are available to manufacturers whereby new products can be developed on the one hand, and on the other hand quality can be improved or costs can be reduced while quality is maintained. In this context, maintaining quality is defined as assuring the shelf life and sensory characteristics of a product.

The development of new processes for making certain additives is anticipated. Such products are often made to specification so that other processes which are cheaper or safer or in some other way better can be introduced. The manufacture of foods using new processes is more complex, however. Here we are concerned with specific sensory properties which may suffer a minor upset if a different process is employed. Nevertheless, there is sufficient work in progress to make one anticipate changes.

The following developments in the manufacture of food and allied products are considered important:
— further improvements to and control of existing biological processes;
— the use of enzymes, immobilised or otherwise, in certain processes;
— enzymatic and sophisticated methods of analysis for biological parameters,
— the use of immobilised whole cells;
— genetic manipulation of the characteristics of the micro-organisms used or to be used (particularly in the production of foods in which the sensory characteristics are not of predominant interest and in which micro-organisms are already being utilised).

Factors which may retard developments are:
— Lack of suitable knowledge, methods and equipment. For this reason collaboration between educational establishments themselves and between

these establishments and industry is considered necessary. The institution of courses at the University of Agriculture and at Delft with the necessary research facilities will make a positive contribution to acquisition of the lacking knowledge. In this respect, it is fortunate that the government is no longer against universities conducting contract research. Supplying industries will also have to gear themselves to biotechnology (with reactors and control equipment). Their development should be subsidised by the government.

— Consumer acceptance: consumers expect their food to be of unchanging quality. To some consumers the mere fact that other production methods are being used implies that the product has been changed. In some cases there will be minor changes in the sensory quality. A manufacturer will always try to avoid putting his sales at risk.

— Official regulations, more specifically the requirements for toxicology testing of new products. The authorities may also intervene on raw materials or the finished product by quota systems and levies, which may have a great influence on the market situation (as has already happened in the case of isoglucose).

The food and allied products industry will therefore move slowly but surely in developing products and processes involving biotechnology. In view of the great importance of this industry this may be expected to be linked with appreciable financial burdens.

Members of the working group

The working group which produced Chapter IV consisted of the following members:

Dr G. Bakker	DSM, Heerlen
P. van Eerde	Heineken, Zoeterwoude
W.J. Klopper	TNO Central Food Research Laboratory, Zeist
Prof. W. Pilnik	University of Agriculture, Wageningen
Dr J. Stadhouders	Netherlands Institute for Dairy Research, Ede
Dr C.T. Verrips	Unilever Research Laboratories, Vlaardingen
J.H.F. van Apeldoorn (secretary)	Netherlands Study Centre for Technology Trends, The Hague

Guest contributors:

Dr J. van der Lijn	Douwe Egberts, Utrecht
P. Woldendorp	Avebe, Veendam

References

1. CBS Statistisch Zakboek (1980)
2. Voedingsmiddelenjaarboek 1979 - 1980 VAB. P.C. Noordevliet BV Zeist
3. V. Mohr, Enzyme Technology in the Meat and Fish Industries, Proces by Chemistry, Aug/Sept 1980, p.18
4. F.M. Driessen, J. Ubbels, J. Stadhouders, Continuous manufacture of yoghurt. Optimal conditions in kinetics of the fermentation process. Biotechn. and Bio Eng. *19* (1977) 821.
5. F.M. Driessen, J. Ubbels, J. Stadhouders, Continuous manufacture of yoghurt. Procedure and apparatus for continuous coagulation. Biotechn. and Bio Eng. *19* (1977) 841.
6. H. Lolkema, J. Blaauw, kaasbereiding. Landelijke Stichting Beroepsopleiding Levensmiddelenindustrie, Apeldoorn.
7. H.A. Veringa, G. van den Berg, J. Stadhouders, An alternative method for the production of cultured butter. Milchwissenschaft *31* (1976) 658.
8. F.L. Davies, M.J. Gasson, Genetic modification of dairy cultures F. Doc 83, 1980 FIL/IDF, Brussels.
9. M.L. Green, Immobilized enzymes in milk treatment, Milk Industry *82* (1980) 25.
10. P.F. Fox, Enzymes other than rennet in dairy technology, J. of Soc. Dairy Techn. *33* (1980) 119.
11. S.G. Coton, Subject, Whey Technology, The utilization of permeats from the ultrafiltration of whey and skim milk. J. of Dairy Techn. *33* (1980) 89.
12. P. Zwaginga, P.J. Alderlieste, T. Robbertsen, Production of factory made cheese with rennet of microbioal orgin, NIZO Ede R74 (1969).
13. J.H. Nelsen, Symposium: Application of enzyme technology to dairy manufacturing. Impact of new milk clotting enzymes on cheese technology. J. Dairy Sci. *58* (1975) 1739.
14. M. Sternberg, Microbial rennets Adv. Appl. Microbiology *20* (1976) 135.
15. P. Zwaginga, P.J. Alderlieste, T. Robbertsen, Production of factory made cheese with a mixture of pepsin and calf rennet, NIZO Ede R77 (1969).
16. G. van den Berg, E. de Vries, Bovine rennet for the manufacture of Gouda cheese, NIZO Ede R103 (1976).
17. M.J. Taylor, T. Richardson, Advances in Applied Microbiology *25* (1979) 7-35.
18. A.H. Rose, Economic Microbiology II. Academic Press London (1978).
19. R.V. MacAllister, Nutritive sweeteners made from starch. In: Advances in carbohydrate chemistry and biochemistry, *36* (1979) 15-56.
20. F.S. Kaper, Voedingsmiddelentechnologie *12* nr. 5, (1979) 18-25.
21. C. Bucke, Recent developments in production and use of glucose and fructose syrups. In: C.A.M. Hough, K.J. Parker and A.J. Vlitos, Developments in sweeteners - 1 London (1979) 43-68.
22. R.E. Gramera, Die Stärke *30* nr. 1 (1978) 20-23.
23. M.L. Skotnicki, D.E. Tribe, P.L. Rogers, Applied and Environmental Microbiology *40* (1980) 7-12.
24. F.B. Kolot, Process Biochemistry, Oct./Nov. 1980.

25. W. Wöhrer, W. Hampel, M. Röhr, Abstracts VI Int. Fermentation Symposium 1980, London, Ontario.
26. K.G. Gupta, A.K. Jain, S. Dhawan, Biotechn. and Bio Eng. *21* (1979) 649-657.
27. W. Hartmeier, Biotechnology Letters *1* (1979) 5.
28. V.L.S. Charley, Some advances in food processing using pectic and other enzymes. Chemistry and Industry *20* (1969) 635-641.
29. F.M. Rombouts, W. Pilnik, Pectic Enzymes. In: A.H. Rose, ed., Economic Microbiology, Microbial Enzymes and Bioconversions, Academic Press *5* (1980) 227-282.
30. W. Pilnik, F.M. Rombouts, Pectic Enzymes. In: J.M.V. Blanshard and J.R. Mitchell, eds, Polysaccharides in Food, Butterworths (1979) 109-126.
31. F.M. Rombouts, W. Pilnik, Enzymes in Fruit and Bevetable Juice Technology. Process Biochemistry *13* nr. 8 (1978) 9-13.
32. F.M. Rombouts, W. Pilnik, Enzymes in the fruit juice industry. Proc. Intern. Microbiology and Food Industry Congress, October 7-12, 1979, Paris.
33. L.S. Young, Preparation, characterization and performance of an immobilized multi-pectic enzyme system. Ph.D. Thesis, Cornell University, USA (1976).
34. W.H. Hanisch, P.A.D. Rickard, S. Nyo, Poly (methoxygalacturonide) Lyase Immobilized via Titanium onto Solid Supports. Biotechn. and Bio-Eng. *20* (1978) 95-106.
35. E.J. Vandamme, L. de Mey, De produktie van 'single-cell protein' - fabels en feiten. Het ingenieursblad *4* (1980) 37-47.
36. Eiwit-symposium, voedingsmiddelentechnologie *10* 41 (1977) 17-31.
37. John E. Kinsella, Functional properties in novel proteins, some methods for improvement. Chemistry and Industry March 5 (1977). 177, 182.
38. Ajinomoto achieves bacterial amino-acid process breakthrough. European Chemical News, December 8 (1980) 27.
39. DNA technology tagged as incentive for US Firms in amino acids output. Chemical Marketing Reporter. November 24, 1980.
40. New amino-acid technology, European Chemical News, October 13 (1980) 30.
41. D.W. de Jong et. al, Homogenised Leaf Curing, Bright Tobacco. Beitrage Tabak Forschung *8* no. 2 (1975) 93-101.
42. T.C. Tso, Tobacco as potential food source and smoke material. Beitrage Tabak Forchung *9* no. 2 (1977) 63-66.

V. Pharmaceuticals

1. Introduction

In this chapter an attempt will be made to analyse how biotechnology in general and recent developments in the technique of genetic engineering in particular can contribute to the production of pharmaceuticals for medical purposes. We can safely assume that there is a need for specific pharmaceuticals based on natural action. These will be substances, of course, which are produced from natural sources and consequently responsive to a biotechnological approach. A list of major medical needs which can be met by this approach will be discussed in detail in section V.3.
A good example of biotechnological production of natural drugs is the production of antibiotics from micro-organisms by fermentation [1]. The fact that there are more than 50,000 different types of known micro-organisms and for instance, more than 3,000 different antibiotics have been isolated from cultures from one single group of micro-organisms (the actinomycetes, consisting of about 600 species) illustrates the enormous potential of the fermentation technique. There are of course other natural products of micro-organisms besides antibiotics which are of major importance to the pharmaceutical industry. It goes without saying that in order to make full use of this potential, extensive screening programmes will be needed to isolate effectively those micro-organisms which can make new products with a defined action. Even though programmes such as this are expensive and time-consuming, it is still lucrative, as is evident from the successes achieved in Japan, for instance. Yet, in this area, we cannot expect genetic engineering to provide any new major possibilities. What we can do is increase the production of well-known substances by introducing various copies of a given gene into the micro-organism. This would enable us to manufacture more products per micro-organism. The question here is whether or not this will also be economically attractive.

The situation is very different in the case of natural products which can only be derived from human or animal material. The limiting factor is often the availability of these materials and the amount of product which can be isolated from them. Cell or tissue culture techniques are worth considering here, since the culture of cell types which are difficult to grow has met with more and more success in the past few years (due to the use of specific growth factors). These kinds of cells have the greatest potential for application because in many cases they make specific products. Yet the use of cell cultures in biotechnological processes raises many questions. The amount of product which is made is often

so negligible that cells have to be cultured in great quantities. Mass cultures, if and when possible, remain very expensive and time-consuming. The contribution of cell culture to biotechnology is slight, chiefly due to the high expense involved. Yet we must not rule out the possibility that when recombinant DNA technology becomes generally applicable in animal and human cells, the role of the cultured plant cell and mammalian cell in biotechnology will increase considerably. Cell culture will also be able to contribute in the transition period preceding production of cell-specific products from micro-organisms by means of genetic engineering and the fermentation technique.

One product category illustrating the vast importance of cell culture is the production of highly specific (monoclonal) anitbodies. So-called hybridomas are used for this purpose. A hybridoma is cell which really consists of two types which have been fused into a single cell. One fusion partner is capable of producing antibodies but cannot be cultured while the other fusion partner cannot produce antibodies but can be cultured easily. The combination of these two cell types yields a cell which not only grows well and produces antibodies but, what is more, can yield much larger quantities of specific antibodies than can be attained from laboratory animals. This absolute specificity of monoclonal antibodies is extremely important in areas such as diagnosis. It should be clear from the above that the strength of genetic engineering is not restricted to improving existent processes. The breakthrough afforded by genetic engineering lies in the production of substances which are either difficult to isolate or which are so scarce that potential application is still beyond reach. An important example in this respect is human hormones (hormones are substances which regulate certain specific activities in the body). The advantage of having species-specific (human) hormones is that side-effects produced by foreign (animal) hormones can be avoided. Insulin, which is used in the treatment of diabetes, is isolated from the pancreas of a pig or cow, and injection of this preparation can lead to hypersensitivity and possibly resistance. In recent years a number of human hormones, including insulin, have been produced from micro-organisms on a laboratory scale. Once we succeed in making an active human product from a micro-organism, further research will be needed to increase production. The next step will be development into an industrial process. Even though it is theoretically possible to produce hormones in this way, there is one restriction. It seems that many proteins carry side chains consisting of all kinds of sugar molecules and, what is more, more than one protein chain may be present in an active protein. Since one gene is needed for each protein chain and the number of genes involved in building sugar chains is still unknown, it is too early to expect that complex substances of this type will be made from micro-organisms in the near future. It is essential that we first acquire more knowledge about the mechanism of sugar chain synthesis in order to be able to manipulate it. Obviously the production of such complex proteins requires the combined action of many genes and much research will be needed to identify, isolate and harness all of these genes in one organism. Meanwhile, a simpler solution seems to lie in the possibility of taking the code-bearing gene for the protein chain and building it into a cell which is

already equipped to make sugar chains and which also grows well. Another possibility is to use the hybridoma technique, fusing a product-producing cell with a cell which grows well. A final possibility is that with any luck the sugar side chain will prove to be unnecessary to the protein's biological activity. This was the case, for instance, in the production of the virus-killing substance interferon by genetic engineering. In theory it is possible to test whether or not sugar chains are necessary to biological activity, but we must also take into account the fact that these sugar chains can play an important role in transport within the body and are perhaps instrumental in maintaining the stability of the protein complex. Should this prove to be the case, the result will still be a product which is inferior to the original protein, which means that more will have to be produced to meet the need for larger doses.

The next aspect is also important and can be illustrated by the production of human hormones. The exact action of quite a few known hormones has not been studied, either because there is so little available or because no good test has yet been developed. Genetic engineering furnishes us with the potential to meet this need. Due to the lack of proper knowledge and great scarcity there are, as a result, no applications for this kind of substance. Yet we foresee that most hormones will be applied widely once sufficient amounts are available and their action is understood.The same applies to a number of other human proteins.
Another category of products for which genetic engineering will be of vital importance is the group of human and veterinary vaccines. Vaccines usually contain killed or only weakly infectious viruses or micro-organisms which, when administered to man or animal, result in the production of antibodies but do not aggravate the disease in question, or only to a slight degree. The next time they encounter the fully infectious virus or micro-organism, the provoked antibodies will suppress the disease. These antibodies are provoked, however, against structures (usually proteins) lying on the external surface of the virus or micro-organism. Hence it should suffice to vaccinate using only those proteins which in themselves are not infectious. For instance, the highly infectious virus causing foot-and-mouth disease among cattle would no longer have to be cultured from animal cells were one able to produce the coat proteins in micro-organisms by genetic engineering. This would also eliminate the risk of infection for people culturing the virus. In fact, these external structures have been successfully produced in micro-organisms on a laboratory scale in several cases. The polio virus is another example, for one would no longer have to use monkey kidneys, now so vital in producing the virus. Apart from the scarcity of monkey kidneys, it would be an obvious advantage not to have to use large numbers of laboratory animals.

Finally, a few remarks about the production scale which is needed for profitable production of micro-organisms through genetic engineering. At the moment there is a maximum volume (10 litres) in which cultures can be made. This limit was imposed in connection with the safety aspects of the process. This volume will undoubtedly prove to be very small should one decide to begin production.

It is to be expected that this volume limit will be raised, either in specific cases or in general, once the process has been proved safe. Another problem is the matter of the quantity of the product which will be needed to meet potential applications. Some products will probably have to be produced in kilograms or tonnes, while gram quantities will suffice for others. The quantity produced will largely depend on the active concentrations required for each treatment, duration of treatment, treatment frequency, and the number of patients to be treated.

These factors can be estimated for some products but are difficult to estimate for others. Usually the need increases once a product has appeared on the market. Once a treatment has been initiated successfully, it must be continued and the pharmaceutical industry must guarantee production capacity. Among the considerations which have to be taken into account before putting a certain product into production in an economically responsible way, the amount of substance to be produced will probably weigh more heavily at this moment than its medical urgency.

Chapter organisation

A description of the pharmaceutical industry is given in section 2. Various product categories are discussed in section 3. This organisation was chosen because it allows for ample presentation of current knowledge pertaining to production per group. A summary of the product categories to be discussed and the anticipated method of production is given in Table 1.
Factors influencing future developments of biotechnology in this sector will be dealt with in section 4. Final conclusions will be found in section 5.

2. The pharmaceutical industry

The pharmaceutical industry is very active in the field on biotechnology. It foresees new applications which will enable it to produce antibiotics more efficiently and to produce valuable or so far unavailable natural products profitably, particularly protein hormones and vaccines. Much research is also being done on replacing chemical steps by microbiological ones [2].

Research and development (R & D)

The pharmaceutical industry spends a great deal of money of R & D; to be specific, 8-10% of its turnover. This is by far the highest percentage within the chemical industry (approx. 3% of the turnover). This high R & D outlay is primarily needed for research on new drugs. The cost of developing a new drug, the manufacturing process and registration amount to some Dfl. 100 million, and it takes around 10 years to develop a product from its initial synthesis to market introduction. A great deal of pharmacological, toxicological and clinical research is needed in order to meet the strict registration requirements. Each country stipulates its own specific requirements.

R & D also includes scaling up from laboratory synthesis to manufacture in a pilot plant, as well as finding the proper dosage form in the shape of a tablet, capsule or injectable.

Table 1. Product categories and production techniques

Current and expected applications

Genetic engineering can be applied in all product categories, with the exception of short peptides.

Product category	Fermentation	Tissue culture
2.1. Proteins		
short peptides	-	-
hormones	-	+
immunomediators	(+)	+
immunoglobulins	-	+
2.2. Enzymes		
for diagnostic purposes	+	(+)
for therapy	+	+
2.3. Enzyme products		
alkaloids	+	+
steroid hormones	+	-
antibiotics	+	-
2.4. Vaccines		
active vaccination	+	+
passive vaccination	-	+

\- = no
\+ = yes
(+) = possible

Production

Production is carried out in two stages: preparation of the active ingredients, and processing then into a packaged drug. Consequently, there are two chief departments.

— Plants producing the active ingredients on a large scale.
. Chemical plants employing chemical multi-stage synthesis to produce, for example, thousands of kilograms of active substance for oral contraceptives for millions of consumers. Production requires multi-purpose reactors with volumes of 1,000 to 5,000 litres.
. Microbiological plants producing antibiotics such as penicillin. Production requires large bioreactors, usually 100-200 m^3, and extraction equipment to isolate the active ingredients from the culture broth.
. Extraction plants extracting animal material such as insulin from a pancreas, or vegetable material such as morphine from poppy capsules.

Extraction is done in a simple vessel with stirrer or in a moving band extractor. After isolating the crude product, there are a number of purification steps, in which chromatography over columns is often employed.

— Pharmaceutical plants for processing the active ingredients, together with multifarious additives, into ointments, creams, syrups, tablets, capsules or injectables and delivering the drugs in their final packaging.

Quality control plays a very important role in production. Since the active ingredients are the raw materials for drugs, they must meet a vast number of requirements which have been specified precisely in the national Pharmacopoeia. All intermediate products are subjected to a proper scrutiny as well. The pharmaceutical factories, in particular, must meet the strictest requirements set by good manufacturing practice for accuracy and sterility while preparing the products, and all specialties must pass a thorough inspection.

The Dutch pharmaceutical industry

There are four pharmaceutical industries in the Netherlands manufacturing pharmaceuticals. The following table gives the most essential data.

Manufacturer	Turnover 1979 (Dfl. x million)	International position
Gist Brocades	1,216	Average (at the top in the area of fermentation)
Akzo Pharma	1,274	Average
Duphar	400	Small
ACF	399	Small

Biotechnology plays a prominent part at Gist Brocades, but is of less importance at Akzo Pharma and Duphar.

Table 2 gives a survey of the nine product groups of pharmaceuticals which are manufactured in the Netherlands, specifying the industry, number of processing stages, reactor size, number of products and tonnes per annum.

3. Product categories

3.1 Proteins

The following is a list of major medical needs which, it is hoped, will be met by means of new biotechnological process routes and new techniques. Some of these needs pertain to proteins about which little is known, owing to the fact that only very small quantities are available for studying their exact working. In those cases in which possibilities for application are presumed to have great

potential, as is the case with interferon, producing these proteins will probably be financially feasible, if only for research objectives alone.

Table 2. Pharmaceuticals manufactured in the Netherlands per product group

Product group	Firm	Process stages	Reactor (m^3)	No. of products	Tonnes per annum
Synthetic pharmaceuticals	A, AP, DU	1-10	0.1-10	26	1-200
Steroids	AP, DU, G	1-20	0.1-5	46	0.1-10
Polypeptides	AP	25-80	0.01-1	3	kilograms
Antibiotics	G	microb.	150	12	several thousand
Biochemicals from animal material	AP	extraction	1-30	13	0.1-10
Opiates and	AP	extraction	1-50	10	0.1-20
quinine products	A	extraction	up to 20	2	100-200
Vaccines	DU, G, R	culture	eggs	62	
	AP		roller bottles		
Diagnostics	AP			16	

A = ACF
AP = Akzo Pharma
DU = Duphar
G = Gist Brocades
R = Netherlands Public Health Institute

Short peptides

Peptides are small proteins which are built up from less than 40 amino acids. They are relatively inexpensive to make using chemical synthesis. For this reason, the production from micro-organisms by means of genetic engineering will not be able to compete with the synthetic products. Yet a small peptide was produced in *E. coli* by means of genetic engineering in 1977 [3]. This particular peptide-somatostatin is 14 amino acids in length and the amino acid sequence is known precisely. What is more, sensitive assay methods are available to indicate the product. The important fact here is that a piece of DNA, corresponding to the known amino acid sequence, has been made entirely synthetically. Its importance lies in the fact that it allows us to detect yet unknown genes in a complete DNA preparation. In fact, if a part of the amino acid sequence of a protein is known, one can detect the entire genetic code by means of a short fragment of synthetic DNA by using the characteristic that complementary fragments of DNA hybridise with one another. These types of synthetic DNA fragments might find application in prenatal diagnostics of hereditary disorders. For instance, a synthetic gene would not be able to hybridise with a deviant cell lacking this particular gene.

Insulin is a hormone which is supplied in large amounts by the pharmaceutical industry. A short historical survey of insulin production will illustrate the importance of what may be the first widescale industrial application of the recombinant DNA technique for the production of a human hormone.
Insulin was discovered in 1921 by the Canadians Banting and Best. This hormone is produced by the pancreas and is essential to the regulation of glucose metabolism in the body. Insulin is the most important means of treating diabetes *mellitus*. In the Netherlands there are about 100,000 diabetes patients regularly using insulin. This hormone is isolated from the pancreas of a cow or pig. Insulin is a small protein containing 51 amino acids. Bovine insulin has three amino acids differing from human insulin, while porcine insulin only has one. Since many diabetics need two to three insulin injections a day, antibodies can form in the body which diminish its effect. The dosage must therefore be increased as years pass. Porcine insulin is less likely to form antibodies than bovine insulin because it deviates less from the human hormone. Immunological complications can also arise from possible impurities in the preparation, such as other pancreas proteins. Even though there is a very pure product on the market, only the human hormone is truly ideal. Since human insulin could only be obtained from a human pancreas, this ideal insulin was but an idle hope until recently. It is now possible to make human insulin from porcine insulin by means of hemisynthesis. Alanine, the terminal amino acid in porcine insulin, is removed enzymatically, and threonine, the terminal amino acid in human insulin, is linked to it. The first publication on this dates from 1966, but methodology has improved so much since then, particularly in the use of a highly specific bacterial enzyme which removes alanine only. The threonine is linked under influence of the same enzyme in a suitable solvent. In 1980, the Japanese reported that they had achieved a yield of 50%. One bothersome problem has always been the removal of closely related residual products. NOVO is known to be making a concentrated effort on this approach. A second way of producing human insulin is by applying genetic engineering. Genentech succeeded in doing this about two years ago. This firm has granted a license to Eli Lilly, the world's largest insulin manufacturer. According to recent reports, human insulin is already been prepared in 1,000 litre reactors and Eli Lilly has announced that he is planning to invest $ 40 million in factories in the United States and England [4]. Reports on the date when this preparation will be put on the market vary from 1982 to 1985. In July 1980, 17 healthy volunteers were treated with the preparation in a London hospital.

Preparing insulin from *E. coli* is a complex process, and it will not be easy to develop it into a profitable operation. Specifically, it requires a large number of stages to attain the final product. Insulin is composed of an A-chain having 21 amino acids and a B-chain having 30 amino acids; both chains are linked together by a number of disulfide bridges. At Genentech, the A and B chains have been produced separately in *E. coli* and the genes both for the A as well as the B chains have been made synthetically for this purpose [5]. Eli Lilly has

developed a secret and unique process for linking these purified A and B chains. The motive for making human insulin is self-evident, but Eli Lilly also foresees a shortage of bovine and porcine pancreas in the next 20 years owing to the increasing number of diabetics, particularly in developing countries.

Another human hormone, the growth hormone, has also been produced recently on a laboratory scale using genetic engineering [6]. This is a complex protein, consisting of 191 amino acids. Growth hormone is currently used for treating patients with growth disorders and pregnant women in whom the foetus is not growing properly. Growth hormone has a potential use in treating broken bones to accelerate the healing process. Since the effect of the growth hormone lies in its stimulating the production of another hormone, somatomedin, this hormone could also be of interest. Another important protein is the growth hormone releasing factor. By identifying this presumably short peptide, we might be able to bypass direct administration of the growth hormone and thus the production of this complex protein.

The hormone erythropoietin is important in the production of red blood cells in the body and is produced by the kidney. Patients with a chronic kidney insufficiency, producing too little erythropoietin, and patients without kidneys (approx. 1,400 people in the Netherlands), producing no erythropoietin, are subject to serious anaemia. There is an urgent need for erythropoietin to treat these patients, for a kidney transplant remains the only other alternative.

Another example of a human protein hormone with a potential application is thrombopoietin. There are some indications that this hormone stimulates the production of platelets (thrombocytes). Thrombocytes are vital for checking bleeding. There are few deficiencies of this hormone on record, but then again it is difficult to diagnose because there are no good methods for measuring the amount of thrombopoietin present. This situation could be improved by producing thrombopoietin on a laboratory scale to improve the assay methods.

The hormone gastrin certainly has a potential application, since patients with ulcers have a disturbance in gastrin secretion. This hormone is so scarce, however, that few studies have been done on its action and no study on its potential use.

Finally, human chorionic gonadotropin (HCG) and human menopausal gonadotropin (HMG) could be produced by genetic engineering. Both of these hormones are currently isolated from the urine of women who are either pregnant or in the menopause. One way in which HMG is applied is to stimulate ovulation in certain types of infertility due to inadequate secretion of the follicle-stimulating hormone (FSH). HCG is used, among other things, as a substitute for the luteinising hormone (LH) in pregnant women at risk of miscarriage. Direct production of the hormones FSH and LH is also of course a possibility.

Hormones, other than protein hormones, are also found in the body. These are the steroid hormones. These substances are not built up from amino acids like the protein hormones but from an entirely different class of building blocks. The steroid hormones are discussed in section V.3.3.

Immunomediators

Immunomediators are proteins which serve to modulate the immunological system. As matters stand at present, we are only able to identify a number of these modulators, not their structure. For instance, we are able to identify Interleukin I and II, both important in the interaction of various types of lymphocytes (a type of white blood cell) during an immunological response. We are also aware of certain factors produced by the thymus. These factors modulate the maturation of this type of cell having immunological properties. It appears that these substances will contribute greatly to the treatment of primary as well as secondary immunological deficiencies, perhaps proving instrumental in combatting tumours by means of activating the natural killer cells.
An immunomediator which is understood somewhat better is interferon [7, 8]. Interferon is used to combat virus infection as well as a disease, juvenile papillomatosis of the larynx, whereby warty growths in the throat can lead to loss of the voice and possibly suffocation (asphyxia). Interferon might also be applied in future to control certain types of cancer. However, a large amount is needed for each treatment and there is a shortage of interferon for basic and applied research. One problem is that there are multiple types of interferon. The main types are Type I interferon (IFN Alpha) and type II interferon (IFN Beta). It was recently discovered that type I interferon consists of at least five different, closely related proteins having few sugar side chains [9, 10, 11, 12]. Much more research will be needed before we are able to identify which type has which activity. Furthermore, production for research objectives alone will undoubtedly be commercially feasible.

Immunoglobulins

As was stated in the introduction, the hybridoma technique enables us to produce monoclonal antibodies. Theoretically it is possible to obtain antiserum for every type of antigen in this way. This opens up great perspectives for research, as well as for diagnostic purposes. For instance, diagnosing cancer by means of 'cancer detecting antigens' will be greatly improved by the use of monoclonal antisera. Tissue typing for organ transplants is another important use of the tissue-specific antisera (anti-HLA sera). A third potential use of the hybridoma technique might lie in producing human (monoclonal antibodies, which are currently only available from random donors or immunised volunteers [13]. There are, however, certain specific problems here. The most important needs at this moment are for the following preparations: anti-rhesus D for the prevention of haemolytic anaemia of the newborn (breakdown of red blood cells) and for diagnostic purposes; Australia antigen (AU-antigen,

causing hepatitis B); and finally, anti-tetanus for combatting or preventing tetanus infections in, for instance, victims of road accidents.

Other proteins

Several proteins will be discussed in this section which do not belong in one of the above sections. These proteins are all used in the field of medicine and the majority are isolated from human blood plasma. Antihaemophilic globulin (coagulation factor VIII) is vital for the prevention and treatment of haemorrhaging in patients having a hereditary tendency to excessive bleeding (haemophilia A). It is also used to treat haemorrhaging in patients having Von Willebrand's disease. The total annual production of the regional blood banks and the Central Laboratory of the Blood Transfusion Service is not sufficient to meet the needs of all the patients in the Netherlands (approx. 1,000). Moreover, there is a highly fluctuating additional need among these patients for factor VIII; for instance, for light surgery such as tonsillectomies and pulling teeth and molars. Another coagulation factor which is being used is factor IX. This is used for patients with haemophilia B (approx. 150 patients in the Netherlands) for the same objectives as described above. Factor IX is not as scarce as factor VIII, however.

Antithrombin III is a natural inhibitor of the coagulatory system and is used, among other things, for hip operations, where it is used to prevent blood clots which can lead to blockage of a blood vessel (deep venous thrombosis). Other than this, it is also used for hereditary deficiency of antithrombin III and for haemorrhaging of varicose veins in the oesophagus as a result of hepatic cirrhosis (poor functioning of the liver due to connective tissue formation).

Tissue activator activates the dissolution of fibrin which is present in blood clots. It can be used for patients with thrombosis (spontaneous formation of local coagulation of the blood) to dissolve clots which are blocking off a blood vessel. Here lies a large area for application. Urokinase and streptokinase are being used for this at the moment. One disadvantage of streptokinase is that it is a bacterial enzyme which might produce antibodies. It can also cause haemorrhaging.

Fibronectin is a protein which attaches itself to cells and ensures that they remain properly within their connective tissue. It can be used to hasten the healing of a wound, burn or excessive damage to tissue due to infections and following surgery.

Human albumin is a protein which is often used to treat shock as a result of infection, serious burn or albumin deficiency which might be due to kidney disorders, hepatic cirrhosis or malnutrition. It is often used after surgery, as well. In spite of the fact that albumin is, quantitatively speaking, the most important protein from blood plasma, this product remains scarce due to its multifarious applications.

All of the above products could be produced by genetic engineering, and there is a specific demand for production of the scarce products. Secondly, it is difficult to predict whether or not blood donors will continue giving blood without wanting to receive cash compensation. This has already happened in many countries. This would result in spiralling costs of products isolated from blood plasma, making production by means of genetically engineered micro-organisms even more attractive.

3.2 Enzymes

3.2.1 Diagnostic applications

Most chemical responses in humans and animals can only occur after interacting with enzymes. Metabolic processes and clotting are two such responses. Many enzymes are synthesised in the cell in which they are used, while others must be transported elsewhere. Some enzymes only appear in specific organs, while others appear in a variety of organs. These are localised in cells and only small quantities can be found in circulating blood. Should an organ contract a disease, it is possible that there will be a dramatic increase in enzymes in the blood, owing to increased permeability or damage of the cell membrane.

A damaged organ can be located by measuring organ-specific enzymes or an 'enzyme specimen', a combination of various enzymes which become organ-specific when combined. This has often proved to be an excellent tool for evaluation in differential diagnosis. Enzymes are particularly important in diagnosing diseases of the heart, liver and pancreas. These diseases are widespread in industrial countries. There has been a noted increase in liver disease with the rise in alcohol consumption. Alcohol consumption in the Netherlands increased from 2.5 litres per inhabitant to 8.5 litres of pure alcohol between 1970 and 1977 [14]. The number of cirrhosis patients increased from 510 to 730 in the same period and the number continues to grow [15].

Enzymatic analysis has also been used succesfully for monitoring therapies. Many changes in a clinical picture distinctly influence the height of enzyme activity in the serum tested. We are thus able to recognise acute stages of a chronic disease as well as recovery.
Another field for enzymatic analysis is in detecting congenital enzyme defects. Early detection can increase life expectancy as well as prevent lifelong disability.

Enzymatic analysis has also become increasingly important in the clinical, chemical laboratory. Enzymes can be used to determine various substances. Nearly 2% of the Dutch population are known diabetics [15]. The percentage for other west European countries is 2-3%. An annual increase of 5% has been predicted for western Europe. The use of enzymes for blood sugar analysis is a widely acclaimed and internationally accepted method. In recent years,

enzymatic responses can even be read off test strips. This makes it possible to screen people on a large scale at low cost. In recent months, there have been international reports of test strips which would enable diabetics to check themselves, which is but one step away from receiving more personally tailored treatments in the future.

The number of victims of heart disease, blood disease and circulatory diseases has doubled in the last 50 years. International epidemiological studies clearly indicate that lipid metabolic defects are one of the main causes, certainly placing them among the 'risk factors'.

There have been major advances in our biochemical understanding of the relationship between lipid metabolism and arteriosclerosis in the past few years.
Enzymatic analysis is far superior to routine analytic methods in this area, which were less specific and more time-consuming. The arrival of enzymatic analysis in clinical chemistry is most certainly a milestone in diagnostics, for if therapy is initiated early enough, benefits are not only economical (less sick leave, fewer people laid off work) but personal (less sickness).
Since enzyme concentrations in the body are low and their action needs to be measured as a standard for biological activity, enzymatic analysis met with little laboratory success before certain technical advances had been made. To carry out a large number of analyses with complicated analytic procedures requires specially trained laboratory asistants. The laboratory itself must also be properly equipped in order to take advantage of technical advances. The physician also bears certain responsibilities. In establishing a diagnosis, he must keep up with the latest scientific findings. Since our knowledge is increasing rapidly, this will require a form of permanent education.

New developments have led to techniques employing enzyme markers instead of radio-isotopes or radio-immunoassays. Most analyses of the thyroid gland can be done without danger of radiation. Consequently there is less danger of radiation in hospital laboratories and more protection for the environment.

Enzymatic analysis is not restricted to clinical chemistry, for it is also used in food analysis. Traditional methods can no longer compete with current scientific standards and may at times even impede development. Some countries have changed their laws to allow for such development. This has led to greater accuracy in less time, which means more certainty at less expense.

3.2.2 Therapeutic applications

The amount of enzymes required for diagnostic purposes is generally small, and type-specificity is of secondary importance. The situation changes, however, when enzyme deficiencies are treated with enzymes. This form of therapy not only requires large quantities of a specific enzyme, but each enzyme has a limited lifespan (the period of time before half of the activity of the

administered enzyme has ceased, varying from several hours to several days) and must be administered to the patient for his entire life.The administered enzyme must often be human in origin because foreign proteins elicit immunological defense responses. Enzymes of human origin will probably be produced in the future through recombinant DNA technology. It has to be borne in mind that various cellular processes are required for the synthesis of enzymes. Many of these processes cannot take place in *E.coli* since its enzymes are not equipped for this synthesis. For example, in cells of higher organisms, including man, sugar groups are linked to certain protein molecules, making the molecule operational. Genetic engineering with mammalian cells may provide an answer in the future.
The synthesis of these molecules is one problem, but the therapy itself is too. The first experiments, done about 10 years ago, looked very promising. Yet research has had many setbacks this past decade. In addition to the aforementioned immunological defense responses and the limited lifespan of the administered enzyme, there are still a number of other problems to be solved [16]. Many enzyme deficiencies cause defects in the central nervous system, so the administered enzyme must reach the brain cells. This presents a major problem when administering the enzyme intravenously, for it must pass the 'blood brain barrier'. The blood brain barrier is a membranal system which can only be permeated by gases and small molecules. A great deal of fundamental research will be necessary to solve all of these problems.

3.3 Enzyme products

Enzyme products are classified according to primary and secondary metabolites. Primary metabolites are small molecules found in living cells, the intermediate or final product of metabolism. These final products are the building blocks of essential macromolecules such as DNA, proteins, cell wall polymers, membranes and co-enzymes. Primary metabolites are essential to the cell's metabolism, division and growth.

Primary metabolites are also transformed into secondary metabolites in many kinds of cells [17]. Although these secondary metabolites are not directly involved in the organisms' growth, they play an important role in the adaptation of the organism to its environment, thus increasing its chance of survival. Secondary metabolites are the product of a series of specific enzymes in which various primary metabolites are transformed into one specific secondary metabolite. The pharmaceutical industry is interested in the pharmacological action of these secondary metabolites, particularly their potential to prevent or cure disease. The chemical structure of secondary metabolites can differ greatly (alkaloids, steroids, cyclical peptides, glycosides, derivatives of beta-lactams, amino acids, sugars and fatty acids). The pharmacological action of these substances is also highly divergent and comprises anti-tumour, anti-leukemic, anti-bacterial, anti-coagulatory and hormonal activities, to name but a few. The action of the secondary metabolites bears little relation to their chemical structure.

In the fermentation industry, secondary metabolites are produced primarily from micro-organisms, since these organisms can be produced inexpensively and techniques are available to increase production capacity by means of mutation and selection [18]. Only a few secondary metabolites are produced commercially by means of animal or plant tissue cultures, since tussue culture is expensive and productivity is low. Production is only economical for indispensable and scarce products which can command a high price. Many secondary metabolites are produced by plants, such as the steroid/raw materials diosgenine and sitosterol, as well as various raw materials for the alkaloids.

New developments in producing secondary metabolites will be less spectacular than they were for proteins. Modern molecular biology techniques will aid in improving production processes or in developing new ones. Developments will also progress less quickly, since biosynthesis of these molecules often involves complex enzyme systems, each being the product of many genes. Specific advances will mainly depend on a thorough knowledge of the enzymes involved in biosynthesis as well as their regulation. By breaking through the regulation and increasing the various enzyme activities, the metabolism of the production organisms can be changed in such a way that the secondary metabolites are secreted in very high concentrations. Some steroid and antibiotic fermentations yield as much as 30 to 50 grams of product per litre of fermentation broth [19].

It is too early to tell whether or not the production of secondary metabolites by means of tissue culture will encounter competition from fermentative processes based on manipulated micro-organisms. The latter involves transferring genetic information from the higher organism into bacteria and ensuring that all the enzyme systems involved in the biosynthesis of the secondary metabolite are also active in the new environment. It is also possible that genetic engineering techniques now developed for bacteria may also be applied to the higher organisms used in tissue culture. Such a system has already been developed for yeast. Rather than transferring genes to bacteria, production might be improved more efficiently by developing systems in the organisms which are used in tissue culture, and only amplifying those genes which are involved in the biosynthesis of the metabolite in their own cellular environment. Commercial prospects will depend largely on developments in molecular biology and the complexity of biosynthesis of the desired product.

3.3.1 Antibiotics

Antibiotics are the most well-known secondary metabolites. They are one of the most important products of the fermentation industry. Between three and four thousand antibiotics have already been described. They are sought and found primarily in micro-organisms. Spore-producing micro-organisms, such as *Actinomyces* species and bacilli, are the main producers of many types of antibiotics. These antibiotics involve varous mechnisms and are active against

different micro-organisms. Of all known antibiotics, less than 100 are produced and only about 10 of those on a large scale [17].

Some fermentation industries produce more than one million kilograms of one antibiotic, having a market value excereding $ 26 million.
Future developments are expected to include:
— screening for new antibiotics;
— improving process and production strain.

Screening is done for the purpose of obtaining new antibiotics with certain improved characteristics. For instance, the development of pathogenic micro-organisms which are resistant to various antibiotics has led to the development of new antibiotics and semi-synthetic antibiotics, which are chemical derivatives of antibiotics. Likewise, cephalosporin C was the only known cephalosporin for some time. Owing to the development and application of various sensitive test organisms in screening, a new series of natural cephalosporins has been discovered. They and their chemical derivatives are currently being produced and developed. Much time and money are being spent, particularly in Japan, in developing and applying screening systems to unearth natural substances having divergent biological activities.

Improving processes and strain material will continue to enhance production in the fermentation industry. Sugar is the most important substrate for the growth of the micro-organism and biosynthesis of the secondary metabolite in many fermentation processes. The conversion rate of sugar to the desired product is an important factor in the productivity and cost of the process. In producing primary metabolites, such as citric acid, 90% of the sugar is converted into the product, while percentage recovery varies between 30% and 60% in amino acid production. Biosynthesis of secondary metabolites is more complicated than for most primary metabolites, and the sugar conversions are usually lower than for primary metabolites. In penicillin fermentations, 10% of the sugar is now being used for biosynthesis of the penicillin molecule [18].
The production of secondary metabolites, such as antibiotics can be broken down into various stages, each being carried out by different enzymes.
Usually there are more than ten different enzymes involved in the biosynthesis of an antibiotic. Genetic information for the biosynthesis of these enzymes is carried by at least ten different genes. Should recombinant DNA techniques become available in the production organism for penicillin (the mould *Penicillium chrysogenum*), the production capacity of the organism could be improved by transferring the various enzymes involved in biosynthesis. Development of recombinant DNA techniques for the various production organisms would be most advantageous. A system has already been developed for *Streptomyces* species.

3.3.2 Steroids

Biotechnology plays a fundamental role in the production of various steroid combinations [19]. Several years ago this amounted to more than two million kilograms of crude, raw materials which had been transformed to commercial products, having a market value of $300 million. Currently, several groups of steroids are being used for purposes such as suppressing immune responses during organ transplants, reinforcing muscle tissue, healing infections and preventing ovulation (the pill). Steroid production begins with crude animal or raw vegetable materials, such as sitosterol which is gained from soya beans. Partially breaking down this sitosterol molecule, by using a *Mycobacterium*, for example, yields substances such as androstane and adrostanedione. These substances are then changed by chemical and microbiological conversion into the desired product, such as hydrocortisone (raw material for male hormone) and oestrone (raw material for female hormone).

Microorganisms are often used in these conversions to perform stereospecific responses. In this way, for instance, the fungus *Aspergillus ochraceus* is able to transport a certain group to a specific place in the steroid while another fungus transports a mirrored pattern of the same group (stereospecific hydroxyl actions). The production of steroid hormones involves many response stages, usually five to fifteen per steroid. The majority are chemical conversions. Yet microbiological conversions are indispensable in converting major steroid hormones, such as the corticosteroids; this involves one or two conversions per cycle.

New organisms might be sought for these stages so that it would no longer be necessary to depend upon micro-organisms. If recombinant DNA techniques do enable us to transfer genetic information to one micro-organism and get it to work, this would mean that a series of responses which are now carried out separately could be done in a single conversion. This would lead to a greatly simplified procedure and possible higher overall conversion output.

Should it prove possible to combine various enzyme systems in one organism, then one organism could conceivably convert crude, raw materials, such as sitosterol into hydrocortisone and by another organism into oestrone.
A series of entirely different enzymes would then have to be active in one organism, selectively breaking down and oxidising the molecule stereospecifically. Various micro-organisms must be designed and constructed for each product, each carrying out a different series of specific reactions. This may seem to be asking for too much, but in future we will certainly be looking for combinations of conversions which can be united in a single micro-organism. How these organisms are used, as cells in suspension or as immobilised cells on a carrier, will be a question of process improvement.

3.3.3 Alkaloids

In contrast to antibiotics, most alkaloids are not produced by micro-organisms, but by plants. Alkaloids comprise a very heterogenous group of nitrogenous substances which are usually produced from plants. They have been used from ancient times for their medicinal properties. More than a thousand different alkaloids have been isolated and new ones are still being discovered. Their effects are highly divergent, which is evident from their current use as anti-cancer agent, insecticide, psychedelic, pain-suppressant and anti-hypertensive.
The major products such as opiates and quinine are produced at a reasonable cost price (Dfl. 500 — 1,000 per kilogram) and in reasonable amounts (see Table 2). The more exclusive substances are quite expensive. Factors such as low product concentrations per plant, temporary unavailability of the product on the world market, political upheaval, etc. are motivating research on new production methods.
Much research is being done at the moment on plant tissue culture in particular [20]. For instance, the anti-tumour alkaloids, vancristin and vinblastin, and the anti-hypertensive alkaloid, amalicine, are already being produced on a laboratory scale.
The same developments can be expected in the production of alkaloids as were described above for the other secondary metabolites. Recombinant DNA techniques and knowledge of biosynthesis and of regulatory mechanisms will be used to improve both the classic plant as well as tissue culture and fermentative production. Developments will depend largely on the amount of research which is being done on the products. Should developments follow the course of antibiotics, tissue culture and fermentative processes will probably play a role in the production of alkaloids in the coming years.

3.4. Vaccines and antisera

Developments in the area of genetic engineering and new tissue culture techniques will make numerous new vaccines and antisera possible in the coming decade. One thinks first and foremost of products against viral and bacterial infections, which up till now have been difficult or even impossible to prepare. Vaccines and antisera could also be made which can now only be prepared by means of potentially hazardous (risk of infection) or extremely expensive production methods.

3.4.1 Human applications

Hepatitis B

This contagious disease often leads to liver damage. Since this virus is highly resistant to physical influences and can be transferred through body fluids (serum, saliva, etc.) hospital staffs and patients taking dialysis for renal failure belong in the highest risk group. Medical interest and society's need for an

active vaccination for large-scale use is very great. In western Europe, the hepatitis B antigen is present in the blood of 1% of the population.
Since the hepatitis B virus cannot be cultured easily to produce vaccine, the only therapy thus far has been the use of human immunoglobulin. This is prepared from plasma containing antibodies (passive immunisation). This can only be applied once the contagion is suspected, for these antibodies have only a brief lifespan in the body.
The discovery of the Australia antigen, one of the important surface proteins of the hepatitis B virus, opens up the possibility of developing an active vaccine. It was recently shown that vaccine containing the inactivated Australia antigen does indeed furnish virtually complete protection from hepatitis B infection [21].

The Australia antigen can only be derived from plasma of chronic carriers of the antigen, and then only on a laboratory scale. Genetic engineering might make it possible to produce the Australia antigen on a large scale and with no risk of infection. In order to do this, the gene for this surface protein is introduced into a bacterium cell and is then brought to expression. This has already been done with a non-surface protein of the hepatitis B virus [22]. There is every reason to believe that this can also be done with the Australia antigen.

Influenza

Influenza is a viral infection which is primarily dangerous to older people and to those in high risk groups: patients with respiratory disorders, patients with kidney defects, diabetics, etc.
A vaccination is given annually with virus cultured in embryonated eggs. The problem is that new strains appear almost yearly, so that the vaccine has to be adapted. The World Health Organisation has built up a world-wide network in order to detect new strains promptly. This organisation publishes advice each year on the strains which should be in the vaccine.
There is little potential for genetic engineering in this area. The gene for the surface protein haemagglutinin has been inserted into a bacterium and brought to expression [23]. For research purposes this offers interesting prospects of learning more about the genetic structure and consequently about genetic changes which haemagglutinin undergoes in nature. Yet the continually changing strains make this method too cumbersome for vaccine production compared to the growing of a virus in embryonated eggs. The present method produces sufficient vaccine within the desired period.

Malaria

In order to fight malaria we need a vaccine against the parasite which causes the infection. This means that parasites will have to be cultured *in vitro*, which has been impossible up until now. Only recently has a method been developed for culturing the malaria parasite continuously [24]. This method involves culturing the parasite on red blood cells (erythrocytes) which are regularly

refurbished with fresh erythrocytes. A great deal of research is still needed before proper culture conditions have been developed and vaccines are available.

Herpes

Viruses from the herpes group cause various disorders in man, such as chicken pox, shingles and mononucleosis. The herpes virus often remains latent in the body following infection and rears its head again later, sometimes even causing carcinoma. Much research is still needed on these viruses and their molecular biological action before they can be used in vaccines. For instance, a patient with recurrent herpes regularly produces an amount of antigen, without forming sufficient antibodies and without building up sufficient protection against this virus. For this reason a herpes vaccine containing a relatively small amount of antigen is not likely to provide protection.

Adeno virus

Adeno viruses cause, among other things, infection of the respiratory system and some kinds of conjunctivitis. Some 1-5% of respiratory disorders are caused by adeno virus. From an epidemiological perspective, a vaccine can do little more than mildly alleviate this type of disorder, even when given in massive doses. A further complication is that some viruses, analagous to those in the herpes group, have carcinogenic properties, which means they will not be used so readily for vaccines.

Immune sera (antisera)

The developments of monoclonal antibodies, produced by hybridoma cell lines, have shown that all kinds of specific antibodies can potentially be produced. These antibodies are needed as a diagnostic tool for distinguishing between syndromes more specifically. Monoclonal antibodies can also be used for passive immunisation (see immunoglobulins). However, it may not be suitable for human use, for hybridoma cell lines result from a fusion with a tumour cell. This will probably raise questions about the safety of hybridoma products. It is therefore imperative to study this extremely carefully.

3.4.2 Veterinary applications

Foot-and-mouth disease

A vaccine for food-and-mouth disease is badly needed (the total herd of livestock in the Netherlands is treated). A vaccine is currently being made at only one location in the Netherlands (Lelystad). Production requires the use of the virulent virus, with all the attendant risks of its escaping the factory. What is more, the culture method is very expensive.
A method analogous to that for hepatitis B vaccine is the introduction and

expression of genes for the surface proteins of foot-and-mouth virus in a bacterial system [25]. Should this prove feasible on a production scale, it will no longer be necessary to work with the virulent virus and production costs will drop considerably.

Bacterial vaccines

Bacteria such as *E. coli* are not harmful to man but can cause infections in animals. The bacteria which are now used as source material for vaccines can be changed with the aid of genetic engineering. Bacteria could thus be obtained which produce fewer enterotoxins (intestinal toxins), greatly improving the quality of the vaccine material. Another possibility is to manipulate the bacterium in such a way that it contains more attachment proteins, enhancing their effect as vaccine (inducing greater immunity). Should it prove possible to manipulate bacteria in such a way that they considerably increase their production of attachment proteins, these proteins could then be isolated from the culture medium and used as a vaccine. This vaccine would then no longer need to contain bacteria.

Immune stimulators and adjuvants

Certain parts of the cell membrane of bacteria have the property of protecting the receiver against all kinds of infection: a-specific immune stimulators. These membrane parts can also be used to reinforce the characteristics of a small quantity of specific vaccine (adjuvant function). It would therefore be attractive to genetically manipulate bacteria in such a way that these bacterial substances are produced by the bacteria in much greater quantities.

Antisera

Highly specific antisera for passive immunisation can be produced by hybridoma cell lines. Potential hazards plays less of a role here than in human applications. It is probably not economically feasible, however, since the cost price for veterinary products plays a much greater role than for products intended for human use.

3.4.3 Conclusion

The most striking examples of developments to be expected in the next few years are a vaccine against hepatitis B and a vaccine against foot-and-mouth disease, both by means of a recombinant DNA technique. A great deal of research still needs to be done before the other vaccines discussed will be ready for use.

4. Factors affecting development

4.1 Social factors

Health care is one of society's most important tasks. The development and application of new diagnostic methods, cures and drugs are essential aspects of this task. For instance, one of the most significant breakthroughs in health care was the discovery of antibiotics to combat bacterial infections in humans and animals.

Biotechnological production methods have proved extremely valuable in the pharmaceutical industry, both in improving production efficiency of given micro-organisms and in making it possible to synthesise combinations which are otherwise extremely difficult or impossible to make.

Standards have been laid down for the development of new drugs which cover a number of factors. These have influenced the development of biotechnological processes used in preparing drugs. Many pharmaceuticals have undesired side-effects. Rapidly increasing biomedical knowledge has acted as a stimulus for the development of more refined (specific) drugs and cures. New techniques, including recombinant DNA, are making a substantial contribution.

Drugs of animal origin, such as insulin, may often cause an undesired immune response in man. This problem can be remedied by making human insulin from *Escherichia coli* bacteria through genetic engineering.

Society also sets ethical standards for the development and production of drugs. Alternative production methods will make it possible to use fewer test animals in the preparation of pharmaceuticals. This is but another indication of the potential of biotechnology.

4.2 Research

Molecular biological research is responsible for the most spectacular innovations in products and processes at the moment. Much of the experimental molecular biological research in the United States is being subcontracted to small and average-sized firms such as Genentech and Cetus. They are both highly specialised and have had years of experience. The actual production of pharmaceuticals involving genetically engineered micro-organisms is then done by large pharmaceutical concerns. The Dutch pharmaceutical industry might also choose to follow this route. A CIII facility is currently being installed in the TNO Medical Biological Laboratory, which already has a CII facility at its disposal. This will enable the pharmaceutical industry to have projects developed by TNO on a contractual basis.

A number of other firms and university laboratories have also been equipped to work on CI and CII levels. Further collaboration would not only promote the efficiency of biotechnological research but greatly enhance coordination in this area.

Obscure permission procedures for doing recombinant DNA research are a serious impediment to the industry. There is also a limitation on reactor volume, 10 litres of culture fluid with manipulated organisms, which is too little for most applications and too restricted to make the process in any way economic.

4.3 Registration

As a rule, new drugs must be registered before they can be sold in a country. The manufacturer must prove that they are safe and effective. This of course requires many pharmacological, toxicological and clinical studies. This also applies, in principle, to existing drugs which are prepared by a new method. Producing specific antisera from hybridomas (2.4.1) opens interesting new perspectives. Yet for human use, this production method is too new and is not yet generally accepted. Registration authorities will first have to study this method critically. It is of primary importance to completely eliminate any potential danger of transferring an oncogenic (cancer-causing) factor from the hybridoma cell lines. This could mean that a number of long-term toxicity tests will have to be done before the health officials consider these antisera safe.

When registering protein products which have been produced by means of manipulated bacteria, safety and purity must be proved. The extent to which this must be done is still uncertain. This means that the first products will have to bear the brunt. A second problem lies in the effectiveness of the protein product. The structure of a protein product manufactured in this way lacks the carbohydrate part. Sources show that the carbohydrate part is instumental in the protein's capacity to induce antibody production.
This does not necessarily mean that these proteins would be less effective, but this must be supported by conclusive evidence.
Should the protein engineered from bacteria prove to be identical to the current product, there will be little reason to doubt its effectiveness.
It is unclear whether or not the above points mean that the (clinical) research programme for registration will need to be as extensive as that for entirely new drugs.
A shortened programme might be possible in some cases, but this will differ per product and per country.

4.4 Training

The pharmaceutical industry needs college graduates for research and development of new biotechnological processes. Delft University of Technology and Wageningen University of Agriculture regard it as part of their task to provide biotechnological training. The main emphasis at Delft is on process

technology, and at Wageningen on the molecular sciences. The first class of biotechnologists graduated in the spring of 1980 from Delft University of Technology. A study programme in biotechnology commenced at the University of Agriculture in September 1980.

Fundamental research in molecular biology is expected to yield the most advances. Molecular biology as a discipline is of recent date. The training in this discipline is good. The university curricula 1979/1980 which were submitted for a SON grant, show that recombinant DNA research has been made part of the training and research.

In view of the simplicity and diversity of biochemical and genetic engineering, recombinant DNA is eminently suited for practical training.

The *ad hoc* committee on recombinant DNA activities has proposed that *Escherichia coli* K-12 be used for recombinant DNA research, since it has been thoroughly studied and in principle only requires a low safety standard (CI). This would enable university staff and students to conduct research without severe restrictions. *Escherichia coli* K-12 bacteria have been used in studies for decades to gain fundamental knowledge about processes in the living cell, so that this proposal is good from a didactic point of view as well.

A few points will now be made about training research workers in cell biology techniques. Even though Dutch universities offer no specific training in cell biology, many research teams regularly use cell biology techniques.

The hybridoma technique is being used, for instance, by various research teams at Erasmus University in Rotterdam. It would be most advantageous if research teams which have worked intensively with tissue culture techniques could also acquire a thorough grasp of techniques involving animal vectors (such as SV-40). In this way cloning in animal cells could also be studied and taught in the Neterlands.

It seems unlikely that every specialisation will be represented in the Netherlands, due to its size (fungus genetics being a case in point). Nor does it seem likely that the demand from the pharmaceutical industry for qualified personnel will exceed supply for the next few years; that is, if there are no deep budget cuts in higher education.

5. Summary and conclusions

This section summarises the prospects of applying recent results of fundamental research. In the past, organic chemical and microbiological products played an important role in the development of pharmaceutical preparations. In the future, modern biochemical analytic techniques will be used primarily to detect natural resistance substances. These substances will then be produced by means of tissue culture or specially constructed micro-organisms. Bio-

logical products and production methods will soon play an important role in pharmacy and the pharmaceutical industry; a number of production processes are currently being developed.

Dutch universities are fairly well advanced in the area of molecular biology, which means that various departments and research teams will soon take advantage of these new possibilities. It is the task of the universities to continue investigating and teaching new advances.

International success in a given area depends on cooperation between various departments of one or more universities. This is because one project may often require highly divergent, strongly specialised techniques and knowledge. Proper coordination, in turn, requires strong and dynamic project supervision and decision-making power. Yet the general tendency is for the various departments within the university to explore the various possibilities within their own branch rather than to place their knowledge and inventiveness at the disposal of joint projects aimed at developing new products.

The established pharmaceutical industry will have a hard time taking advantage of all of the new advances because it also has to make certain choices, and it takes time to gather full, specialised know-how. Especially now that leading scientists have shown their awareness of industrial uses for their molecular biological knowledge, related to advances made in recombinant DNA research, by teaming up in many cases with new firms such as Genentech, Biogen, Cetus, etc.

Industries which are putting these findings into production or have developed them themselves, are now being confronted with multifarious, obscure requirements on research and production facilities. In the Netherlands, regulations limit recombinant DNA research to a 10-litre scale. Registration of these products will also be a lengthy and expensive process.

If the Netherlands hopes to maintain an industry based on innovative research, it will need legislative and administrative government bodies sensitive to innovation. Dutch universities and industries will need to equip themselves with the most modern research and production facilities. Good government supervision in the area of legislation and administration must share the same dynamic policy as that which is conducive to innovative research, if the Netherlands wishes to remain a society based on innovation.

The Dutch public will witness a new generation of pharmaceutical products in the coming years which have not been developed on a chemical basis, but discovered in man himself and produced by specially constructed cell lines or even from bacteria. Clinical tests are already being carried out on these products.

Members of the working group

The working group which produced Chapter V consisted of the following members:

Dr M.N. Hamers	Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam
Dr H.A.P. de Jongh	Diosynth (Akzo Pharma), Oss
Dr A. de Leeuw	Gist-Brocades, Delft
Dr H. Pannekoek	Leiden State University
Dr W.F. van Voorthuizen	Duphar, Weesp
Dr A. Westerveld	Erasmus University, Rotterdam
J.H.F. van Apeldoorn (secretary)	Netherlands Study Centre for Technology Trends, The Hague

Guest contributor:

Dr U.K. Geisler	Boehringer Mannheim, Amsterdam

References

1. H.B. Woodruff, Natural Products from Microorganisms. Science *208* (1980) 1225—1229
2. H.A.P. de Jongh, Chemisch Magazine. November (1980) 741—743
3. K. Itakura, T. Hirose, R. Crea, A.D. Riggs, H.L.Heyneker, F. Bolivar, H.W. Boyer, Expression in *Escherichia coli* of a Chemically Synthesized Gene for the Hormone Somatostatin.
4. N. Wade, Recombinant DNA: Warming up for Big Pay off. Science *206* (1979) 663—665
5. R. Crea, A. Kraszewski, T. Hirose, K. Itakura, Chemical Synthesis of Genes for Human Insulin. Proc. Natl. Acad. Sci USA *75* (1978) 5765—5769
6. D.V. Goeddel, H.L. Heyneker, T. Hozumi, R. Arentzen, K. Itakura, D.G. Yansura, M.J. Ross, G. Miozzari, R. Crea, P.H. Seeburg, Direct Expression in *Escherichia coli* of a DNA Sequence Coding for Human Growth Hormone. Nature *281* (1979) 544 — 548
7. S. Nagata, H. Taira, A. Hall, L. Johnrud, M. Strueli, J. Ecsödi, W. Boll, K. Cantell, C. Weissmann, Synthesis in *E.coli* of a Polypeptide with Human Leukocyte Interferon Activity. Nature *284*, 316 — 320
8. T. Taniguchi, L. Guarente, T.M. Roberts, D. Kimelman, J. Douhan, M. Ptashne, Expression of the Human Fibroblast Interferon Gene in *E.coli*. Proc.Natl.Acad.Sci.USA *77* 5230 — 5233
9. R. Derynck, J. Content, E. De Clerq, G. Volckaert, J. Tavernier, R. Devos, W. Fiers, Isolation and Structure of a Human Fibroblast Interferon Gene. Nature *285*, 542 — 547

10. T. Taniguchi, N. Mantei, M. Schwartzstein, S. Nagata, M. Muramatsu, C. Weissmann, Human Leukocyte and Fibroblast Interferon are Structurally Related. Nature *285*, 547 — 549
11. G. Allen, K.H. Fantes, A Family of Structural Genes for Human Lymphoblastoid (leukocyte-type) Interferon. Nature *287*, 408 — 411
12. S. Nagata, N. Mantei, C. Weissmann, The Structure of one of the Eight or More Distinct Chromosomal Genes for Human Interferon-α Nature *287*, 401 — 408
13. C.M. Croce, A. Linnenbach, W. Hall, Z. Steplewski, H. Koprowski, Production of Human Hybridomas Secreting Antibodies to Measles Virus. Nature *288*, 488 — 491
14. Ned. T. Geneesk. *124* (1980) 2193
15. Statistical Yearbook CBS (1980)
16. J.M. Tager et.al, in Birth defects: original article series, *16*, 1, (1980) 343 — 359
17. A.L. Demain, The astonisching synthetic versatility of micro-organisms. 13th International TNO conference (1980) 30 — 40
18. J.F. Marin, A.L. Demain, Control of Antibiotics Biosynthesis. Microbial Reviews *44* (1980) 230 — 251
19. K. Kieslich, Industrial aspects of biotechnological production of steroids. 13th International TNO conference (1980) 83 — 96
20. E.J. Staba, Plant tissue culture as a source of biochemicals (1980) CRC Press Inc.
21. Szmuness et al, Hepatitis B vaccine. Demonstration of efficacy in a controlled clinical trial in high-risk population in the United States. New England Journal of Medicine *303* (1980) 833 — 841
22. M. Pasek, T. Goto, W. Gilvert, B. Zink, H. Schaller, P. Mackay, G. Leadbetter, K. Murray, Hepatitis B virus genes and their expression in *E.coli*. Nature *282* (1979) 575 — 579
23. J.S. Emtage, W.C.A. Tacon, C.H. Catlin, B. Jenkins, A.G. Porter, N.H. Carey, Influenza antigenic determinants are expressed from haemgglutinin genes cloned in *Escherichia coli*. Nature *283*, (1980) 171 — 174
24. J.H.E.Th. Meuwissen, De *in vitro* kweek van *plasmodium talciparum* als eerste stap tot ontwikkeling van een malariavaccin. Ned. T. Geneesk. 123/48 (1979) 2057 — 2060
25. H. Küpper et al, Cloning of cDNA if major antigen of foot and mouth disease virus and expression in *E.coli*. Nature *289*, (1981) 555 — 559

VI Chemistry

I. Introduction

Over the past 40 years, the chemical industry has undergone revolutionary development. There are two basic factors involved: firstly, the change-over from largely coal-based to predominantly oil-based chemical technology and secondly, a more than one-hundredfold increase in output. This is illustrated by the following figures: in 1940, world production of synthetic chemicals was 3 million tonnes (59% coal-based) and in 1979, 350 million tonnes (79% oil-based). In the course of this revolutionary development, and even earlier, the old fermentation processes for the production of bulk chemicals such as acetone and ethanol became obsolete.

The declining availability and rising prices of oil and natural gas are reason enough to investigate whether other minerals such as coal can - in conjunction with the appropriate technology - form a realistic basis for the supply of chemical industry feedstocks. Attention is also being paid to the products of photosynthesis - the various types of biomass. Parallel with - but more or less independently of - this trend, there have been great advances in our knowledge and understanding of the activity of living organisms in the biosciences (microbiology, biochemistry and molecular biology). In particular, there has been spectacular progress in the fields of enzyme technology and the modification of the genetic material of organisms - especially the recombinant DNA technique.

Both these developments have led researchers to assess the ways and means by which this knowledge and understanding of the activities of living matter can be exploited in the chemical industry. The areas in which these new approaches could have a significant impact can be subdivided as follows:

- basic raw materials
- product categories
  - existing products form existing raw materials
  - existing products from new raw materials
  - new products

  - energy carriers
  - bulk chemicals
  - fine chemicals.

Process aspects will be discussed where relevant.

1.1 Basic raw materials

As pointed out above, renewable raw materials such as carbohydrates (cellulose, starch, sugar), wood and lignin - from agriculture, forestry or 'mariculture' (cultivation of the seas) - can theoretically be substituted for petrochemical products. Some carbohydrates can be readily converted into alcohol and subsequently into ethylene, but this process is much more difficult with other carbohydrates (for example, cellulose) and above all with wood. The products obtained from these raw materials by relatively simple techniques are virtually identical to those gained by petrochemical means.

The processes whereby the products are gained from these alternative raw materials can be based mainly on biotechnology or mainly on conventional chemistry. Raw materials and processing technologies are classified in matrix form in figure I. Almost all processes used by the present-day chemical industry (e.g. the manufacture of glycol from ethylene) are in the top left-hand part. One process that belongs at top right is that described in a patent by CETUS [1]. for the synthesis of propylene oxide from propylene. At bottom left we have the gasification of wood to synthesis gas to make methanol, and at bottom right the synthesis of alcohol from maize; one possible application of this product is in automotive fuel (gasohol).

PROCESS TYPE → / FEEDSTOCKS ↓	CHEMICAL TECHNOLOGY	BIOTECHNOLOGY
FOSSIL	ETHYLENE → ETHYLENE GLYCOL	PROPYLENE → PROPYLENE OXIDE
BIOLOGICAL	BIOMASS → SYNTHESIS GAS	MAIZE → ETHANOL

Figure 1. Processes and raw materials

1.2 Product categories

1.2.1

If the conditions are right, biotechnology can provide a range of processes that, while using existing raw materials, are better, more efficient and more cost-effective than present chemical processes. They use, for example, the catalytic activities of free or immobilised enzymes or cells.

Some of the ways in which production processes can be improved are as follows:

— improved selectivity
— improved yield
— fewer process stages
— lower energy costs

— simpler separation techniques
— lower investment costs
— fewer hazards for the environment.

The NOVO/DSM process [2] for the synthesis of D-phenyl glycine and the Delft University of Technology process [3] for the synthesis of mannitol are typical improvements of this type; and the economic advantages of such processes are relatively simple to estimate.

Biotechnology may enable an existing product to be synthesised from a new raw material. The interplay between product and raw material can give rise to some of the above process improvements, and the cost advantage of the new raw material can also be an important factor. Strategic and political considerations may be involved as well. Examples: the manufacture of ethylene from 'green' ethanol (e.g. ethanol from maize) and the anaerobic fermentation of grain to isopropyl alcohol and butanol.

On the other hand, biotechnology can also open up ways of synthesising entirely new products. In marketing terms, there are two aspects involved. Firstly, there is the possibility that an entirely new market will be created. This is often the case with the pharmaceutical applications for products obtained by genetic engineering, such as interferon. It can also happen that the new product competes with existing products – for example, isomerose, which is a saccharose substitute obtained from starch, and ethanol, which can be used instead of unleaded gasoline. In the first case, it may be very difficult to assess the marketing potential, and in the second case it is the competition – which can be subject to political and commercial considerations – between the old product and the new that will decide the matter.

1.2.2
Chemical products can also be subdivided into energy carriers, bulk chemicals and fine chemicals.

Energy carriers

We consume a good 80-90% of the fossil materials we use as energy carriers [4]. Recent political and economic circumstances have focussed much attention on whether photosynthesis-based energy carriers can be used, as these are generated at a rate 10 times greater than the rate at which energy is consumed [4]. It is best to view this question in terms of the products, as their number is limited and in almost all cases they are products competing on an existing market. Here, chemical engineering and biotechnological process compete with one another (for example, wood gasification as against digestion and fermentation), so that it is not possible to do justice to this aspect without dicussing chemical process types as well, even though these lie somewhat outside the terms of reference of this study. Photolytic hydrogen production is discussed in a separate chapter.

Bulk chemicals

The volume of fossil minerals used to synthesise chemical feedstocks is much lower than that used to generate energy. For bulk chemicals too, we can distinguish between types of product. The first distinction to be made is whether or not the products can fit into present petrochemical synthesis routes. Let us first take ethylene; this can be manufactured from oil but also from 'green' raw materials ('agrification' of petrochemical synthesis routes). Another instance would be the use of biotechnological processes in existing bulk chemical synthesis routes.

A second approach would be to construct a new chemical processing technology based on the chemical structure of the biological material available, making use, for example, of starch chemistry and sugar chemistry. As a relatively wide range of individual products are involved here, it is best to think in terms of product types. On the one hand, we can look for substitutes (including those obtained from microbiological processes in bulk petrochemicals) for the basic raw materials used at present in the chemical industry, and on the other hand it may be possible to develop an entirely new manufacturing technology for bulk chemicals, based, for example, on carbohydrates and other components derived from living organisms.

Fine chemicals

In the case of fine chemicals, it is simply impossible to look at individual products; the range is for too wide. They can, however, be subdivided into product groups:
— flavourings and odorants
— amino acids
— stabilisers
— insecticides
and many more.

But this categorisation still does not help much, as the category often bears no relation to the type of synthesis problem and the process type. Synthesis can range from selective hydrogenation to selective oxidation; the process can range from fermentation to the use of an immobilised enzyme. For fine chemicals, therefore, it is best to think in terms of process type and the nature of the synthesis problem – particularly when we come to look at entirely new products that do not fit into any product category. Process types can be subdivided into fermentation, the use of free and immobilised cells and the use of dead cells or enzymes.

Fermentation
In the majority of fermentation processes, the production of the catalyst, the living organism, is linked to the synthesis of the product; they take place in one and the same process. Conventional fermentation processes (for example, the

synthesis of antibiotics such as penicillin) differ markedly from those encountered in the bulk chemical industry; batch processing is general, that is to say, continuous processes are rarely used. This preference for batch processing is partly due to the traditional idea of using multi-purpose equipment (enabling various products to be synthesised in turn in one unit). Sterility requirements are also important. Situations can also frequently arise in which the production organism is modified in such a way that it would inevitably disappear by natural selection if continuous processing were used [5].
Fermentation also covers the production of biocatalysts, enzymes or entire organisms that can be used in free or immobilised form for the synthesis of bulk or fine chemicals. These processes will be discussed separately.

Immobilised cells
One refinement is the use of immobilised but otherwise intact cells. This technique is best suited for processes where the formation of the product is independent, or is made to be independent, of growth, although the use of immobilised growing cells should not be ruled out entirely.

One fundamental question is whether product formation can be dissociated from growth or not. For a large number of synthesis problems, there is no demonstrable need for this association [6], even though the formation of the product can sometimes be difficult in a non-growing system. In general, immobilised cells are to be used for the more complex syntheses, such as multistage synthesis with use and regeneration of cofactors such as ATP and $NADH_2$.

One advantage of immobilised cells over fermentation is that product formation is often much more efficient because less energy is required for growth. Moreover, immobilised cells are more suitable for continuous processing.

Immobilised dead cells or enzymes
With these techniques, a preparation is used in which nearly all of the synthetic activity has been eliminated. Only one or a very small number of chemically synthetic operations can be performed. This has the advantage of higher specificity, but a drawback is that it is difficult to carry out complicated reaction patterns in which, for example, cofactors occur.

Enzymology literature [7] describes so many possible syntheses that they are best categorised according to type (oxidation of primary alcohols to aldehydes and acids, of secondary alcohols to ketones and so on).

To conclude: in the case of energy carriers and bulk chemistry, it is best to look at the problem in terms of products and product categories; in the case of fine chemicals, in terms of process type and synthesis problem. The resultant matrix structure is shown in figure 2.

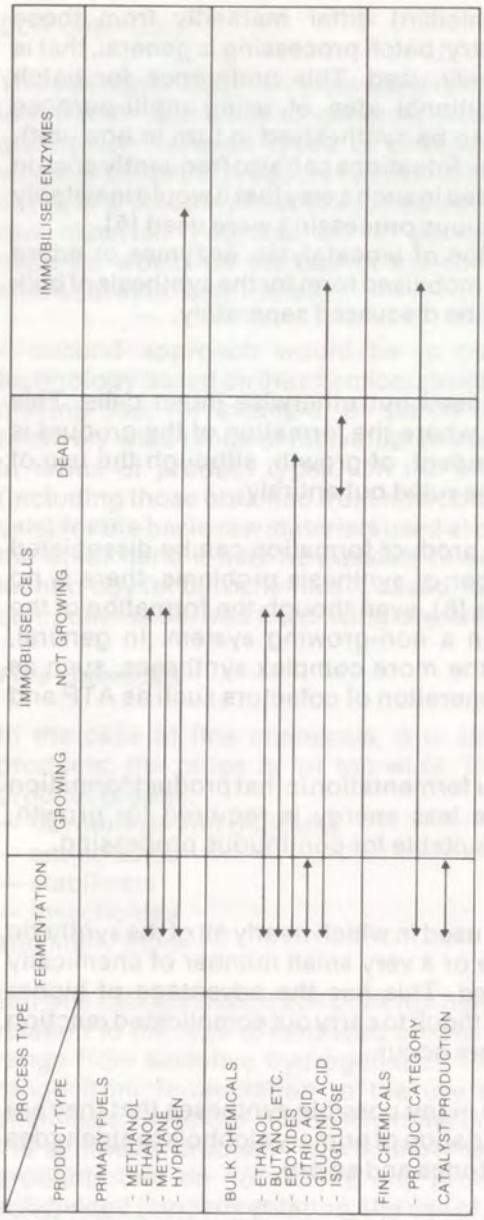


Figure 2. Application areas and process types in chemical processes based on biotechnology

2. Brief structural analysis of the Dutch chemical industry

As a result of its development over the years, the Dutch chemical industry now makes a major contribution towards the high standard of living and the level of employment in the Netherlands. It also plays an important part in the country's balance of trade [8,9,10], as Table 1 shows.

Table 1. Extent of the Dutch chemical industry

	Turnover (in Dfl. x 1 million)	Export (in Dfl. x 1 million)	Employees
1978:	22,000	18,600	94,000
1979:	27,000	24,000	92,000
Proportion of entire industry:	14%	19%	9%

Naturally, if we break down these overall figures, distinct differences between the various sectors of the industry emerge, as we see in Table 2.

Table 2. The sectors of the Dutch chemical industry (1978 figures)

Sector	Turnover (in Dfl. x 1 million)	Employees	Production (x 1,000 tonnes)
Bulk chemicals			
Petrochemicals	8,500	30,000	not available
Fertilisers	1,800	7,800	3,250
Electrochemistry	4,800	2,000	4,500
Final products			
Paints	900	6,300	230
Pharmaceuticals	1,800	12,000	not available
Rubber and plastics processing	3,300	24,400	530
Others	1,000	11,900	

The sectors involved with bulk chemicals are characterised by sophisticated technology and low intensity of labour, whereas those in which low-volume chemicals are produced are more labour-intensive and the technology is perhaps less advanced. Over the entire industry, 75% of the turnover relates to bulk chemicals and 25% to small-scale production.

Characteristic of the bulk chemical sector - in which the petrochemical industry occupies first place - are the integrated production facilities with a well-balanced infrastructure in which raw materials, products and by-products are aligned with one another. The integrated chemical complex has a certain but limited flexibility capable of accommodating fluctuations in the demand for

certain products. However, financial returns are frequently marginal, and operation must be extremely efficient to be economically viable.
The fact that the Netherlands is one of the world leaders in bulk chemicals is mainly due to this operational efficiency, created by a pool of well-trained personnel. It is on bulk chemicals that chemical technology courses at the various professional and higher education establishments concentrate. The geographical location of the Netherlands plays an important part too.

The Netherlands also possesses a relatively large share of the world's fermentation capacity. The fermentation industry generally concentrates on relatively high-grade products on a relatively small scale (usually smaller than, for example, 1975's world-wide penicillin output of 10,000 tonnes). Perhaps this is one reason why process technology in this industry has not followed the rapid pace of development in bulk chemicals process technology - which, if biotechnological processes prove attractive, could well experience further revolutionary progress. The Netherlands, with its educational emphasis on bulk chemicals, the availability of fermentation know-how and the high standing of its university research in microbiology and biochemistry, has a unique opportunity to play a leading part in this development.

The Netherlands has built up a strong position in the chemicals field. There are a number of factors that have brought this about and will, short of unexpected developments, continue to do so: experience, modern production facilities, efficient processing, qualified personnel, geographical location, to name but a few. One way in which this position could be improved still further would be to develop bulk chemicals production along the lines of a complex in which products would be further processed as well.

In principle, biotechnology could make its presence felt both in bulk chemicals and the more specialised areas of the chemical industry. The pros and cons of biotechnological and chemical processing will have to be carefully weighed from case to case; but far from always is there sufficient information available for such decisions to be taken. For some products, the biotechnological synthesis is just not feasible (for example, ethylene oxide and polyethylene), while it is a more or less realistic option for others (e.g. propylene oxide). However, there are firm prospects that biotechnology will come to play an important part in chemical processes throughout the chemical industry within the next 10 years.

3. The potential of biotechnology in chemistry

3.1 Sources of raw materials

Table 3 shows the dry yields (in tonnes per hectare per year) of natural ecosystems and certain agricultural crops. It can be seen that these yields vary widely from crop to crop, including those grown agriculturally. These variations are due to differences in climate and agricultural techniques, to

different varieties of crop and to the fact that the harvested, useful part differs from plant to plant (for example, the grains of rice and wheat but the stems of sugar cane). We see that in the Netherlands the average yield of potatoes is eight tonnes per hectare and for sugar beet 11 tonnes per hectare.

Table 3. Yields of some natural ecosystems and agricultural crops

SYSTEM	NET PRODUCTION LEVEL (T/HA/YEAR)
Naturel systems	
. Spruce forest, Germany	15
. Grassland, New Zealand	30
. Tropical forest, West Indies	approx. 60
. Algae on coralreef Marshall Islands	50
. Seaweed, Novo Scotia	20-25
Agricultural systems	
. Sugar beets, Netherlands [10]	11
. Potatoes Netherlands [10]	8
. Wheater, Netherlands [10]	
straw	3.7-4.2
grains	4.3-6.2
. Corn (maize), USA	10-14
. Corn silage, USA	approx. 27
. Soybeans	2.5-4.5

These crops - like all other vegetation - live and grow by using sunlight to convert carbon dioxide and water into sugars (carbohydrates) with the aid of chlorophyll, their green pigment. This process is termed photosynthesis. (It

should be noted that 20-40% of the sugars produced are oxidised to CO_2 and water to provide the plant with energy for its own growth and maintenance.

If we consider this process in terms of energy conversion, we see that sunlight - radiated to the earth's surface at rates ranging from 100 to 500 W/m² - is converted to harvestable vegetable matter having energy contents of 0.65 to 1.5 W/m². Although this means that the efficiency of photosynthesis is something like 1%, it is estimated that, over the entire world, sunlight is being converted into vegetable matter at a net rate of 95 terawatts per year: about 26 terawatts in hard-to-harvest phytoplanktons, seaweeds, etc., in the oceans, about 40 terawatts in woods and forests and 20 terawatts in other vegetable matter [11,12].

These energy production rates are large in comparison with the world's present annual energy consumption of about 9 terawatts, but not so large when set against the world's predicted annual energy consumption of 20 terawatts by the year 2000 [11].

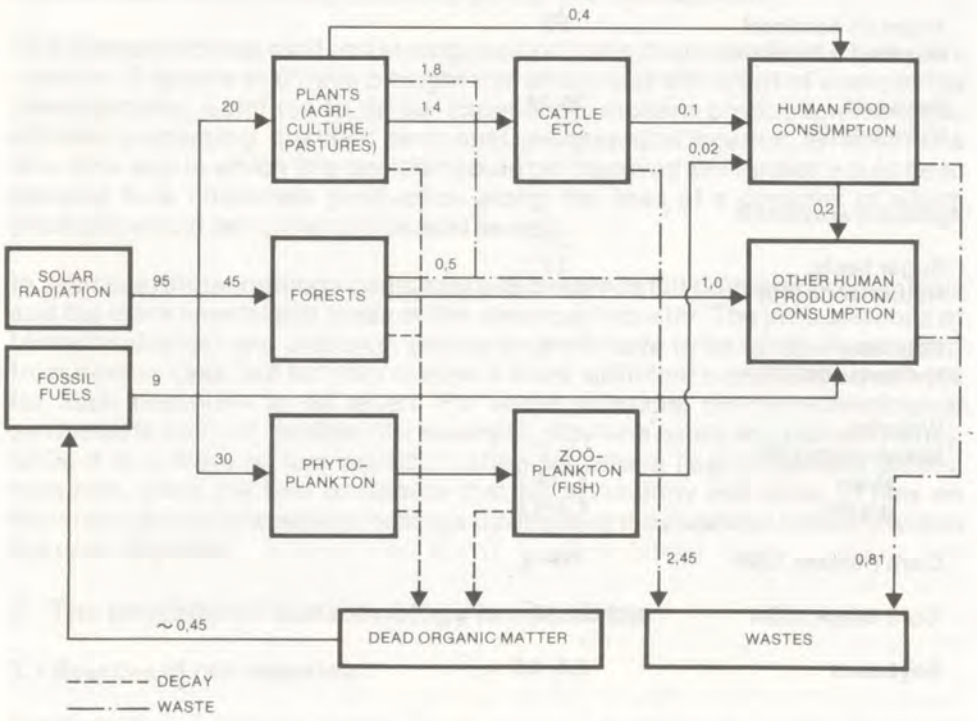


Figure 3. Global energy relations

In agriculture and forestry, solar radiation is thus used by man to produce vegetable matter - biomass. Some of the captured solar energy is consumed as human food, animal feed, fuel, building materials, etc. At the same time, man fishes the seas and so retrieves a small portion of the solar energy which, via the growth of phytoplankton and the marine food chains, is entrapped in fish growth.

Each of these biomass flows is also an energy flow. Figure 3 illustrates these energy flows and those of fossil fuels, measured in terawatts. The thick arrows indicate the flows directly involved in man's consumption of food and other activities. Over the entire world, these flows represent 1.5 TW of plant biomass plus about 1 TW timber and about 9 TW fossil fuels. A great part of the plant biomass is used as feed for livestock and pets. Together with 0.02 TW in the form of fish, this comes to 0.52 TW for food and stimulants, equivalent to 130 W per head of population. Man's activities in forestry, livestock farming, industrial production and consumption create a waste flow of about 1.3 TW. About 70 TW (gross) of solar radiation are required to produce the timber, food and stimulants by photosynthesis. Allowing for the respiration and maintenance of crops and forests (about 30%) and phytoplankton (about 50%), the net consumption of photosynthesis biomass is 37 TW per year [13].

Similar calculations can be performed for the Netherlands [14]. Figure 4 shows the energy balance for that country's agricultural and foodstuffs industries for 1975. Three main areas are to be distinguished in this diagram: agricultural and market-garden production, livestock farming and the foodstuffs industry. The Netherlands has about 1.6 million hectares of arable land; its fossil fuel requirement is about 12 GW, including the fossil fuels required outside the Netherlands for the production of raw materials for animal feed and the transport of these raw materials to the animal feed industry in the Netherlands. The fuel costs for the foodstuffs industry are inclusive of transport and packaging. A significant amount of agricultural produce in the form of forage and animal feeds, together with imported feeds (which are created by photosynthesis elsewhere) is earmarked for livestock farming. For every hectare devoted to animal feed production in the Netherlands (about 1.3×10^6 ha), there are two outside the Netherlands from which raw materials are imported for that purpose.

It is livestock farming which provides dairy products, meat and eggs for human consumption. The foodstuffs industry imports raw materials and intermediate products having an energy content of 2.7 GW and delivers about 4 GW-worth of food, of which half is consumed in the domestic market. Livestock consumes a waste flow from the foodstuffs industry of 1.3 GW, which is quite a good recycling rate, and produces about 6 GW in the form of wastes - manure, methane and urine.

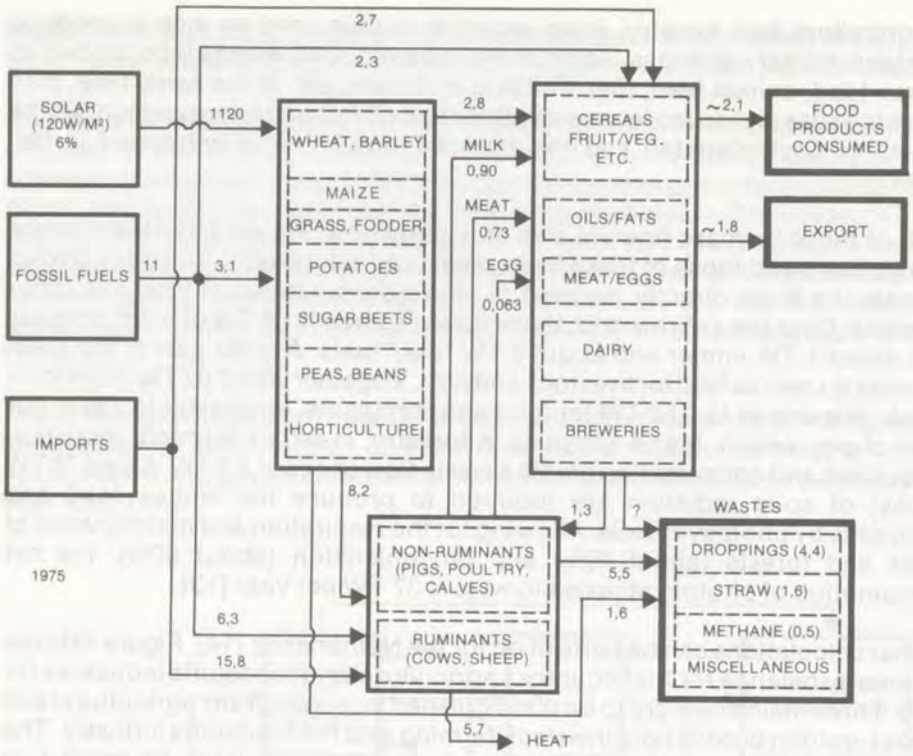


Figure 4. Energy relations in the Dutch agriculture/food industry

Apart from the abovementioned waste products from agriculture and livestock farming, animal and vegetable domestic wastes and domestic and industrial waste water are also potential sources of raw materials [15]. Another possibility, already put into practice, is the cultivation of biomass as raw material for energy production (energy farming) or for non-foodstuffs industries. Such potential raw materials are summarised in Table 4.

Table 4. Potential sources of raw materials

— AGRICULTURAL WASTE *(straw)*
— LIVESTOCK WASTE *(manure)*
— DOMESTIC WASTE *(waste water, household refuse)*
— BIOMASS CULTIVATION FOR ENERGY
— BIOMASS CULTIVATION FOR INDUSTRY

There are, of course, many potential technologies for the manufacture of energy products, substitutes of petrochemical derivates and fine chemicals, based on plant cultivation with subsequent chemical, biochemical or biological conversion processes. They can be dubdivided as follows:

— production of energy carriers such as SNG (substitute natural gas), methanol or ethanol;
— production of base chemicals for the petrochemical industry, such as ethylene, methanol, ethanol, butanol, acetaldehyde, sorbitol and hydroxymethyl furfural;
— the direct use of plant-derived products as raw materials for the chemical processing industry, such as sugar, starch and cellulose;
— extraction of plant metabolites for special industrial or pharmaceutical purposes, such as vegetable oils, fats, alkaloids and resins.

The advantages of these processes are that the energy and raw material sources are renewable and that carbon dioxide is re-used in a short cycle. Unfortunately, in the use of biomass as energy carrier, there are many disadvantages as well. Apart from the possible use of wastes and the production of raw materials by mariculture, energy farming requires substantial arable acreage.

To round off this discussion of raw materials and their availability, the major categories of biomass are summarised in Table 5.

Table 5. Sources of renewable raw materials

PHOTOSYNTHESISED RAW MATERIAL	PRODUCT
Sugar cane	Cane sugar, molasses, cane sugar juice (saccharose)
Sugar beet	Beet sugar, beet sugar syrup
Cereals (rice, maize, wheat, mittel, sorghum)	Maize starch etc.; starch hydrolysate (glucose, maltose, etc); starch hydrolysate with intermediate degree of polymerisation (DP 6-30)
Roots and tubers (potatoes, cassava)	Potato starch etc.; starch hydrolysate; starch hydrolysate with intermediate DP
Trees, straw, fibrous matter	Cellulose, lignin; cellulose hydrolysate (glucose)
Waste flows	Potato juice, spent sulphite liquors, maize steep liquor, Vinasse (distillate from liquor still residue); chaff, maize cobs, brans, coconut shells, leaves, chitin (krill); whey (lactose)

Only to widely varying extents can these raw materials be processed by the technologies currently available. For a number of processes, the starting material must first be broken down into monomers or dimers. For starch, this technology is already well advanced: by acid hydrolysis or, as a more recent

development, enzymatic decomposition with amylase and amyloglucosidase, a substrate is readily obtained that is suitable for a wide range of microbial conversion processes. For saccharose, this indirect route is not necessary: it can be converted straightaway by the action of organisms. The decomposition of cellulose, and in particular of wood, however, still presents many problems: acid hydrolysis is expensive and gives a yield of not more than 50%; enzymatic hydrolysis is impeded by the crystalline structure of cellulose and its cross-linking with lignin. At present, no cost-effective method is known for the conversion of wood and related materials into a fermentable substrate.

3.2 Production of energy from biomass

3.2.1 Carbon-based energy carriers

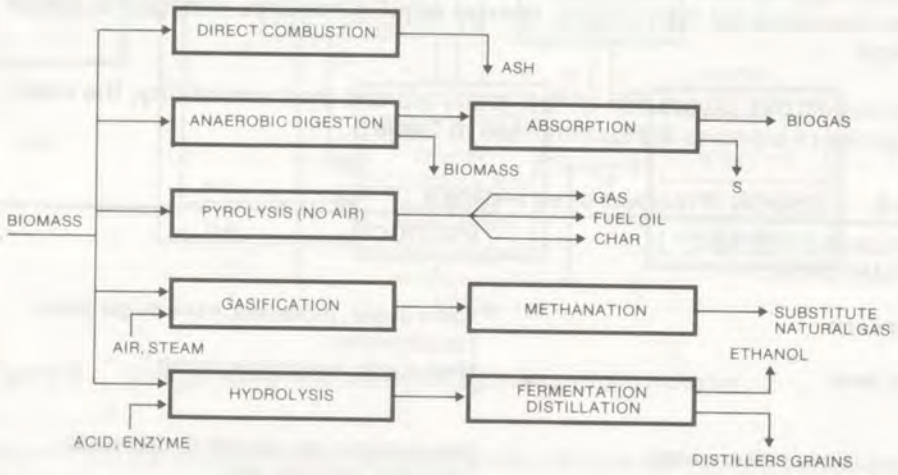


Figure 5. Some technologies for the production of energy carriers from biomass

Figure 5 gives an overview of a number of technologies for the production of energy carriers from biomass.

- Direct combustion (incineration) in steam-raising furnaces. In the short term, this is the most practical way of making efficient use of municipal or agricultural waste.
- Digestion to produce methane. This method is used in the Netherlands, for example to purify the liquid wastes from sugar refineries and to process piggery manure. The methane is used as fuel.
- Thermal decomposition at temperatures of 300-500°C in absence of oxygen. The decomposition products include tar, oil, carbon, methane, water and carbon dioxide.
- Gasification with addition of oxygen, air or oxygen plus steam. The reaction product is a relatively low-BTU fuel gas, but the heating value can be

increased to that of natural gas by including a methanation stage in the process. The production of methanol from agricultural and forestry wastes will, if economically viable, probably be carried out by first producing synthesis gas (a mixture of hydrogen, carbon monoxide and carbon dioxide) by gasification of the waste. At present, methanol is mainly produced from synthesis gas obtained from natural gas or naphtha.

— Ethanol can be obtained from agricultural and forestry wastes by first breaking down the cellulose and/or starch fractions by enzymatic action into maltose or glucose. The sugar solution obtained in this way is then fermented to an alcolhol-containing solution which is concentrated by distillation.

However, there are a number of problems associated with the production of energy from biomass. The arable acreage required is considerable, so that production would come into competition with food production. It is therefore unrealistic to think that the world's energy requirement could ever be met with biomass, but regions with large surpluses of agricultural produce or arable land could well find it worthwhile to adopt these techniques. Heavily forested regions could also be promising. With political and other encouragement, 'agrification' of the energy supply could play an important part in some parts of the world - for example, Brazil and the United States.

Besides, there is a second problem to be considered: the energy balance [16]. Energy farming must deliver a net energy output - but account must also be taken of what this energy costs in terms of fertilisers, biocides and agricultural energy consumption. For most biomass-based energy carrier production processes, the energy balance is negative (see Table 6). In particular, production of ethanol from wood does not seem posible at present for this reason, and also because the carbohydrates in this material are difficult to break down rapidly and completely into monomers.

Table 6. Energy balances for production of ethanol for some crops

RAW MATERIAL FOR ETHANOL	ENERGY REQUIREMENT (GJ/t)	NET ENERGY (GJ/t)
Grain, USA	47	- 28
Straw	222	- 195
Wood, acid hydrolysis	98	- 79
Wood, enzymatic hydrolysis	240	- 221
Sugar cane	15.7	+ 3
Sugar cane, Brazil	22	+ 5.6
Cassava	34.6	- 7.2
	61	- 4.2
Cassava, Brazil	24	+ 3.5

Little research has been done into the efficiencies of the various gasification or pyrolysis processes. In the case of ethanol and methanol, however, it can be argued that an energy balance as such does not give a fair picture. As they can be used to fuel combustion engines, they have a premium value higher than that measured in conventional energy terms. If energy from a low-grade source such as coal or wood can be upgraded to a premium-value fuel, this will tend to offset an unfavourable energy balance. This argument also applies for the conversion of coal into methanol, for example.

For the Netherlands, the prospects for agrification of the energy supply do not appear very promising. It would not be possible to increase arable acreage to such an extent that, say, 5-10% of the domestic energy requirement could be satisfied from biomass.What could be grown and how much land would be required? Poplars (timber), maize or potatoes (starch, alcohol), sugar beets (sugar alcohol)? Electrical power can be generated from wood, and alcohol (ethanol) can be produced from potatoes, maize and sugar beets. A few figures will make the position clear.

The Netherlands consumes natural gas, oil, coal and nuclear energy at a rate of about 90 GW. Imagine that electrical power is to be generated from timber at a rate of 0.7 GW (Dutch electricity consumption, by the way, is over 7 GW). This would call for an amount of wood having a combustion heat content of at least 1.7 GW so that, with fast-growing poplars giving an annual yield of 20 tonnes per hectare, about 77 per cent of the Netherlands' arable land would be required for this purpose alone.

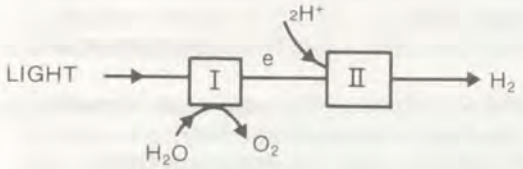
Another example: imagine that, following the Brazilian example, 20 vol% of ethanol were to be blended into gasoline in the Netherlands. The Dutch consume gasoline at a rate of more than 5.2 GW, so no less than 700,000 tonnes of ethanol would be needed per year. This would in turn require 1.2 million tonnes of sugar as starting material, which could be produced by growing either sugar beets on 13-22%, or potatoes on about 35%, or maize on 68% of the Netherlands' arable land.

There are, however, opportunities for producing energy from waste in the Netherlands. At a rough estimate, about 2 GW of energy products could be gained from all domestic waste, straw and livestock waste together. Since these waste materials are scattered all over the country and have a low energy yield to weight ratio, collection, storage and transport costs will weigh heavily against their actual use as raw materials.

3.2.2 Biophotolytic hydrogen production

Because hydrogen is a particularly clean and environmentally acceptable energy carrier, attention is being increasingly devoted, on a worldwide scale, to research into the biophotolytic production of that gas. Photocatalytic hydrogen production with the aid of inorganic systems (semi-conductors, coordination compounds) will not be discussed in this section.

Biophotolytic hydrogen production proceeds in accordance with the following diagram:



It has been known for some time that hydrogen can be produced on a small scale by exposing an aqueous suspension of isolated chloroplasts (I) to light in the presence of certain electron carriers and the enzyme hydrogenase (II). The idea underlying this process was derived from the process of photosynthesis.

Photosynthesis is a process of initial charge separation under the influence of light in a reaction centre containing an electron donor compound (D), an electron acceptor compound (A) and a light-absorbing pigment (P). This proceeds in a number of stages, finally resulting in charge separation (as in a battery). The energy required for this charge separation is taken from the absorbed light. *In vitro* the DPA complex is arranged in such a way that the electrons transferred to A cannot, under normal conditions, return to D. The net outcome of this reaction is that water is split into hydrogen and oxygen. In most cases, the hydrogen is not liberated in the gaseous state but is used as reducing agent for carbon dioxide, which is thus converted into carbohydrates (sugar). In bacterial systems, hydrogen production is possible. For light energy to be fixed in the form of chemical energy, stored in carbohydrates, eight electrons are needed for each CO_2 molecule converted, so that the theoretical maximum energy efficiency is about 12%. Under field conditions, the actual measured efficiency is much lower, about 1%, owing to many losses in the system, the most important of which is respiration.

Photosynthesis has a number of important properties:
— it is an effective collector system for diffused sunlight;
— the photochemical activity is arranged so that charge separation is irreversible;
— biophotonic conversion of light, so that even red light (which has a smaller energy content than blue light) is able to split the water;
— a multipigment system, adapted to suit the solar spectrum, can be used.

There are quite a few problems entailed with the theoretical arrangement described above, of which the most important are:
— the instability of the biological components;
— deactivation of the hydrogenase by the oxygen liberated.

The intermediate electron carriers used are also oxygen-sensitive in many cases. In the laboratory, this can be counteracted by adding the oxygen-

removing system glucose-glucose-oxidase. In the Netherlands, a number of groups - working closely together - are attempting to optimise this laboratory system. The aims in mind are:
— to produce hydrogenase on a large scale;
— to stabilise the hydrogenase so that it can be bonded to a fixed carrier (while retaining its activity);
— to investigate the mechanism and structure of hydrogenase in order to imitate it by means of model compounds (increased stability);
— to increase the hydrogenase yield with the aid of recombinant DNA;
— to search for other, better (non-oxidising) electron carriers.

Research in laboratories abroad has already demonstrated that synthetic iron-sulphur cluster compounds (analogues of the active site of hydrogenase), bonded to an albumin fragment, also exhibit hydrogenase activity. However, practical applications are not to be expected for five or ten years to come, despite the ever-increasing efforts devoted to this purpose in the Netherlands and elsewhere.

Nonetheless, many leading researchers are convinced that commercial production of hydrogen by photolysis (either biotechnologically or by inorganic catalysis) will indeed be a practical option within five years or so, and that developments over the past two or three years have proceeded much faster than had been predicted some three years ago [17].

3.3 Biotechnology and base chemicals

3.3.1 Introduction

In discussing the applications of biotechnology in bulk chemicals, a distincion will be made between processes in which the raw material is of fossil origin and those in which biomass or its constituents are used as raw material. Process types will also be subdivided into conventional chemical (for bulk chemicals, often petrochemical) methods and those in which use is made of micro-organisms or parts thereof.

3.3.2 Petrochemistry and biotechnology

Petrochemistry as it exists at present is based on the use of oil and natural gas as raw materials. These are converted into a relatively small number of base chemicals - for example, ammonia, methanol, ethylene, butadiene, propylene, benzene and xylene -that are used to synthesise a very wide range of chemical products. It is typical of the processes in petrochemistry that they often take place in the gaseous phase, that temperatures and pressures are very high and that heterogeneous catalysis plays an important part. It is basically conceivable that biological synthesis routes - for example, the CETUS epoxide synthesis process [1] - could be substituted for petrochemical synthesis routes. The advantages that could be gained by the use of biochemical or microbiological

routes include the following:
— higher speeds;
— use of cheaper or less toxic auxiliary sybstances (aids);
— greater energy efficiency in that lower temperatures and (possibly) lower pressures can be used.

Although these types of process could offer definite advantages in large-volume chemicals production as well, we should not be over-optimistic as to their chances of being put into commercial practice in the short or medium term for the following reasons.
— Although researchers are experimenting with enzymes in environments other than aqueous ones, there has as yet been little experience of and progress in the use of enzymes or micro-organisms in water-free or virtually water-free systems. Moreover, the products and starting materials often have limited solubility in water, which tends to restrict the volume yield. Moreover, the compounds involved often have a denaturing effect on the enzymes or are toxic to the organisms. There are many fundamental problems to be overcome, and the experience acquired so far has been limited.
— Major developments, that have hardly yet started, will be needed in biochemical reactor technology before full-scale use can be made of complex multi-phase systems.

At present, most experience has been gained on conversions in the methane - methanol - formaldehyde - formic acid series. This forms the basis of Single Cell Protein technology. In this field, fundamental knowledge is steadily being acquired, but the enzyme systems appear to be so complex that no practical applications should be expected in the short to medium term.

Taken all round, biotechnological applications do not seem likely for the time being in the bulk chemical industry. However, the American oil industry has expressed interest in the CETUS epoxide and other processes: whether because feasible processes are expected, or to acquire experience in a field of general potential interest, remains to be seen.

3.3.3 Base chemicals from biomass

There are a number of conversion technologies available for the manufacture of base chemicals from biomass:
— thermal methods, whereby the biomass is converted into a synthesis gas consisting of carbon monoxide, carbon dioxide and hydrogen;
— chemical conversion routes, in which use is made of the specific properties of sugar structure;
— fermentation processes;
— enzymatic conversion routes making use of sugar structure.

An important distinction should be made between products that can be

incorporated into existing petrochemical synthesis routes and those that form the basis for an entirely new type of chemistry.

Production of chemicals for existing petrochemical processes

Conversion via synthesis gas

The production of synthesis gas from biomass does not actually lie within the scope of this paper, but the use of renewable raw materials in the chemical industry cannot be properly discussed or the prospects of biotechnological processes assessed without touching on this process as well.

Synthesis gas – a mixture of carbon monoxide, carbon dioxide and hydrogen – can be produced from all sorts of carbon-containing raw materials. In present technology it is formed from oil and natural gas, as the first step in the synthesis of ammonia and methanol, for example. Biomass can also be converted into a sort of synthesis gas, which means that, in principle, it too can be used as a starting material in the chemical industry. Wood, starch and saccharose can be used for this purpose, although many sorts of biomass have too high a moisture content and must first be dried.

One of the products that can be obtained from synthesis gas is methane: shift conversion of carbon monoxide to carbon dioxide and hydrogen is followed by methanation (reduction of the carbon dioxide with the aid of hydrogen). This conversion can be performed in the gaseous phase at high temperatures and pressures or, with a microbial process [18], in the liquid phase at low temperatures and pressures. Few data are as yet available on microbial technology, so that these two types of process are difficult to compare. In principle, the microbiological process can also be used for the methanation of synthesis gas obtained by the gasification of coal, and the use of biomass-based synthesis gas will have to be compared with this route as well.

One of the most fundamental raw materials that our society uses is ammonia. The largest consumer is the fertiliser industry, but it is also used for the manufacture of explosives. The starting material for ammonia production is nitrogen, together with natural gas, synthesis gas or other hydrocarbons to supply the hydrogen required, so it should also be possible to produce this chemical from biomass by pyrolysis, gasification or anaerobic conversion. In the last case, the ammonia is a by-product of the organic nitrogen-containing fermented material, which can be bonded by absorption in sulphuric or nitric acid. Because of its high nitrogen content, the residual biomass can be used as fertiliser. On the other hand, production from biomass of substitute natural gas (which is the best starting material for the Haber-Bosch ammonia process in terms of capital investment and energy consumption) cannot compete with that of coal-based SNG. If ammonia can be synthesised at lower pressures and temperatures than are usual at present[1] (as biochemists think could be done if

[1] 25×10^6 Pa and 720 K

better biocatalysts become available), it will, in the future, be possible to attain greater efficiency levels in ammonia production.

Direct methanation of biomass
Direct conversion of biomass into methane is also possible. The process is basically the same as that used for the purification of waste water by anaerobic digestion. Methane is formed at a low temperature in the liquid phase by means of acids and a microbiological synthesis gas consisting of hydrogen and carbon dioxide. The advantage of using biomass-based methane instead of ethanol in petrochemical routes is that the separation problems involved with ethanol are eliminated.

In present-day waste water purification technology, methane is produced at a mere rate of 5 m^3 per m^3 reactor volume per day, but this is a poor indicator of the maximum rates at which methane could be feasibly generated in a microbial process. With the use of hydrogen and carbon dioxide, it could be produced at a rate of 5 m^3 per m^3 reactor volume per hour, even with the present, highly rudimentary, state of the art.

The use of fermentation ethanol in the chemical industry
About 4% of world consumption of fossil fuel goes towards supplying the chemical industry with feedstocks. In the Netherlands, which has a highly developed bulk chemical industry, the percentage is significantly higher. The carbohydrate fractions of many plants (sugar cane, cassava, potatoes, grains and sugar beets, in that order, are the most promising candidates) can be fermented into ethanol by chemical or enzymatic hydrolysis, which again represents a potential use of biomass in conventional petrochemical routes. At present, there is no equivalent process for the use of wood for this purpose.

Yeasts (such as *Saccharomyces cerevisiae*) and bacteria (such as *Zymomonas mobilis*) can convert monosaccharides and disaccharides into alcohol. By means of this process, up to 0.51 tonnes of alcohol can be produced from 1 tonne of hexose. However, the fermentation and subsequent ethanol isolation stages require a great deal of energy, so a cheap source must be available for this purpose. In the case of sugar cane, the fibrous by-product (bagasse) can be incinerated, and perhaps the non-fermentable fractions of other crops – as well as wood, coal, etc. – could also be used in this way.

Ethanol can be processed into ethylene, acetaldehyde, butadiene, butanol, etc., by existing catalytic techniques. In this way, the petrochemical industry could be kept going 'on green' – that is, on feedstocks originating from biomass. Evaluation of the economic advantages of this approach has progressed further than in the case of the other alternatives discussed above.

Price comparisons between petrochemical and fermentation ethanol process have been made for a wide range of materials, albeit based on the Brazilian situation, which is strongly biased towards the latter alternative (Table 7).

Table 7. Price comparison for petrochemical and fermentation-ethanol production of base chemicals (first quarter of 1979)

Product	Price $/tonne	
	Fermentation ethanol	Petrochemical
Ethylene	782	393
Acetaldehyde	495	484
Acetic acid	575	563
Octanol	1,445	1,204
Butadiene	1,181	563

Although the price differences are small for some chemicals, a petrochemical industry based on fermentation ethanol cannot yet be considered feasible, even under the highly advantageous Brazilian conditions. But it could well be that, in the long term and in regions with surpluses of agricultural produce or potentially arable land, this technology will prove promising. In any case, the following considerations should be borne in mind.

— Political factors can affect the availability and price of fossil raw materials at very short notice. Depending on local conditions, 'green' technologies will become interesting when oil costs $ 20 [19] or $ 30 [20] per barrel.
— If the carbohydrate substrate is produced from waste, which may even have a negative value, the ethanol route becomes much more attractive. This could well be the case if ways were developed of using wood as a source of glucose. However, little progress has been made on this so far.
— Developments in the technology for processing ethanol by fermentation.

Conventionally, ethanol is produced by fermentation in a batch process, but this has a number of disadvantages when applied on a commercial scale:

— low average output per unit volume;
— batch growth causes the organism to have a high average growth rate, which adversely affects the glucose-to-ethanol conversion efficiency (approx. 85% [6] of theoretical maximum);
— the separation technology used is energy-intensive.

The first two drawbacks could be eliminated by developing a continuous processing technique in which the organism's mean growth rate is suppressed by recirculation of the biomass or by the use of immobilised micro-organisms. This could increase volume production and the alcohol yield could approach the theoretical maximum.

To counteract the third disadvantage, less energy-intensive separation systems and methods for increasing the ethanol concentration in the process will have to be developed. With the present state of the art, it is hardly possible to get ethanol concentrations greater than 12%. For higher concentrations, organisms with better solvent tolerance are needed; perhaps these could be obtained with genetic techniques.

Another important way in which the efficiency of alcohol production could be improved could be to create, for example by genetic means, organisms with a less efficient energy balance [6], or even in which energy production is dissociated from the biosynthesis. This can be partially achieved by using the bacterium *Zymomonas sp.* instead of yeasts. Metabolic energy conservation is twice as low in bacteria than it is in yeasts, which is why a great deal of attention has been paid to *Zymomonas* techniques in recent years.

It is interesting to estimate the amount of arable land that would be needed to produce ethylene from biomass ethanol. For an ethylene output of 500,000 tonnes per year (comparable to that of a large naphtha cracker), 820,000 tonnes of ethanol derived from 1,500,000 tonnes of sugar would be needed. If produced from sugar beet, this would require about 16-28% of all argricultural land (1.6 million ha) in the Netherlands; if produced from potatoes, about 40% and if produced from maize, about 85%. Moreover, these figures are on the low side as they do not allow for differences in soil fertility or the need for crop rotation. In countries such as the Netherlands, therefore, the production of ethylene from biomass is extremely unlikely, now or in the future. On the other hand, the Brazilian company Salega Industrias Quimicas is planning the construction of a 120,000 tonnes per year ethylene plant embodying the Rhône Poulenc/Litwin process and using biomass as feedstock.

At a conference held in Toronto in 1978 to discuss the supply of raw materials for the chemical industry, the expectation was expressed that, by early next century, gas, oil, coal and tar sands would still be the main raw materials for the large-volume chemical industry, but that the use of biomass as raw material on a commercial scale would then gradually become more and more attractive. It follows that serious study must be devoted to the improvement of existing techniques and the development of new technologies.

Production of specific base chemicals

Apart from the petrochemical routes involving ethanol, methane or synthesis gas, specialised anaerobic processes are available for the production of certain base chemicals such as acetic acid, propionic acid, butyric acid, lactic acid, oxalic acid, fumaric acid, citric acid, ethanol, isopropanol, butanol, butanediol, glycerol and acetone. These processes can be highly selective and have high energy efficiency. Apart from ethanol production, fermentation to butanol and acetone or isopropanol is of interest. If oil prices climb to about $ 20 per barrel [21, 22], these processes would become competitive. The following quotation, although already overtaken by events, is revealing: '*...dass die hochwertigen Produkte Butanol und Aceton aus Zucker bei einem Rohölpreis von $ 17/barrel wettbewerbsfähig werden könnten, also möglicherweise ab Mitte der 80er Jahre* [22]. It is questionable whether evaluations based on oil price limits are in fact realistic. For example, it has yet to be seen whether rising energy prices will cause the Hibernia process for the direct hydration of propylene and butylene to be displaced by these microbiological routes.

3.3.4 Carbohydrate chemistry

Introduction

The carbohydrates starch, cellulose, saccharose and lactose can be converted by carbohydrate chemistry: a group of chemical processes in which the starting material and end product are carbohydrates. An important use of carbohydrates is in what are termed derivatised products. Derivatising often involves the conversion of some of the chemically reactive hydroxyl groups of the starch or cellulose molecules into other groups – for example, cellulose esters and ethers, starch esters and ethers and cross-linked starches.

Until 1960, carbohydrate chemistry was completely dominated by chemical conversion methods, but since then biotechnological methods have also come to be used for some processes; in particular, the production of starch hydrolysates (i.e. starch that has been broken down into relatively low-molecular material) and the conversion of glucose to fructose. This can be seen as the substitution of a biotechnological method for a chemical method (see figure 1). As already outlined in the introduction, biotechnological processes have the following advantages [23].

— Fewer by-products are formed (higher selectivity), so taste and odour are not impaired so much and purification costs are reduced. As the enzymes have greater selectivity, a wider range of products with closely defined properties can be achieved.
— Investment costs are lower because of the lower temperatures and pressures and the absence of highly corrosive hydrochloric acid.
— Energy costs are lower.
— As no hydrochloric acid is used, there is no salt to remove after neutralisation.

Because of these advantages, the proportional use of biotechnological methods for starch hydrolysis has risen from around 5% in 1960 to around 90% in 1980.

Enzymes and carbohydrate conversion

There are three groups of enzymes already playing, or expected to play, an important part in carbohydrate chemistry: isomerases, transferases and hydrolases.

An isomerase [24] can convert one type of carbohydrate monomer into another; for example, glucose isomerase, an enzyme which can convert glucose into fructose (see section IV.3.10).

Transferases are able to transfer a monomer or polymer carbohydrate onto an existing polymer; one or more monomer units of the chain are replaced by one or more units of another type. For example, the enzyme glucosyltransferase

which can make saccharose and glucose from maltose and fructose; the branching enzyme which makes amylopectine (a branched polymer) from amylose (a straight-chain polymer); and the enzyme cyclodextrineglucosyltransferase which makes a cyclic compound from starch.

The third group are hydrolases. These are able to cleave the bonds between monomer units or groups of monomer units, often in very specific ways. We know alpha-amylase, beta-amylase and amyloglucosidase, each of which converts starch into oligomers (polymers with a limited degree of polymerisation) or monomers according to its own pattern. The debranching enzyme converts branched starch constituents, the amylopectine molecules, into straight-chain amylose molecules. Cellulase is able to convert cellulose into glucose. The enzymes invertase and lactase convert saccharose and lactose respectively into mixtures of the monomers of those disaccharides.

The enzymes are generally obtained from micro-organisms, but can also be obtained from plants. They can be used in two different ways: either as dissolved enzymes or as immobilised biocatalysts. These uses have already been discussed in section II.4.
There are both advantages and disadvantages attached to the use of immobilised enzymes. Such enzymes are often more expensive to produce so that, even with recycling, the treatment costs per unit product can be high. Particularly when used in polymer or oligomer substrates, the limitation of diffusion in immobilised enzymes can cause problems. This is one reason why immobilised amyloglucosidase is as yet unable to compete with the dissolved form of that enzyme.

At present, commercial preparations of immobilised glucose isomerase and lactase are available on the market. Alpha-amylase, beta-amylase and amyloglucosidase are used almost exclusively in dissolved form, partly because of their low price.

Hydrolysis of polymer and oligomer saccharides

In natural starch, there are basically two types of bond between the glucose units of the polymer. The type that occurs most is the alpha, 1-4 bond: about 25% of starch consists of the straight-chain polymer amylose which has this type of bond only. The alpha, 1-6 bond occurs much less frequently and forms the basis for the branchings in the branched polymer amylopectine, which constitutes about 75% of the starch.

Since 1940, amylases have been used in the starch industry for the manufacture of syrups of partially hydrolysed starch. Since 1960, the enzymes have been made with the aid of micro-organisms and have dropped in price so dramatically that conventional acid hydrolysis is virtually obsolete.

There are three amylases used in the starch-processing industry [25].

— Alpha-amylases. The property of these enzymes is that they attack the starch chains relatively randomly (endo-enzymes), cleaving alpha, 1-4 bonds only. The branch points of the amylopectine chains are not attacked. In this way, the starch molecule is rapidly chopped up into relatively large chunks. The extent to which the starch is broken up is expressed by the Dextrose Equivalent (DE) of the hydrolysate. The DE is equal to 100 minus 100 times the fraction of the original inter-glucose bonds still intact. Thus, starch has a DE of O, glucose a DE of 100. As the alpha-amylase continues its activity, the following products are formed.
  — Starch with DE 0 to 3. In these products, the starch chain has been broken at a few points so that the viscosity is low and starch solutions with high dry-matter contents can be produced. This process, starch liquefaction, is used in the starch processing and paper industries.
  — Maltodextrin with DE 3 to 20.
— Beta-amylase occurs in plants, from which it is extracted in the form of malt or malt extract. This enzyme separates maltose step-by-step from the terminals of the starch molecules until a branch point is reached that the enzyme is no longer able to attack. Starch is split by beta-amylase into maltose and what are termed limit dextrins (large molecules containing all branches).
— Amyloglucosidase occurs in various micro-organisms from which it is isolated. This enzyme can hydrolyse both the bonds in the straight chain and those on the branch points. Theoretically, this enzyme is able to convert starch into glucose completely, but in industrial practice a conversion level of 95-97% glucose is attained with this enzyme. With the former technique, acid hydrolysis, it was only possible to achieve conversion levels of 87% glucose.

By using one or more types of amylase (in some cases in conjunction with acids), it is possible to produce starch hydrolysates of desired composition (as regards contents of glucose, maltose, maltotriose, etc.). The most important starch hydrolysates are summarised in Table 8 [26]; these products are manufactured on a very large scale, with total world output amounting to about seven million tonnes per year.

So far, we have discussed some biotechnological processes that have already been developed. Future developments may be expected in the following fields [27]:

— The debranching enzyme isomylase is able to hydrolyse only the 1-6 bonds of the amylopectine molecules. In this way, amylopectine is converted into short straight amylose chains with about 25 glucose units. From starch, isoamylase activity creates a mixture of long and short amylose molecules which can be separated from one another if need be. So far, no commercial application has been found for the low-molecular amylose. If such a mixture of straight-chain amylose molecules is treated with beta-amylase, a mixture

Table 8. Starch hydrolysates and their properties [26]

PRODUCT	DE	PRODUCTION METHOD	APPLICATIONS
Liquefied starch	0-3	α-amylase activity at 60-100° C	Starch processing; paper industry.
Malto-dextrins	3-20	Continued α-amylase activity	Foodstuffs.
Glucose syrup	20-70	Hydrolysis with acids, α-amylase, β-amylase and/or amyloglucosidase	Fermentation substrate, foodstuffs.
Dextrose	100	α-amylase to DE 10-20; amyloglucosidase to DE 96-98; crystallisation of dextrose	Fermentation substrate, foodstuffs.

is obtained of maltose (e.g. 90-95% dry matter) and maltotriose (3-4%). Compared with glucose, maltose is less sweet, less hygroscopic, less alkali labile and better able to withstand elevated temperatures (less discolouration). In Europe, no large-scale use is made of this method; in Japan, hovever, pure (crystallised) maltose is produced industrially, and in the USA, the debranching enzyme is used for the industrial production of what is called high-maltose syrup (80% maltose dry matter).

— By using specific enzyme preparations (alpha-amylases and/or by isolation from sugar mixtures, it is possible to produce starch hydrolysates consisting chiefly or totally of one of the following sugars: maltotriose, maltotetraose, maltopentaose or maltohexaose. However, so far no commercial application has been found for these sugars; their properties and possible applications have not yet been sufficiently investigated.

— Various glucose products can be made by enzymatic hydrolysis on unmodified starch granules with specific types of alpha-amylase. The advantages of this method are: lower viscosity of reaction medium; higher starch concentrations; lower heating costs; lower reaction temperature (purer product).

— In the decomposition methods used up to now in the starch processing industry, the bond between the glucose units is broken and the bond's free energy is lost. In the cell, enzymes occur, phosphorylases, which conserve most of the energy of the bond with the aid of inorganic phosphate in the form of glucose-1-phosphate. This can be converted with the aid of phosphoglucomutase into glucose-6-phosphate. These molecules, which carry a

significant amount of free energy, can in principle be used in all kinds of synthesis processes, such as combination with other monomers, to give a reaction the impetus required. However, no concrete applications have yet been developed.

— Certain new types of alpha-amylases are able to convert gelatinised starch almost completely (e.g. 99%) into glucose. At present, this enzyme is very expensive, but industrial application may well be possible in the future.

So much for starch. The possibilities of converting other carbohydrates, particularly lactose (from whey) and cellulose, into other, useful carbohydrates are also being investigated. Since 1975 the enzyme lactase has been used in immobilised form: by hydrolysis of lactose, galactose syrup is obtained, thereby doubling the sweetening value. In France, 12,000 tonnes of galactose syrup are produced per year.

The situation as regards decomposition of cellulose has already been discussed. It does not seem likely that cellulose-based glucose syrups will be able to compete with starch-based products in the foreseeable future. There are still too many problems attached to the purification of the cellulose and the breaking down of its crystalline structure.

3.4 Biotechnology and fine chemicals

3.4.1 Industrial synthesis of fine chemicals

From a limited arsenal of compounds, living organisms synthesise a large number of often highly complex chemicals. At the same time, a large number of conversions take place in order to supply energy for the synthesis. It is probably no exaggeration to state that the total arsenal of syntheses that occur in nature covers practically all chemical synthesis processes conceivable.
The synthesis processes that take place in nature almost always occur at low temperatures and pressures, with water as solvent in most cases. The reaction rate required is obtained by the catalytic action of often very specific enzymes. In the chemical industry, fine chemicals are often synthesised at elevated temperatures and pressures, while solvents other than water form the reaction medium. The reactivity of the catalysts used is often the deciding factor for the choice of temperature.

For biotechnological methods to be applied in the synthesis of fine chemicals, biocatalysis must be weighed against chemical catalysis. The reasons why biocatalysis can be preferred to chemical catalysis are similar to those already discussed.

— The catalytic activity is sufficient even at low temperatures and pressures, resulting in low investment and operating costs.
— The greater specificity achievable with biological catalysis is very attractive – for example, the DSM/NOVO process for the production of D-phenyl glycine, one of the two possible stereo isomers of a certain amino acid.

— In principle, biocatalysis permits the use of organic solvents to be avoided. Water is, however, virtually the only solvent used, although there are signs that research is starting on the use of enzymes in solvents other than water. This would open up a whole new range of possibilities.
— Use can often be made of less aggressive reactants: for example, chlorination with chloride instead of with chlorine gas.

Out of all the possible applications of biotechnology in the chemical industry, those for the synthesis of fine chemicals are the most promising for the immediate future. On laboratory scale, the many syntheses possible with the aid of biocatalysis are being investigated in both academic and industrial research institutes, although it is difficult to gain an idea of what is going on inside industry because such matters are regarded as commercial secrets. In publications, topics of interest are only discussed in general terms.

Discussion of possible biotechnological applications in the synthesis of fine chemicals is made difficult by the fact that highly specialised knowledge of organic chemistry is needed to understand this topic. This is one reason why it is not so practicable to talk in terms of products, as was done in preceding sections of this chapter, but in more general terms; but this field is none the less interesting for that. On the contrary, with the appropriate research, many applications could be developed over the next 10 years or so.

Forms in which biotechnology can be used in the synthesis of fine chemicals

The use of biotechnological methods in the chemical industry will certainly not lead to complete substitution of biocatalysis for chemical catalysis. In many cases, one or more stages in an existing chemical synthesis route will be replaced by more suitable biocatalytic process stages, leading to hybrid, biocatalytic/chemical catalytic processes.

The possible uses of biocatalysis in industry can be subdivided, according to the problems involved, into three main groups.
a. The reaction takes place in the biological system without what is termed a cofactor being needed. Hydrolysis and isomerisation are typical of such reactions.
b. The reaction takes place with a cofactor that can be regenerated by the biological system itself - in most cases, with the aid of oxygen or perhaps some other electron acceptor. Such regeneration can take place relatively easily because energy is liberated at the same time.
c. The reaction takes place with a cofactor that cannot be regenerated with oxygen or another simple electron acceptor, possibly because this regeneration requires energy. For this purpose, a biological energy source that can be used by the organism must be available.

The form in which the biocatalyst is used is important. In chemical syntheses, a purified enzyme will rarely be used, for cost and other, more stability related, reasons. In many cases, a raw preparation, partially intact cell or even a living cell will be used. The advantage of living cells, particularly in reactions of types b and c above, is that cofactor regeneration can take place in the cell itself, although there is the risk that this will entail some sacrifice of the selectivity desired. For each possible application, a distinction can be made between use of the enzyme, use of the dead cell and use of the living cell in free and immobilised form.

In the latter case, the biocatalyst is enclosed in or attached to a suitable carrier material. This has the following advantages.

— The catalyst can be re-used, although the cost advantage of this is somewhat offset by the costs of the immobilisation process. The cost of the carrier is a particularly crucial factor.
— The catalyst can be easily separated from the product flow.
— Continuous processing is possible.

Development of a process incorporating a biocatalytic stage involves the following steps.

— Indentification of the synthesis problem and the advantages that a biochemical approach could permit (comparison with chemical methods).
— Selection of an organism containing an enzyme (system) capable of bringing about the synthesis desired. A number of techniques are available.
  — Mutation and screening;
  — Mutation and screening plus selection under a certain 'selection pressure', i.e. an environment in which the organism will very probably only be able to survive if the desired synthesis is present in the organism. (Although selective modification of the genetic material by modern genetic engineering techniques could well be of assistance, conventional mutation techniques in conjunction with planned selection are expected to remain predominant for the near future.)
  — Increasing the activity and quantity of the desired enzyme in the organism by mutation and selection, selection under suitable 'pressure' or additional replication by genetic engineering of the piece of DNA coding for the enzyme system desired.
— Selection of the form of biocatalyst (immobilised or free enzyme, dead cell or living cell) and the nature of the cofactor regeneration system.
— Production of the biocatalyst: is it to be integrated into the process or gained from an external source?
— Integration of the biocatalytic process stage into the overall synthesis process, e.g. the choice of reactor or the problems associated with the simultaneous use of conventional chemical catalysts and biocatalysts.
— Further development work: experimental work, pilot plant.

In the final analysis, it is only economic or overriding social considerations that will favour the biocatalysis option in a given process stage. Of course, the

specific limitations of biocatalysis should not be ignored either: the stability of the catalytic activity, the limited range of temperatures and pHs and the availability of suitable enzyme preparations. Only where the advantages of using enzymes are the greatest will use be made of them to start with. But there are definite grounds for some degree of optimism.

As already pointed out, it is hardly possible to discuss specific reactions or product groups in this field; the only meaningful subdivision is according to types of reaction. Ditstinction is made between six main groups and a large number of subgroups (see section II.4.).

Perhaps the biotechnological synthesis of fine chemicals will permit welcome expansion of the Dutch chemical industry, which at present has a bias towards bulk chemicals. Such applications, which will require the combined efforts of researchers in the fields of organic chemistry, microbiology, biochemistry, chemical engineering and fermentation technology, are particularly crucial for the Netherlands.

3.4.2 Carbohydrate-based fine chemicals

Synthesis of polysaccharides
The chemical synthesis of polysaccharides from lower sugars is not undertaken on an industrial scale, but it has proved possible to produce them commercially by the microbiological fermentation of sugars [28]. The major problem here is the high viscosity of the fermentation medium. As a result, low concentrations must be used, which makes isolation and purification of the polysaccharide a difficult and expensive business. At present, some polysaccharides are manufactured by fermentation on a small scale; these processes are outlined below.

Dextran
The glucose polymer dextran is formed by certain micro-organisms from saccharose. The product is used as blood plasma substitute. World production is low (500 tonnes per year) but the price is high (Dfl. 20 to Dfl. 30 per kg).

Xanthan gum
The bacterium *Xanthomomonas campestris* is able to convert glucose or glucose syrup into the polysaccharide xanthan gum (built up from glucuronic acid, glucose and mannose). Production was commenced around 1960 in the USA (Kelco). At present, world production amounts to about 15,000 tonnes per year; the product is mainly used in drilling fluids (as thickening agent) and in foodstuffs. One application could be in tertiary oil recovery; oil is ejected by thickening the water in certain strata.
Xanthan gum is characterised by high viscosity in low concentrations. The high viscosity is not affected by temperature changes, salts, acids, etc. Its price is high (Dfl. 10 to Dfl. 20 per kg).

Pullulan [29]
Around 1975, the Japanese company Hayashibara commenced industrial production of the polysaccharide pullulan. This polymer is formed by the micro-organism *Pullularia pullulans* from glucose syrups and is made up of maltotriose units. Pullulan can be mechanically formed as a synthetic plastic material, from which films and fibres can be readily manufactured. It can be used as a self-supporting film for foodstuffs (biodegradable); foodstuffs can also be coated with pullulan by immersion in or spraying with pullulan solutions. At present, pullulan is only manufactured on a commercial scale in Japan by Hayashibara (4,000 tonnes per year).

Bacterial alginate
Tate & Lyle (UK) have developed a process for the manufacture of a polysaccharide similar to the alginate isolated from seaweed. Bacterial alginate (made up from guluronic acid and mannuronic acid) is produced by the bacterium *Azotobacter vinelandii* on a saccharose-containing substrate. Tate & Lyle have apparently commenced manufacture on a modest scale. Potential markets are foodstuffs and textiles (thickening of printing inks).

There are many microbiological polysaccharides, apart from those outlined above, that are discussed in the literature but have yet to be manufactured commercially. A major objection to microbiological polysaccharides is their relatively high price (Dfl. 10 and more per kg) which makes them uncompetitive with cheap water-soluble polymers (starch derivates, cellulose derivates, natural gums, polyvinyl alcohol). Only in very special applications where the specific properties of some microbiological polysaccharides are uniquely suitable will there be any real chances of marketing success. No major growth is expected in the production of microbiological polysaccharides.

Modification of polysaccharides
Certain enzymes (transferases) catalyse the transfer of a carbohydrate molecule onto another carbohydrate molecule or onto another part of the same carbohydrate molecule. Reactions of this type occur in various forms in carbohydrate chemistry [30] and some have industrial potential.

Cyclodextrins
From starch, the enzyme cyclodextrineglycosyltransferase can produce cyclodextrins. These are ring-shaped oligosaccharides with six, seven or eight glucose units per ring. Cyclodextrin formation from starch involves on the one hand the breakdown of starch molecules and on the other, a transfer mechanism. Glucose units are relinked with one another. Cyclodextrins are able to form enclosed compounds with various inorganic and organic substances, whereby the guest molecules are stabilised or isolated. Various companies have produced cyclodextrins industrially; at the moment they are being manufactured in Japan on a modest commercial scale.

Oligoglucosylfructosides (Coupling Sugar)
The enzyme glucose transferase is able to hydrolyse starch and to transfer (chemically bond) the starch hydrolysis products to saccharose or fructose. In this way, a syrupy mixture is obtained of the sugars G1F, G2F, G3F, G4F, etc., consisting of glucose (G1), maltose (G2), maltotriose (G3), maltotetraose (G4), and so on, each chemically bonded to fructose (F) at its reducing terminal. The sugars do not contain reducing groups. This syrupy mixture is characterised by high sweetness, low viscosity, high colour stability and high heat resistance. This product is manufactured on a limited scale in Japan. Theoretically, it is possible to produce saccharose (= G1F) from glucose and fructose biochemically, taking both the glucose and the fructose from starch. Thus, in principle, starch can be used as raw material for all carbohydrate sweeteners (saccharose, glucose products, isoglucose) [31].

Branched starch [30]
The branching enzyme splits off part of a starch chain by cleaving a certain bond (1-4 bond). The split-off piece is in turn attached by another bond (branch) to another chain. The enzyme plays a part in the make-up of amylopectine in the starch granule. The viscosity stability of starch solutions could be increased by using the branching enzyme to branch the linear starch fraction (amylose). This method is not yet in use in industrial practice, partly because no cheap enzyme preparations are available. To increase the viscosity stability of starch solutions, starch granules are chemically esterified or etherified. This is a very cheap method, partly because the granular starch derivate can be readily isolated from the reaction medium by filtration. Although it would be theoretically possible to esterify starch biochemically, it seems that in these cases the biochemical methods would be much more expensive than the chemical methods.

Decomposition of polymers into phosphorylated decomposition products
The synthesis of phosphorylated sugar should also be included under the heading of fine chemical synthesis; this has already been discussed in para. 3.3.4.

3.4.3 The production of biocatalysts

In many of the applications discussed in this chapter, free or immobilised, living or dead organisms or parts thereof play an important part. The production of these biocatalysts is thus a crucial task in the biotechnological field.
These biocatalysts are mainly produced by conventional fermentation techniques as discussed in the section dealing with enzyme technology (II.4.4).
As more and more uses are found for enzymes, there is an increasing requirement for enzyme production potential. There are certainly opportunities here for the Netherlands, if know-how in fermentation technology can be combined with high-quality microbiological/biochemical research. However, this research should concentrate more on organisms of interest to enzyme

technology. Similar considerations apply for the development of enzymatic activities by planned selection, mutation/selection and genetic engineering.

4. Social and economic factors: conditions affecting development

Availability of energy carriers
The price of energy carriers is crucial, and should be considered from two aspects. Firstly, we have the rising price of conventional energy carriers derived from oil and natural gas. Political developments outside our control and the expected future demand for these energy carriers are responsible for this trend, and the population boom and rise in living standards in the Third World will also make increasing demands on the energy sources available. The second critical factor is the availability of energy sources. Biotechnological processes frequently involve the upgrading of an energy carrier, but for this to be possible, a cheap source of energy (coal, wood) must be available.

Agricultural policy
Many of the raw materials suitable for biotechnology are of agricultural origin. This sometimes has a significant effect on prices which, for political reasons, can differ widely from place to place. This can in turn seriously affect the competitiveness of biotechnological applications in industry in some places: for example, the disadvantageous position of isomerose in the USA and the advantageous position of gasohol in the USA. In general, it is true that political factors can have marked stimulating or demotivating effects on biotechnological developments.
To take a concrete example, we need only look at the consequences of the EEC's agricultural policy for the competitiveness of the fermentation industry, where the prices for the carbohydrate sources that are used as fermentation substrates are fixed. On the other hand, biotechnology can assist the implementation of certain agricultural policies by encouraging the cultivation of crops that can be processed into bulk chemicals and energy carriers.

Social factors
Biotechnology is a sensitive area in so far as environmental, public nuisance and operating permit problems are concerned (witness the recent stagnation in the granting of permits for recombinant DNA research).

Research efforts
There is relatively little research being conducted in industrial, government and educational research establishments into biotechnological applications. Much more must be done if full use is to be made of the possibilities of biotechnology. Active encouragement by the government would be particularly appropriate in view of the innovative nature of biotechnological activities for industry.

5. Summary and conclusions

It will not be feasible for tomorrow's world to generate all its energy from photosynthesis products. Too much arable land would be required and the energy balance would hardly break even. However, production is conceivable of biogas or special energy carriers such as automotive fuels (gasohol), with cheap energy sources (wood or coal) being upgraded into premium-value liquid fuels.

Although the problem of arable acreage would be particularly acute in the Netherlands, this country could well play an important part in the development of process know-how. Its strong points in this connection are its traditional emphasis on microbiology and biochemistry, the relevant experience in large-scale process technology and the scientific higher education establishments (Delft University of Technology, Wageningen University of Agriculture) where integrated biotechnology is taught and practised.

To achieve full-scale production of, for example, ethanol, developments in reactor technology and separation techniques will be essential. For hydrogenase to be used on a large scale for hydrogen production, many difficulties still have to be overcome, even though significant progress is being made. The development of large-scale photoreaction techniques could well be another major bottleneck.

Fundamentally, the same state of affairs exists in base chemical production, except that far less arable land would be required as the production level is only 5-10% of that of energy carriers. Too often, insufficient information is available for assessment of the competitiveness of biological synthesis routes. Fundamental research is still needed on continuous production systems, process intensification, genetically modified production organisms and product separation. Here too, the expertise that has been built up in the Netherlands in the field of biochemistry/microbiology and process technology could enable this country to gain a leading position as supplier of know-how; and here again, a multidisciplinary approach should be encouraged. There are highly important uses of enzymes in conjunction with natural carbohydrates. In this field too, there are many opportunities, and in fact the most important large-scale applications have already been put into practice.

By far the most important applications of biotechnology lie in the field of fine chemical synthesis. One or more chemically problematical process stages in the synthesis of a given chemical could be replaced by a reaction catalysed by an organism or a part of an organism. Hybrid catalysis (a chemical and a biochemical catalyst in one and the same process stage) is also conceivable; the possibilities are boundless. To put such applications into practice, the synthesis problem must be identified, an organism with the desired activity must be selected, a suitable form of the biocatalyst must be produced and, finally, this must all be integrated into the synthesis process. A great deal of

goal-oriented research is therefore required.

The Netherlands has the appropriate industrial and university research potential (fermentation industries, microbiological/biochemical research establishments), but a part of the research potential of the universities should be oriented more towards the development of useful biocatalysts and towards research into what they can achieve in synthesis processes. As regards this applicational research, Dutch experience in process technology is a great asset, although somewhat offset by the lack of a fine-chemicals tradition in the Netherlands.

Members of the working group

The working group which produced Chapter VI consisted of the following members:

Prof. S. Bruin	Wageningen University of Agriculture
Dr J.J. Dahlmans	DSM, Heerlen
Prof. J. Reedijk	Leiden State University
Prof. J.A. Roels	Delft University of Technology
Prof. A.H. Stouthamer	Free University, Amsterdam
Dr H.C. Volger	Shell, Sittingbourne (England)
P. Woldendorp	Avebe, Veendam
J.H.F. van Apeldoorn (secretary)	Netherlands Study Centre for Technology Trends, The Hague

References

1. S.L. Neideleman, J. Geigert, W.F. Amon, E. Liu, B. Wolf, In: Abstracts VIth International Fermentation Symposium, London, Ontario (Canada) July 20 - 25 (1980) 178
2. M.H. Nielsen. In: 13th International TNO Conference: 'Biotechnology a Hidden Past a Shining Future'. (1980) 41
3. M. Makkee, A.P.G.Kieboom, H. van Bekkum, J.A. Roels. J.C.S. Chem. Commun. (1980) 930
4. S. Bruin. Ref. 2 p. 153
5. Enzyme Nomenclature: Biochim. Biophys. Acta, 1 (1976)429
6. J.A. Roels. Biotechnol. Bioeng. *22* (1980) 2457
7. B. Sikyta, Z. Fencl. In: A.C.R. Dean, D.C. Ellwood, G.G.T. Evans, J. Melling (Eds.), Continuous Culture Soc.Chem. Ind. London *6*(1976)158
8. Bedrijfstakverkenning Chemische Industrie. Ministerie van Economische Zaken (1981)
9. Chemisch Magazine (1980) 687-764
10. Statistisch Zakboek CBS (1980)
11. World Energy Resources 1985 - 2020, W.E.C. IPC Science & Technology Press (1978)

12. Renewable Energy Prospects, Proc. Conf. Non-fossil Fuel and Non-nuclear Fuel Energy Strategies, Honolulu, USA, Pergamon Press (1979)
13. B.A. Stout, C.A. Myers, A. Hurand, L.W. Faidly, Energy for World Agriculture, FAO Agriculture Series No. 7, Rome (1979)
14. S. Bruin, Biomass as a source of energy, Extern IX (1980), nr. 4, 235 - 275
15. L.L. Anderson, D.A. Tillman (Eds.) Fuels from waste, Acad. Press, New York (1977)
16. P.B. Weisz, J.F. Marshall, Science *206*, (1979) 24-29
17. M. Calvin, G. Porter New Scientist (1980) 585.
O.O. Sullivan, Chem. Eng. News (1981)66
18. D.L. Wise, C.L. Cooney, D.C. Augenstein. Biotechnol. Bioeng. *20*(1978) 1153
19. W.J. Sheppard, E.S. Lipinsky. In: J.L. Hickson (Ed.) Sucrochemistry A.C.S. (1977)336
20. E. Gwinner, Bio-energie, Biomassa, Biotechnologie. 1.c. Verlag Handelsblatt (1978)56
21. In ref. 3
22. Ref 4 p. 59
23. R.V. MacAllister, Nutritive sweeteners made from starch. In: Advances in carbohydrate chemistry and biochemistry, Vol. *36*, (1979) 15-56
24. F.S. Kaper, Voedingsmiddelentechnologie *12* nr. 5 (1979) 18 - 25
25. K.G. de Noord, J.J.M. Swinkels. Polytechnisch Tijdschrift *27* (1972), 518 - 523
26. W.C. Bus, H. Reuvekamp, J.J.M. Swinkels, Consudel *33* (1976), 829 - 837
27. C. Bucke, Recent developments in production and use of glucose and fructose syrups. In: C.A.M. Hough, K.J. Parker and A.J. Vlitos, Developments in Sweeteners - 1 London (1979) 43 - 68
28. C.J. Lawson, Chemistry and Industry, March (1976) 258 - 261
29. S. Yuen, Process Biochemistry, (1974) 7 - 9, 22
30. H.A. Spa, F.S. Kaper, H. Hokse, Polytechnisch Tijdschrift - Procestechniek *33* nr. 6 (1978) 318 - 322
31. R.E. Gramera, Die Stärke *30* nr. 1 (1978) 20 - 23

VII Waste treatment

1. Introduction

As human society has evolved so it has created an increasingly complex network of material flows, from the input of primary raw materials to the output of manufactured products and wastes (figure 1).

The nature and quantity of those wastes is governed by the technologies used. The burden on the environment can be alleviated in a number of ways.

— *Clean technology*
A production process is described as clean if the technology involved produces the desired products from the raw materials while generating little or no waste, or only benign waste. Biotechnology can undoubtedly make a valuable contribution in this area.
Biotechnological processes generally take place under moderate conditions (low temperature, low pressure, aqueous solution).Enzymatic conversions are often highly specific, leaving few by-products to be disposed of.

— *Clean products*
After it has been used for a certain length of time every product is discarded and becomes waste. The amenability of the product to treatment as waste should be taken into account when selecting the raw material and the technology to be used. Products which present few or no waste processing problems can be regarded as clean products.
Biotechnology has a role to play here, for example in the manufacture of naturally degradable plastics and in the production of biocides for use in agriculture.

— *Recycling*
Waste materials and discarded products can sometimes be processed into usable products. In practice there are numerous intermediate forms between waste treatment (purification) and re-use. Examples include composting, methane fermentation, and the production of Single Cell Protein from agricultural waste.

— *Purification*
The object of purification is to convert unusable, polluting residuals into materials which can be discharged harmlessly to the environment.

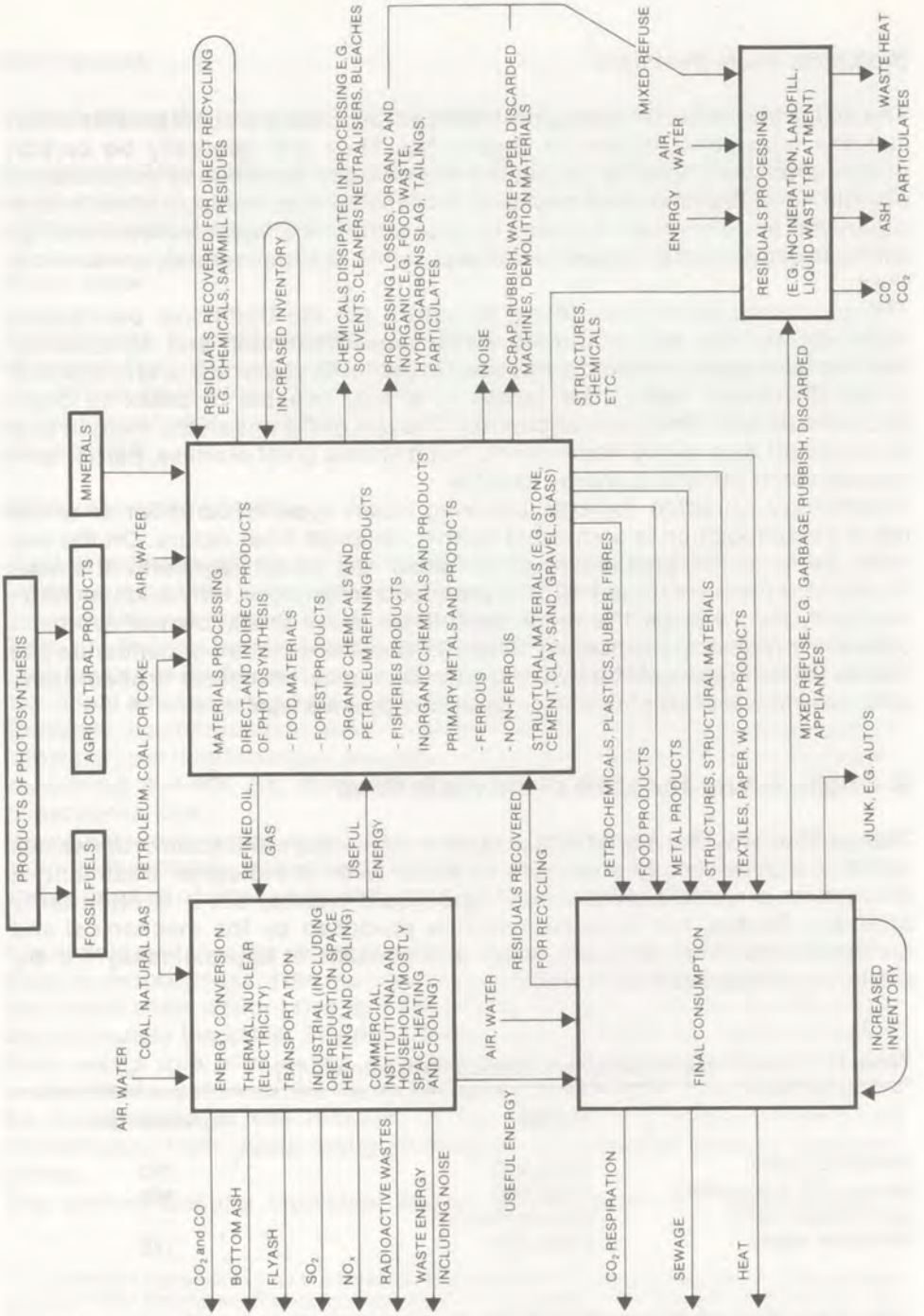


Figure 1. The interaction between materials, energy and the environment [1]

The main candidates for biological treatment processes are compounds which suit the enzymatic system of organisms. They will generally be carbon compounds, but phosphorus, sulphur and nitrogen compounds would also be eligible. This restriction also applies to the biochemical manipulation of micro-organisms and enzymatic systems, but one of the challenges for biotechnology will be to use natural processes as far as possible, or to deliberately deviate from them.
The processes which are closest to nature are the traditional purification methods: aerobic and anaerobic waste water treatment, and composting. Aerobic waste water treatment has been in use for 65 years now, and in that time it has developed many new facets. It is also capable of breaking down phosphorus and nitrogen compounds. The use of the anaerobic method is of more recent date in the Netherlands, but it shows great promise, particularly because of its attractive energy aspects.
Technology drirected towards breaking down specific compounds which resist decomposition is somewhat further removed from nature. On the one hand there is the breakdown of cellulose and lignin (agricultural waste, domestic refuse) combined with the production of glucose, fatty acids, alcohol, methane, etc., and on the other hand there is the breakdown of synthetic chemicals (plastics, pesticides). There is also the question of concentrating metals from dilute waste flows using microbiological processes or adsorption, and the removal of sulphur and sulphur compounds from coal.

2. Origin, nature and scale of the waste flows

This section provides a brief review of the origin, nature and scale of the wastes which are theoretically amenable to some form of biological treatment. A distinction is made between solid and liquid wastes, but it is often fairly arbitrary. Sludge, for instance, which is produced by the mechanical and biological treatment of waste water and consists of approximately 4% dry matter, is counted as a solid waste.

Table 1. Solid wastes suitable for biological treatment [2]

	tonnes	kg/inhabitant
domestic wastes	3,500,000	250
sludge (4% dry matter)	5,000,000	360
agricultural waste	several million*	—
industrial waste	2,400,000	175

* Only a proportion can be regarded as waste, the remainder finding a useful application on the farm.

Solid wastes

Table 1 shows the production of some solid wastes in the Netherlands in 1977.

Roughly half the domestic waste is suitable for biological treatment, and the same applies to sludge and agricultural wastes. A proportion of the industrial waste consists of organic matter.

Waste water

A distinction is made between municipal and industrial waste water. It is estimated that total waste production in the Netherlands amounts to 30 million population equivalent,* of which approximately two-thirds is currently subjected to treatment. It is expected that the treated percentage will rise to 90% by around 1985.

Municipal waste water

Most sewerage systems accommodate both waste water and rainwater. The waste water comes from households, and often from industry as well. The industrial stream may have undergone physico-chemical pre-treatment, since limits are imposed on discharge into a municipal sewerage system, covering such factors as acidity and heavy metals content. One general characteristic of municipal sewage water is that it is highly amenable to biological treatment. Moreover, its composition remains fairly constant. Total discharge (including rainwater) per inhabitant per day amounts to approximately 200 litres of water, containing 200-400 mg BOD_5/litre, 40-80 mg nitrogen/litre, and 10-20 mg phosphorus/litre.
Municipal waste water is purified using a combination of mechanical and biological methods, or by biological methods alone. Table 2 lists the number and capacities of sewage treatment plants in the Netherlands.

It is anticipated that the increase in the volume of municipal waste water will at most be proportional to the increase in population. Individual water consumption might show a slight rise, but the BOD and nitrogen contents will remain approximately the same. The phosphorus content would be roughly halved if there was a total elimination of phosphates in detergents. The content of undesirable substances like heavy metals will probably fall. There will probably be an increase in the removal of the organic nutrients, nitrogen and phosphorus, from waste water in existing and planned sewage treatment plants.
The cost of treating municipal waste water (approx. Dfl. 35/p.e./year) is

* A population equivalent (p.e.) is a measure of the pollution of waste water based on the average pollution with biodegradable, oxygen-consuming substances produced per inhabitant per day. One p.e. is equal to 54 grams biological oxygen demand in five days of decomposition (BOD_5) plus 10 grams of nitrogen.

currently running at around Dfl. 700 million a year. It would therefore take only relatively minor improvements in biotechnology to have a fairly substantial financial impact.

Table 2. Number and capacities of sewage treatment plants in the Netherlands in 1980, with projections for 1985 [3]

capacity (p.e.)	1980 *number*	1985 *number*
less than 5,000	189	128
5,000-25,000	161	183
25,000-100,000	110	137
more than 100,000	47	68
Total capacity in million p.e. (including industrial installations)	17	25

Industrial waste water

The nature of industrial waste water and process water differs from one industrial sector to another, and consequently there is a wide divergence in composition, concentration and scale.
The waste water from most industrial sectors is eminently suitable for biological methods, although it may have to undergo pre-treatment. The pre-treatment stages include sieving, the removal of fats and oil, neutralisation and the removal of heavy metals, colouring agents, etc. In view of the specific nature of the waste water it may sometimes be necessary (textile plants being a case in point) to add nutrients like nitrogen and phosphorus in order to promote optimum biological treatment. In some cases, though, nitrogen and phosphorus are already present in large or even excessive amounts (sugar industry, chemical industry, intensive animal rearing).
The BOD content of industrial waste water is almost invariably higher than that of municipal waste water. Values of several thousand mg BOD/litre are not uncommon, and may be as high as 20,000-30,000 mg/litre in the case of the pharmaceutical industry. This means that biological treatment techniques other than the aerobic method may be applied. The main variant is anaerobic treatment, possibly followed by aerobic post-treatment.
The scale of the industrial waste water which is theoretically suitable for biological treatment was around 14 million p.e. in 1980 (see Table 3).

At the moment there are some 180 industrial waste water treatment plants with a total capacity of approximately 5 million p.e. As in the case of municipal sewage treatment plants, there is a wide spread in capacity, ranging from less than 5,000 p.e. to more than 100,000 p.e. The volume of industrial waste water is not expected to increase in future, but there may be higher concentrations of BOD, etc., due to more efficient water use. There will be increasing use of anaerobic

treatment, possibly combined with aerobic post-treatment. The volume of industrial waste water from a particular concern and the costs of internal treatment, or of direct discharge to the sewerage system, are the main derterminants for the way in which the problem of waste water or process water is tackled.

Table 3. Sector distribution of industrial waste water in the Netherlands [3]

	Biodegradable substances, residual discharge in million p.e.	
Sector	1980 (estimate)	1985 (or later) (estimate)
potato flour	3.5	0.15
paper, cardboard	0.4	0.2
dairy	0.6	0.55
sugar	0.07	0.04
textiles	0.4	0.3
yeast/alcohol	0.1	0.1
slaughterhouses	0.7	0.6
chemicals	2.1	1.1
agriculture	0.1	0.1
other	6.3	6.6
Total	14.3	9.7

3. Types of process

3.1 Biological treatment of waste water using living organisms

The object of the biological treatment of waste water is to eliminate from the aqueous phase dissolved biodegradable matter (organic and inorganic) which resists sedimentation, and if necessary to render it harmless in a subsequent treatment stage. To date biological purification has concentrated on the elimination of substances which require oxygen for their decomposition, but in recent years there has been growing interest in such processes as biological dephosphating.
Bacteria play a dominant role in biological purification processes. All bacteria require energy and carbon for the synthesis of new cell material. In addition, every living organism requires inorganic elements such as nitrogen, phosphorus, sulphur, and various trace elements. Organisms can be differentiated according to the type of carbon source which they use for cell synthesis:
— autotrophic organisms use CO_2 as the carbon source;
— heterotrophic organisms use organic carbon.
Autotrophic organisms obtain their energy from the sun (photosynthesis) or from inorganic oxidation-reduction reactions (chemoautotrophic).
Heterotrophic organisms obtain their energy from the oxidation of organic matter, employing either molecular oxygen (aerobic organisms) or oxygen incorporated in the substrate in an otherwise oxygen-free environment

(anaerobic organisms). In the latter case a proportion of the organic matter is fully oxidised, while another portion is completely reduced to methane. Some organisms can exist in both anaerobic and aerobic environments.

The mechanism of purification

Bacteria can only take up matter dissolved in water. If the waste consits of both dissolved matter and macromolecules, hydrolytic enzymes (which are secreted by various types of bacteria) are required in order to reduce the size of the particles. A typical example of this is the splitting of cellulose into smaller sugars (paper and agricultural waste). Only then can the organic matter be processed further intracellularly.
Biochemical oxidation does not always have to lead to CO_2. It can be limited, depending on the circumstances and the type of micro-organism, to the formation of such substances as volatile fatty acids, which are excreted by the organism. Those fatty acids in turn form the specific substrate for another group of bacteria. The breakdwon of complex organic matter therefore requires the participation of many different kinds of bacteria. If the organic matter is to be eliminated as fully as possible it is important that this chain of operative bacteria remains intact, otherwise there will be a build-up of a particular intermediate product. The slowest link in the chain determines the overall speed of the process.

The speed and efficiency of the process are affected by the following factors.

Temperature

Depending on the temperature range within which an organism operates best, one speaks of psychrophilic (cryophilic) organisms (0° C to approx. 30° C), mesophilic organisms (20-50° C), and thermophilic organisms (40-100° C). Most biological systems will tolerate temperature fluctuations, but it should be remembered that the various links in the decomposition chain can be affected in different ways. Aerobic organisms can operate at low temperatures of approximately 5° C. The ideal range for mesophilic anaerobic organisms is 30-40° C. In other words, anaerobic systems require energy for heating.

Acidity

Most organisms are active in the pH range of 6.5-7.5, the limits being 4.0-9.5. Some organisms, however, can exist under extreme pH conditions. They include *Thiobacillus*, which is capable of oxidising H_2S to H_2SO_4 at a pH of 1.

Inhibitors and noxious substances

Below a certain concentration many substances act as stimulants, and as such they are essential growth factors. Above a certain concentration practically every substance is noxious to an organism. The degree of harmfulness depends largely on the type of organism, the presence of other components, the presence of sufficient energy to enable the organism to

survive in a hostile environment, and the measures taken to allow the system to adapt to the substances in question.

The kinetics of purification

The elimination of organic matter in sewage treatment is generally a chain process. There is a wide range of substrates in the waste water, and a great variety of micro-organisms which carry out the conversions. As a result it is not easy to describe the conversion processes involved. Use is made of characteristic process parameters, such as the maximum rate of substrate consumption, sludge build-up and sludge breakdown.
In most biological systems the maximum rate of substrate consumption is of the order of 5-10 mg substrate chemical oxygen demand (COD) per mg sludge dry matter per day.
In aerobic process the sludge build-up per unit of substrate can be 10-15 times greater than in anaerobic processes. This is because aerobic organisms convert a larger fraction of the substrate into cell material. Anaerobic organisms convert a greater fraction of the substrate into secondary products, such as fatty acids and methane.
Sludge breakdown (death of the organisms) mainly occurs when there is a temporary lack of substrate. Breakdown is many times slower with anaerobic organisms than with aerobic organisms, which may be an important factor in systems where the supply of the waste stream is not continuous.
These process parameters are temperature-dependent.

Organisms other than bacteria

Although bacteria see to the elimination of organic matter in the waste water, numerous other micro-organisms play a role in biological processes. Protozoa and rotifers, for example, consume bacteria, and so they play an important role in reducing the amount of sludge dry matter when there is continued aeration in aerobic activated sludge installations.

3.2 Aerobic waste water treatment

Aerobic micro-organisms

The treatment of waste water or process water with aerobic biological methods involves a mixed micro-organism population. Its make-up depends on numerous factors, such as the nature and composition of the water to be treated, temperature, the residence time of the water and, above all, the residence time of the sludge in the system.
The sludge consists of organic and inorganic matter. The sludge from municipal waste water contains 70-80% organic matter, composed of living cellular matter, dead cells, biopolymers, and the like. The active biomass content depends to a large extent on the loading of the system. Measurements of the DNA content have shown that in very low-load activated sludge systems

(0.05 kg BOD/kg sludge/day) the active biomass accounts for less than 10% of the organic matter. With a sludge load of 0.5 kg BOD/kg sludge/day the active biomass can amount to 50% of the organic matter.
The sludge produced in very low-load activated sludge systems is very largely mineralised or stabilised. The micro-organisms are in the autolytic or death stage.
The micro-organisms found in aerobic biological processes belong both to the strictly aerobic types (such as *Nitrosomonas*) and to the facultatively anaerobic types (such as *E. coli*).
The morphology of filamentous micro-organisms is an important factor in the process. A relatively large population of filamentous micro-organisms in the activated sludge process has a very adverse effect on the separation between sludge surfaces and purified waste. For years *Sphaerotilus natans* was regarded as the most important filamentous micro-organism in waste water treatment. The nature and composition of the waste water largely determine which types of filamentous micro-organisms occur.
There are variations in the growth rates of the different groups of micro-organism. The fairly slow-growing nitrifying micro-organisms can only maintain themselves adequately at temperatures above 10° C if the sludge load is no more than 0.2 kg BOD/kg sludge/day. The nitrification capacity of the sludge falls off rapidly at a higher load or a lower temperature. Purification amounts to more than 90% if the sludge load is in excess of 0.5 kg BOD/kg sludge/day. At 1 kg BOD/kg sludge/day purification is around 75%.

Aerobic systems [4, 5, 6].

In treatment systems using aerobic micro-organisms there is a distinction between oxidation beds (trickling filters) and activated sludge systems. Trickling filters have been in use for centuries. Originally they took the form of basins filled with large blocks of lava. The waste water was admitted into the basin and was drained off again a few hours later. However, the process took up a great deal of space, results were only moderate, and there were considerably difficulties in operating the system. In 1893 Corbett developed a continuous method which was essentially the same as that in use today.
In this system the waste water continuously trickles down through the oxidation beds. The beds contain a packing material (lava, coke, stones or plastic) to which a layer of slime has attached itself. Part of this slime layer consists of biologically active matter which, with oxygen, is capable of converting organic pollutants into CO_2, water and nitrate.
Oxygen is supplied by drawing air through the packing material, either with or against the water flow.
Crude waste water containing suspended matter first passes through a primary clarifier before being fed to the oxidation bed.
Until 1970 many of the sewage treatment plants built in the Netherlands had oxidation beds, but from then on there was a switch to activated sludge systems. Oxidation beds are still used in large treatment plants, where they precede an activated sludge system.

The activated sludge system was another British development, being introduced in 1914 by Ardern and Locket. They discovered that bacteria flocs formed when waste water was aerated for a certain length of time. Once the flocs had been removed it was found that the waste water was far less polluted than it had been before aeration. These bacteria flocs were later referred to as activated sludge.

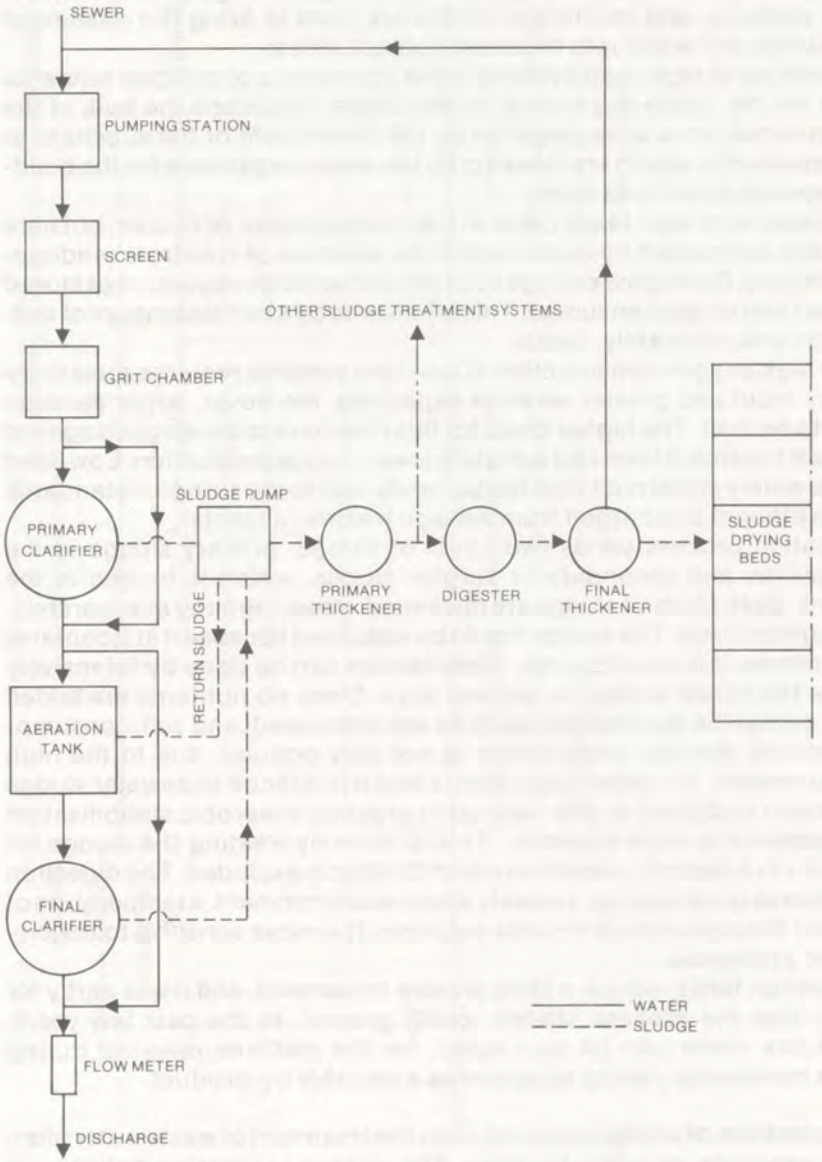


Figure 2. Flow diagram of an activated sludge plant

Figure 2 illustrates the principle of an activated sludge system. This is what is known as a traditional activated sludge plant. The sedimentable particles in the incoming sewage are removed in the primary clarifier, and only then is the waste water admitted to the aerobic biological section. The activated sludge is separated from the water in the final clarifier and is returned to the aeration tank. There has to be a good supply of oxygen if the biological stage of the process is to operate properly, and mechnical means are used to bring the mixture of activated sludge and water into intensive contact with air.
The main feature of high-load systems is the abundance of external nutrients (substrate) for the micro-organisms. Under these conditions the bulk of the oxygen consumption is accounted for by the breakdown of the substrate to simpler components, which are taken up by the micro-organisms for the build-up of compounds specific to them.
Substrate respiration also takes place in low-load systems, of course, but here oxygen is also consumed by organisms in the absence of substrate (endogenous respiration). During the endogenous phase the reserve substances stored in the cellular matter are consumed. This is followed by a certain amount of self-consumption and, ultimately, death.
A relatively high oxygen consumption in low-load systems requires a relatively high energy input and greater aeration capacities. Moreover, larger aeration tanks have to be built. The higher costs for this item have to be weighed against the increased treatment level and a slightly lower sludge production. Low-load systems are widely preferred in the Netherlands, due to the stringent standards imposed on effluent discharged from sewage treatment plants.
The purification process yields two kinds of sludge: primary sludge in the primary clarifier, and secondary or surplus sludge, which is formed in the aeration tank. Both kinds of sludge are thickened, either together or separately, before being stabilised. The sludge has to be stabilised because it still contains numerous putrescible constituents. Stabilisation can be done by intensively aerating the thickened sludge for several days. Since no nutrients are added during this period the putrescible residues are consumed, and self-consumption also occurs. Aerobic stabilisation is not very popular, due to the high energy requirement. An added objection is that it is difficult to dewater sludge which has been stabilised in this way, so in practice anaerobic statibilisation (sludge digestion) is more common. This is done by treating the sludge for several weeks in a sealed space from which oxygen is excluded. The digestion process requires good mixing, a weakly alkaline environment, a temperature of 30-35° C, and the presence of mineral nutrients. It is more sensitive to toxicity than aerobic processes.
Sludge digestion tanks require a fairly sizable investment, and it was partly for that reason that the process started losing ground. In the past few years, however, it has come into its own again, for the methane released during digestion is increasingly being regarded as a valuable by-product.

The large quantities of sludge obtained from the treatment of waste water often require the provision of costly facilities. The various processing options are outlined in figure 3.

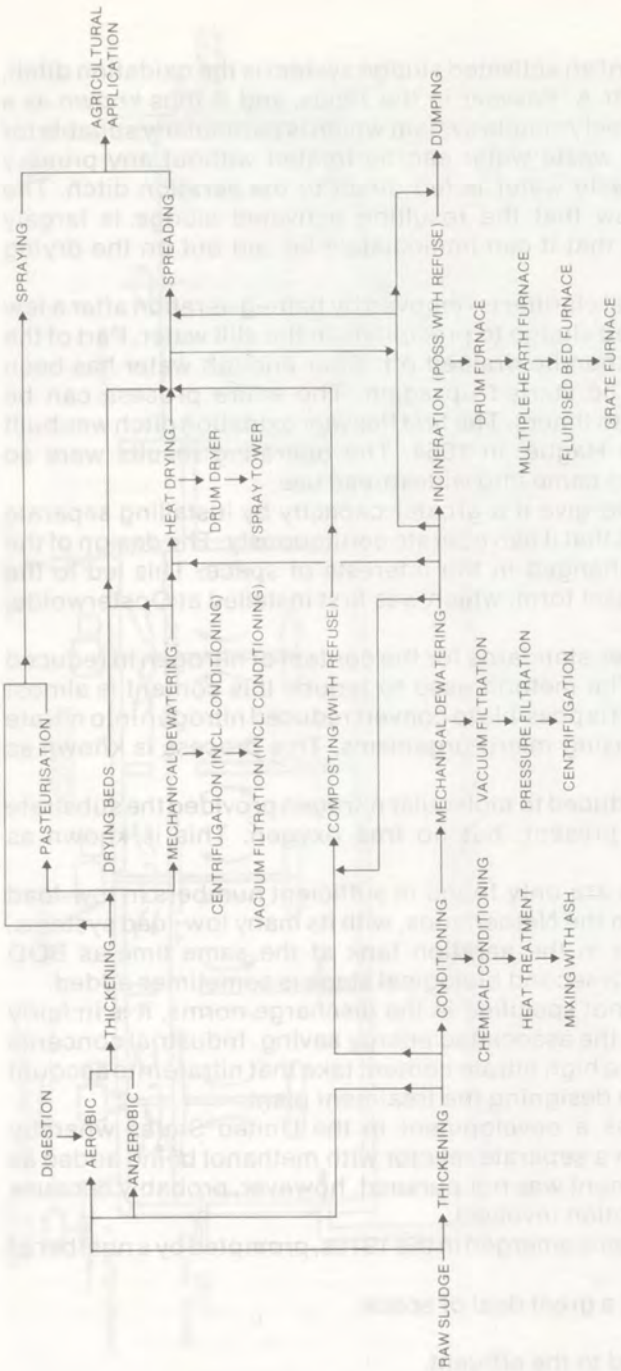


Figure 3. Commonest methods of sludge processing

One special configuration of an activated sludge system is the oxidation ditch, which was developed by Dr A. Pasveer in the 1950s, and is thus known as a Pasveer ditch. It is an extremely simple system which is particularly suitable for smaller communities. The waste water can be treated without any primary clarification. The crude waste water is fed direct to the aeration ditch. The sludge load is kept so low that the resulting activated sludge is largely mineralised, which means that it can immediately be laid out on the drying fields.

The need for a separate final clarifier is removed by halting aeration after a few hours, allowing the activated sludge to precipitate in the still water. Part of the purified water on top can then be drained off. After enough water has been removed the aerator can be started up again. The entire process can be controlled automatically with timers. The first Pasveer oxidation ditch was built at Voorschoten, near The Hague, in 1954. The operating results were so satisfactory that the process came into widespread use.

The system was modified to give it a greater capacity by installing separate clarifiers, which also means that it can operate continuously. The design of the oxidation ditch was also changed in the interests of space. This led to the development of the Caroussel form, which was first installed at Oosterwolde, near Zwolle.

The Netherlands also applies standards for the content of nitrogen in reduced form (Kjeldahl nitrogen). The method used to reduce this content is almost invariably biological, since it is possible to convert reduced nitrogen into nitrate under aerobic conditions using micro-organisms. This process is known as nitrification.

The nitrate can in turn be reduced to molecular nitrogen provided the substrate and micro-organisms are present, but no free oxygen. This is known as denitrification.

Nitrifying micro-organisms are only found in sufficient numbers in low-load activated sludge systems. In the Netherlands, with its many low-load systems, nitrification can take place in the aeration tank at the same time as BOD removal. In other countries a second biological stage is sometimes added.

Although denitrification is not specified in the discharge norms, it is in fairly widespread use because of the associated energy saving. Industrial concerns producing waste water with a high nitrate content take that nitrate into account as a source of oxygen when designing the treatment plant.

A few years ago there was a development in the United States whereby denitrification took place in a separate reactor with methanol being added as the substrate. The development was not pursued, however, probably because of the high energy consumption involved.

Various new treatment systems emerged in the 1970s, prompted by a number of considerations.

— Existing systems take up a great deal of space.
— Rising energy prices.
— Stricter standards applied to the effluent.
— Stricter standards covering environmental nuisance.

The following are some examples of these new systems.

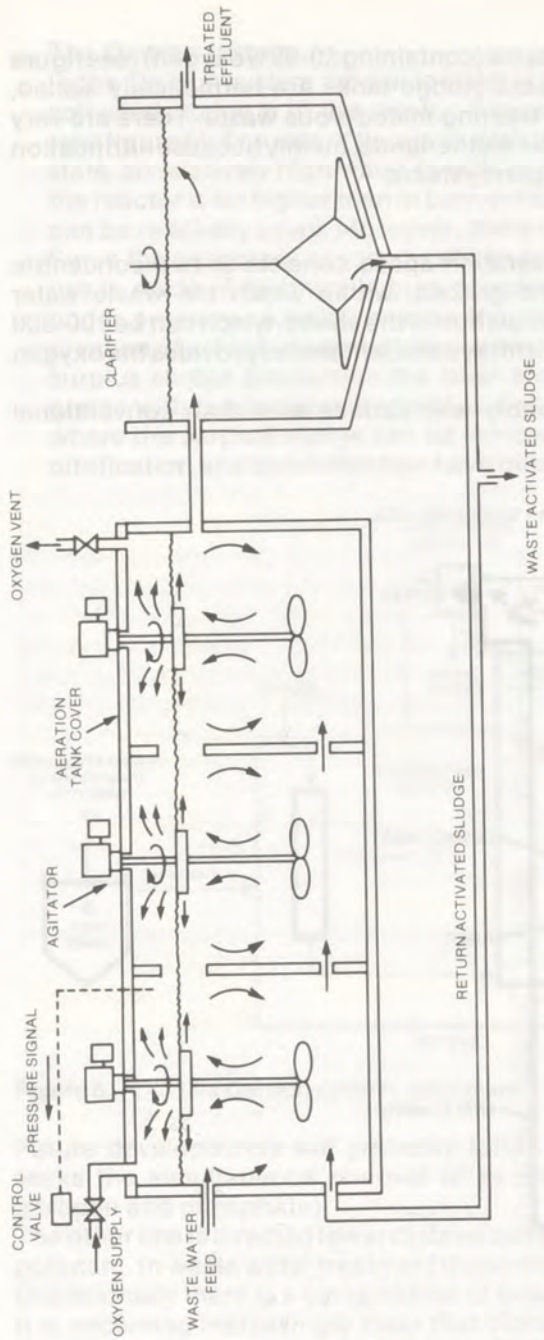


Figure 4. Schematic diagram, three-stage Unox system
From: Cement nr 9, *32* (1980) 515

— The use of oxygen-rich gas mixtures (containing 50-99% oxygen) (see figure 4). In these systems the activated sludge tanks are hermetically sealed, which makes them suitable for treating malodorous waste. There are very few examples of this system in the Netherlands, mainly because nitrification does not take place in pure oxygen systems.

— The deep shaft system
In the deep shaft system the aeration space consists of two concentric cylinders, buried upright in the ground, within which the waste water circulates (see figure 5.). The circulation in the shafts, which can be 100-300 metres deep, is achieved by an air lift system, which also provides the oxygen supply.
The system occupies considerably less surface area than conventional systems.

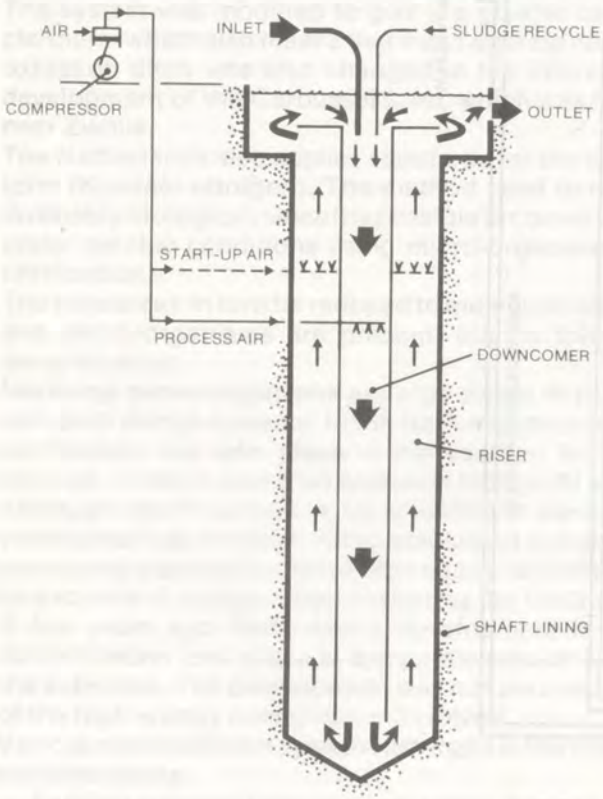


Figure 5. ICI deep shaft system
From: Cement nr 9, *32* (1980) 514

— The Oxitron system
In the Oxitron system the waste water is led upwards through a reactor. The activated sludge is in the reactor, where it attaches itself to grains of sand (see figure 6). The rate of flow is so high that the sand grains are in a fluidised state, so relatively high water speeds can be applied. The sludge content in the reactor is far higher than in conventional systems, so the reactor volume can be relatively small. Moreover, there is no need for final clarifiers.
Since the necessary oxygen has to be added to the waste water all at once, use is made of technically pure oxygen, which is fed into the waste water under pressure. Another reason for using pure oxygen is to prevent the creation of a three-stage system of water, sludge and nitrogen. As a result of surplus sludge production the layer around the grain of sand grows. The grains with the thickest layers of sludge rise to the top of the fluidised bed, where the surplus sludge can be removed from the reactor. BOD removal, nitrification and denitrification have been achieved in pilot plants.

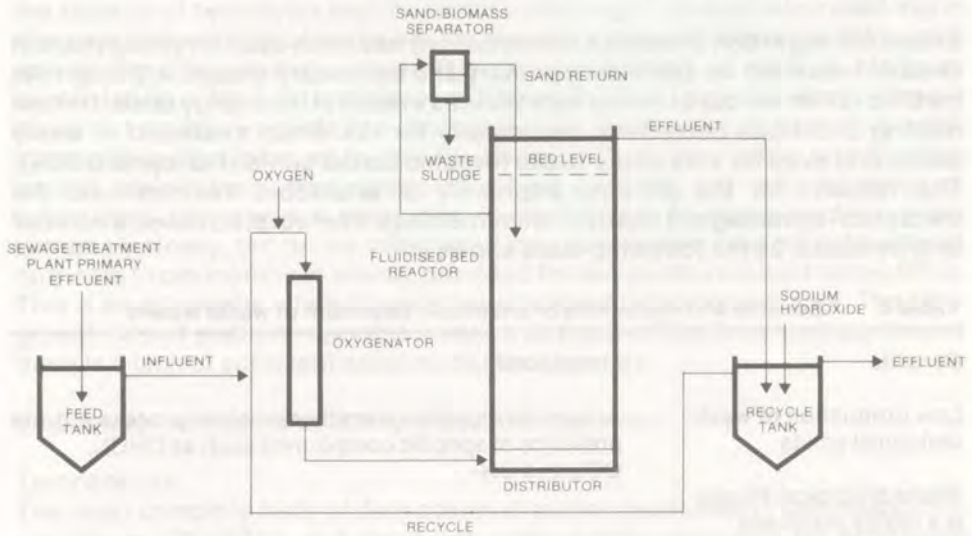


Figure 6. Orillia Oxitron system, pilot plant

Future developments will probably follow a number of directions. One line seeks the simultaneous removal of as many pollutants as possible (BOD, nitrogen and phosphate).
The other line is directed towards developing separate methods for each type of pollutant. In waste water treatment those methods would then be used in series. Occasionally there is a combination of biological and chemical methods.
It is becoming increasingly clear that biological methods are more attractive than chemical or physical methods for BOD and nitrogen removal. Phosphate removal is generally achieved via the chemical route, but here too various research institutes are examining the possibilities of biological methods.

It would also be worth devoting more attention to the possibility of obtaining the desired sludge properties in separate reactors. In this type of method the return sludge would always have to go through a certain treatment stage before being reintroduced into the aeration tank. Contact stabilisation is one method in which this principle is applied to a certain extent, but it is rarely used in the Netherlands.
New systems will invariably have to meet a combination of requirements, in which the concept of social costs is assuming growing importance.
Social costs are both time and location dependent. This section, for example, has been restricted to systems which are used in the Netherlands and other rich countries with a high population density. In developing countries, though, waste water is often treated in simpler and less expensive ways, such as land treatment (100-200 m^3/ha.day), soil filtration (300-750 m^3/ha.day), and oxidation ponds.

3.3 Anaerobic waste water treatment [7, 8, 9, 10]

Anaerobic digestion (methane fermentation) has been used for many years in sewage treatment for stabilising primary and secondary sludge. Although the method rather fell out of favour for a while as a result of the energy crisis it is now making a definite comeback, particularly for the direct treatment of waste water, and even for very dilute waste (organic matter content as low as 0.05%). The reasons for the growing popularity of anaerobic treatment are the theoretical advantages it has over other methods. There are, however, a number of drawbacks, as the following table shows.

Table 4. Benefits and limitations of anaerobic treatment of waste waters

Benefits	*Limitations*
Low production of waste biological solids	Anaerobic digestion is a rather sensitive process, e.g. the presence of specific compounds such as $CHCl_3$, CCl_4 and CN^-
Waste biological sludge is a highly stabilised product that as a rule can be easily dewatered	Relatively long periods of time are required to start up the process, as a result of the slow growth rate of anaerobic bacteria
Low nutrient requirements	
No energy requirement for aeration	Anaerobic digestion is essentially a pre-treatment method. An adequate post-treatment is usually required before the effluent can be discharged
Production of methane, which is a useful end product	

Very high loading rates can be applied under favourable conditions	Little practical experience has been gained with the application of the process to the direct treatment of waste water
Active anaerobic sludge can be preserved unfed for many months	

The anaerobic digestion process

The anaerobic treatment of complex organic matter consists of three phases: hydrolysis (liquefaction), acidification and methane fermentation. Liquefaction is generally the slowest phase of the three. This is particularly true at low temperatures and at pH values lower than 6. We still know relatively little about the kinetics of hydrolysis and the factors affecting it. To a certain extent this is also true of the acidification phase, but since this does not determine the overall rate of the process our comparative ignorance is not so serious. Methane fermentation is the best understood of the three processes. However, since it involves a complex population of various organisms our understanding is still fragmentary, the more so in that the mathematical description of microbiological conversions (particularly in mixed cultures and with mixtures of substrates) still leaves a great deal to be desired. Methane bacteria grow extremely slowly, but on the other hand they can tolerate long periods without nutrients (from months to years), provided the temperature is held below 15° C. This is an advantage when the process is applied in seasonal plants. The slow growth rate of methane bacteria means that there will be long start-up times if there is a lack of sufficient adapted bacterial matter.

Environmental factors affecting anaerobic digestion

Temperature
The most complete body of data covers digestion processes under mesophilic conditions (25-40° C), but there are undoubtedly prospects for processes under cryophilic conditions, particularly for dissolved forms of waste water. In view of the slow rate of hydrolysis at temperatures below 15-20° C this does not apply to complex (undissolved) organic matter. Thermophilic digestion (50-65° C) could prove to be an interesting option for a more rapid and complete digestion of complex organic matter, but as yet there is little practical experience with this sort of process.

Acidity and bicarbonate alkalinity
An optimum anaerobic digestion process requires maintaining a pH of the reaction mixture of between 6.5 and 7.5. In other words, there has to be sufficient bicarbonate alkalinity present in order to neutralise any volatile fatty acids which might be formed.

Inhibitors and toxic substances

Methane bacteria are particularly sensitive to various compounds which are sometimes found in certain types of waste water. These substances, which include chlorinated hydrocarbons and cyanide compounds, are toxic in concentrations as low as 1 mg/litre, where as others, such as formaldehyde, sulphite, sulphide, etc., are only toxic in concentrations above approximately 200 mg/litre, but at that level their effect is often lethal. Oxygen, in particular, is extremely toxic to methane bacteria in pure cultures, but in mixed cultures it is generally less toxic. Other compounds and elements only act as inhibitors when present in very high concentrations. The degree of inhibition depends largely on the latitude given the system to adapt to these substances.

The use of anaerobic digestion for low and medium strength waste which is largely dissolved.

When applying anaerobic treatment processes to low strength waste water it is important to maintain the greatest possible amount of active bacterial matter in the reactor under high organic load conditions. The first attempts to realise these conditions involved the use of an anaerobic activated sludge process (Anaerobic Contact Process; see figure 7.1), in which a well mixed digestion plant is combined with a sludge separation and return system. Operating experience was rather disappointing, mainly because of difficulties with sludge separation. Fortunately the situation has been considerably improved by the development of new and fairly simple anaerobic treatment processes, such as the Anaerobic Filter (AF) and the Upflow Anaerobic Sludge Blanket (UASB) (see figures 7.2 and 7.3).

The UASB process, which is a Dutch development, was first used on a practical scale in 1977 for treating sugar beet effluent. As far as is known the AF process has not yet been used on a practical scale, despite the fact that it was developed 10 years before the UASB. In both processes the waste water is forced upwards through the digester. The AF contains a packing material (plastic, gravel, coke lumps, etc.). The sludge remains behind in the reactor with the packing material. Initially it was thought that this was due to attachment of the anaerobic bacterial matter, but now it is believed that the good sludge retention is mainly due to the excellent flocculation and clarification properties of the anaerobic sludge itself. This, then, is the basis of the UASB process, in which the waste water is simply passed through the anaerobic sludge bed. The formation of sludge which precipitates easily is a precondition for the proper operation of the UASB process. At present it is impossible to say for certain how far this will apply to the various types of waste water, temperatures, loads, etc.
Another condition for the good operation of the UASB process is that the gas/liquid interface in the gas separator has to be large enough to enable the gas to escape easily from the water/sludge phase. The presence of packing material can be useful in retaining finely dispersed anaerobic sludge. This leads to a considerable improvement in the filtration performance of an AF process after an initial working-up period. It is for this reason that the final clarifier in the

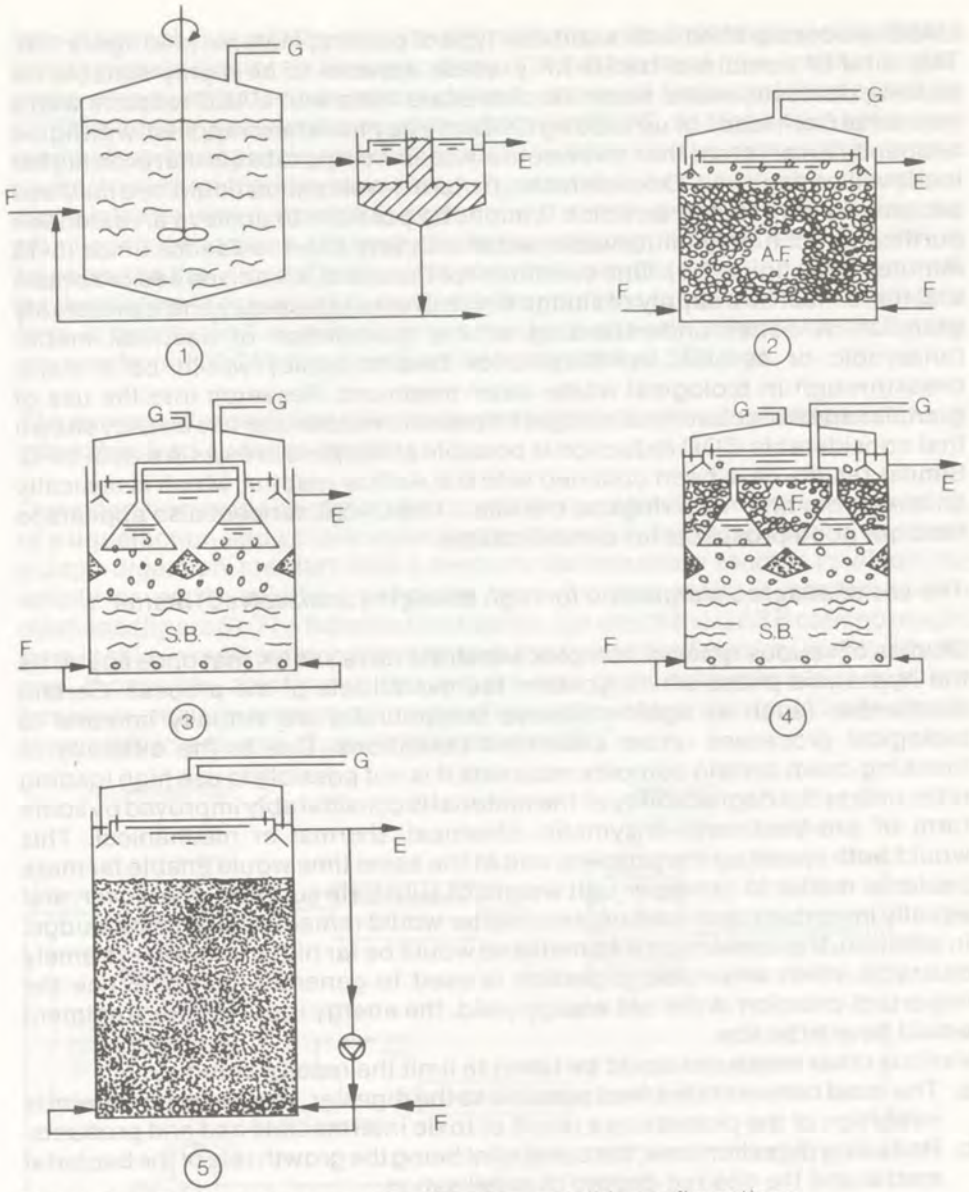


Figure 7. Reactors for anaerobic digestion, possible configurations
1. Anaerobic Contact Process
2. Anaerobic Filter (AF)
3. Upflow Anaerobic Sludge Blanket (UASB)
4. Combined UASB-AF process
5. Fluidised bed anaerobic reactor

A.F. = anaerobic filter, E = effluent, F = feed, G = gas, S.B. = sludge blanket

UASB process is filled with a suitable type of packing material (see figure 7.4). This kind of combined UASB-AF process appears to be highly suitable for treating domestic waste water. In pilot-scale trials with UASB reactors with a volume of 6 m^3, loads of up to 50 kg COD/m^3/day have been applied, with liquid residence times of less than three hours. It seems very probable that even higher loads will be achieved. One interesting future development could be a fluidised bed anaerobic reactor, in which it might be possible to achieve an extensive purification of highly dilute waste water with very short residence times (5-10 minutes) (see figure 7.5). One condition for the use of a fluidised bed process is that there must be a supply of sludge which precipitates easily and is preferably granular. A better understanding of the granulation of bacterial matter (anaerobic or aerobic, autotrophic or heterotrophic) would be a major breakthrough in biological waste water treatment. Research into the use of granular anaerobic bacterial sludge in domestic waste water has already shown that considerable COD reduction is possible at temperatures as low as 5-10^{0}C. Similar results have been obtained with the Anflow reactor, which is basically an anaerobic filter with rings as the filler. The UASB concept also appears to hold out good prospects for denitrification.

The use of anaerobic digestion for high strength (undissolved) matter

Studies of various types of complex substrate have shown that once again it is the hydrolysis phase which governs the overall rate of the process. Certain substances (such as lignin-cellulose compounds) are virtually immune to biological processes under anaerobic conditions. Due to the difficulty of breaking down certain complex materials it is not possible to use high loading rates unless the degradability of the material is considerably improved by some form of pre-treatment--enzymatic, chemical, thermal or mechanical. This would both speed up the process, and at the same time would enable far more bacterial matter to grow per unit weight of substrate supplied. Moreover, and equally important, less inert organic matter would remain behind in the sludge. In addition, the conversion into methane would be far higher. This is extremely desirable when anaerobic digestion is used to generate energy. Since the important criterion is the net energy yield, the energy input for pre-treatment would have to be low.
Various other measures could be taken to limit the reactor volume.

a. The most concentrated feed possible to the digester, where the constraint is inhibition of the process as a result of toxic intermediate and end products.
b. Reducing digestion time, the constraint being the growth rate of the bacterial matter and the desired degree of stabilisation.

Another proposal is to divide the digestion into two stages, separating hydrolysis and acidification from methane fermentation. In this two-stage process it would be possible to apply very high loading rates in the second stage. However, hydrolysis remains the determinant of the overall rate, and it is clear that without pre-treatment etc. in the liquefaction reactor it will be impossible to achieve high loads (short residence times). The advantages of a

two-stage process will therefor be minimal unless the first stage can be carried out extremely simply and inexpensively. For the moment it seems that single-stage digestion processes are preferred, above all because loads of 6-7 kg COD/m^3/day are attainable at a temperature of 30°C. At higher temperatures, loads of up to approximately 10 kg COD/m^3/day can be achieved in certain cases. In view of the intensive research which has been carried out in recent years into the use of anaerobic digestion for energy generation (including the small-scale generation of energy on farms using agricultural residues), it is to be expected that more effective and, above all, simpler digestion processes will be developed in the near future. At the moment the production of biogas on a farm is economic (i.e. without any need for a subsidy) when fermenting the manure from 100-150 cows, or from approximately 1,500 pigs.

The use of anaerobic digestion for low strength waste water composed mainly of undissolved complex matter

Consideration could be given here to a multi-stage treatment system consisting of a liquefaction and acidification reactor (possibly combined with a separate sludge digestion reactor) and a methane fermentation reactor in which the wholly or partly acidified substrate from the first stage is converted into methane (figure 8). The liquefaction reactor, for which the UASB concept might be suitable, would serve both to entrap the suspended matter, and to liquefy and, if possible, acidify it. Since the process could take place slowly at low temperatures, any entrapped matter which was not liquefied could be dealt with separately in a sludge digester operating at 30°C.

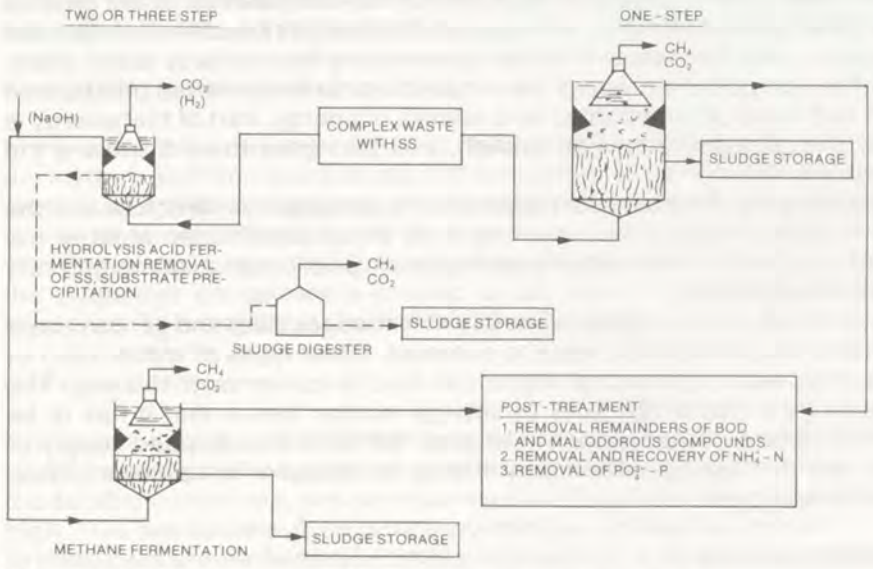


Figure 8. Multi-stage anaerobic treatment of complex wastes (SS = suspended solids)

The need for post-treatment

Anaerobic treatment requires a suitable post-treatment stage if the effluent is to be discharged to surface waters. This is because BOD removal will generally be incomplete, and also because a large proportion of the nitrogen compounds in the waste water leave the system in the form of ammonium. Moreover, the presence of sulphurous compounds leads to sulphide formation, which can create a serious nuisance. In some cases post-treatment can raise the overall treatment costs considerably. Biological processing might be employed to eliminate the ammonium (nitrification, combined with denitrification if possible). The introduction of a pre-acidification stage might be beneficial in the anaerobic treatment of waste waters containing sulphate or sulphite (the latter being particularly toxic to methane bacteria). Aerobic methods could also be used to eliminate sulphides from the effluent from the methane fermentation reactor, but consideration could also be given to a denitrification stage.

3.4 Composting

Introduction

Composting is usually defined as the aerobic decompostion of organic matter to a humus-type product, accompanied by disinfection [11]. The conversions are mediated by various organisms, such as bacteria, *Actinomyces* species (which have the features of both bacteria and fungi), fungi, protozoa and worms.
The dominant type of organism depends on the composition of the organic matter, the size of the particles, the moisture content, the amount of oxygen, the temperature, and the extent to which conversions have already taken place.
During the composting process the organic matter is converted into carbon dioxide and water, accompanied by a release of energy. Part of that energy is used by the organisms for cell growth, and part goes towards raising the temperature.
The possible uses of the compost depend on its composition, structure, and the degree of composting. The main use is as a soil conditioner. Most of the compost produced in the Netherlands is used in public parks, sports grounds and in private gardens.
Domestic refuse contains approximately 45% food residues and garden waste [2], which makes it relatively easy to compost. Other types of waste, such as sewage sludge and agricultural waste, can also be converted in this way. The high moisture content (92-98%) of sewage sludge means that it has to be dewatered before composting. There also has to be an adequate supply of oxygen, and this can be achieved by mixing the sludge with domestic refuse, tree bark or sawdust.

Composting systems
Composting processes can be divided into static and dynamic systems [12].

The static systems can be further subdivided into:
— windrow composting;
— composting of compressed wastes;
— cell composting.

Windrow composting

Windrow composting is a very old system, and in the Netherlands it has been used at Wijster since 1929. The refuse is heaped into rows approximately six metres high, and is turned over two or three times during the decomposition period of 7-10 months. The material is then sieved. A reduction in the size of the refuse components increases the surface area, giving the bacteria more space in which to operate, with the result that biological conversions take place more rapidly. Mechanical pre-treatment, such as mixing the refuse with sewage sludge, paper or sawdust, is made easier if there is size reduction.
Since the reduced refuse is more compact it is difficult to maintain an adequate supply of oxygen, and anaerobic conditions can rapidly build up. In order to prevent this happening the reduced refuse is heaped into rows lower than those used for unreduced waste. These rows also have to be turned frequently during the first few months.
If the rows are high it is technically difficult to turn the waste, and so air is generally drawn through it. Any malodorous substances can be removed from the exhaust gases in a filter plant.
This forced aeration can cut the decomposition time to less than two months. This method of composting domestic refuse with sewage sludge is found at Zell am Zee (Austria) and elsewhere. After the waste has been composted in the aerated rows, post-composting takes place in non-aerated rows for 3-4 months.

Composting of compressed wastes

In this system the domestic refuse is reduced, mixed with sewage sludge (25% dry matter), and then compressed into briquettes. The briquettes are heaped in loose stacks. Within four weeks the dry matter content rises to around 80% due to intense microbial activity and the high temperature generated (up to 70°C). This material can be stored for a long time in the open air. For agricultural use the briquettes are generally ground or are set out for post-composting in windrows. This method is used at Biel (Switzerland).

Cell composting

It is possible to accelerate the decompostion process by maintaining the moisture content at the optimum level throughout the process, by maintaining the aerobic conditions, and by ensuring that the temperature does not rise too high. This can be done in cells incorporating air and water pipelines.
In these cells pre-fermentation takes place within 1-2 weeks, and is followed by composting for 4-8 weeks. An example of this system is found at Calais (France).

An intermediate form between the static and the dynamic systems is the *Klappbodenzell*. In this system the waste is alternately at rest and in motion. Motion is achieved by free fall to a lower floor. The cells are stacked in storeys in a tower (also known as a bioreactor). The reduced waste is fed in at the top of the tower. The residence time is usually one day per floor, and a tower generally has 6-10 floors. Oxygen, carbon dioxide and temperature regulate the air supply. Mixtures of sewage sludge and wood waste are also composted in this way (Rastatt, West Germany) [13].

Dynamic systems

Composting in towers

In the dynamic systems a distinction is made between composting in towers and in drums.
The Kneer system is an example of tower (bioreactor) composting. The waste is fed in at the top. No mixing takes place. The waste descends under the force of gravity, with the composted matter being continuously removed from the bottom. Variations are possible in the way in which air supply and water content are controlled.
At some sewage treatment plants in West Germany (Buchen and elsewhere) a bioreactor is used for composting the sludge. Residence time is 10-14 days. This is followed by post-composting in windrows for approximately six weeks [13].
There are also storeyed towers in which the waste is kept in continuous motion. The floors are fitted with ploughshares which are set so that the waste is moved from inside to outside and back again. The material falls to a lower floor each time. Residence time can be short. Windrow post-composting is necessary in order to obtain a stable product. This Multibacto system has been in use since 1974 at Heidelberg (West Germany).

Composting in drums

Drum composting is used in the Netherlands at Haarlemmermeer and Soest-Baarn. The Dano drums rotate on a horizontal axis. The rotation transports the material continuously from the inlet side to the outlet side. Air and water can be added at the inlet side. A residence time of 2-3 days is usually sufficient. Windrow post-composting is necessary, but there is no need to turn the material.
Composting systems are chosen partly on the basis of the standards required of the compost, and partly on the type of waste to be treated.

Possible developments

The composting process could be accelerated if the organic fraction of domestic refuse was collected separately, or if it could be separated mechanically from the remainder of the refuse stream. Separation would also

help to improve the quality of the compost.
Any increase in the popularity of composting will depend on the market for the product and on the competitive position of the processing technique compared to other biological treatment methods, such as anaerobic digestion (methane production) and hydrolysis (glucose production).

3.5 Other systems and developments

Immobilised micro-organisms

Research is being carried out on the possibility of employing micro-organisms to reclaim useful substances from waste. This can be done more easily with clearly defined waste streams (from industry, for example) than with sewage water. One specific area attracting attention at the moment is the production of organic acids from waste streams containing carbohydrates. Compere and Griffith [14] studied the production of lactic acid by passing whey in a continuous stream through an anaerobic fixed-film reactor. The reactor had been inoculated with a mixture of lactic acid bacteria and lactose-fermenting yeasts. Coulman et al. [15] and Stieber et al. [16] used *Lactobacillus bulgaricus* cells in a similar process in which the cells were immobilised by containment with semi-permeable membranes granting passage to the relatively small molecules but not to the micro-organisms. Hang et al. [17] developed a batch-fed process for the production of citric acid from brewery effluent using the mould *Aspergillus foetidus*. Up to 96% of the available sugar was converted, half of it to citric acid. These and similar processes are still in the bench-scale stage. They have a reasonable chance of success, which will increase as levies on discharge are raised. If they do prove successful the effect will be to transfer clearly defined waste streams, such as whey, to the feedstock category.
Suzuki et al. [18] are working on the development of a biochemical fuel cell, the principle of which is shown in figure 9.
The reactor is filled with *Clostridium butyricum* cells, entrapped in small cubes of agar (or polyacrylamide) gel. The micro-organism produces hydrogen and organic acids from the organic substances in the waste water. The liquid then passes the actual biochemical cell, where the hydrogen causes a negative potential at the platinum-black anode. The fluid finally passes the third reactor in the system, which too contains cells, immobilised on gel-cubicles, this time aerobic, isolated from the activated sludge. Here the acids produced as well as other biodegradable organic products present in the fluid coming from the biochemical fuel cell are converted to carbon dioxide and water.
In principle, immobilised cells are ideal for breaking down compounds for which more than one enzyme is required. One example is the decomposition of phenol by polymer-entrapped *Candida tropicalis* of *Trichosporon cutaneum* cells [19, 20].
There has even been a report of the isolation of a micro-organism (a yeast) which shows a preference for growing on phenol, even in the presence of glucose [21].
The significance of the development of this sort of process lies in the fact that a

great deal of phenol can find its way into waste waters from the production of plastics, pesticides, disinfectants, etc.
Another example is the hydrolysis of Epsilon aminocaproic acid cyclic dimer, which is found in waste water from polyamide plants. The dimer is an unnatural substance which strongly resists biodegradation. Kinoshita et al. [22] isolated a micro-organism, *Achromobacter guttatus*, which is capable of hydrolysing the dimer. They have demonstrated on a bench scale that it is possible to treat the waste water by leading it through a packed bed with cells entrapped in acrylamide gel.

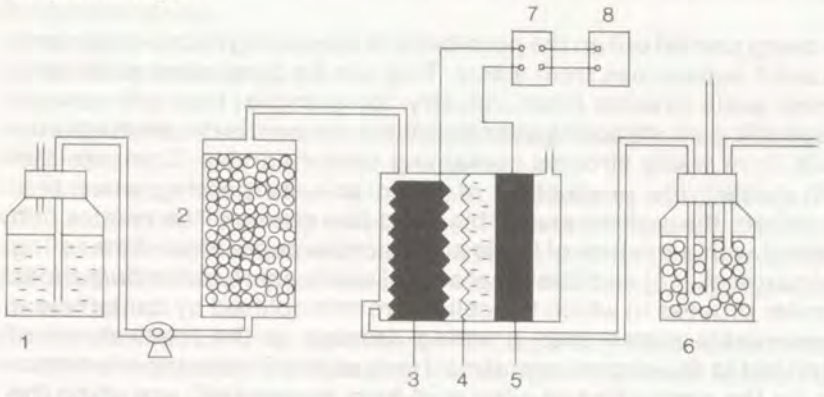


Figure 9. Schematic diagram of biochemical fuel cell system
1. Reservoir for the waste water
2. Packed-bed reactor for immobilised *C. butyricum*
3. Platinum-black anode
4. Ion-exchange membrane
5. Carbon cathode
6. Continuously stirred tank reactor for immobilised aerobic micro-organisms
7. Ammeter
8. Recorder

From: Biotechnol. Bio eng. *8* (1978) 503

Totally inactivated micro-organisms can also play a useful role in the treatment of waste water. The cell walls of micro-organisms (or capsular materials surrounding them) often prove very suitable for fixing metals and thus concentrating them. This is also the case with heat-inactivated micro-organisms, as has been demonstrated in experiments involving moulds [23] and algae [24].
The latter experiments showed that dead micro-organisms are sometimes far better at fixing metals than are their living brethren. The walls of disrupted cells of *Bacillus subtilis* have also proved capable of fixing many metals. Very strong binding has been reported for magnesium, ferric and cupric ions (0.2 mg/mg dry weight of cells from 0.5 mM solutions) [25]. This opens up the prospect of using filtration to remove metals present in low concentrations in aqueous solutions. Immobilisation of the micro-organisms or cell walls makes even

filtration unnecessary. This system is comparable to an ion exchanger. Since the cells are dead no substrate is needed, so the system could be used as a pre-treatment stage.

Immobilised enzymes

Enzymes which are more or less pure can also be useful in the pre-treatment of waste waters. Enzyme isolation is a relatively expensive operation, which is why they are immobilised to prevent them being lost during the treatment process. One example is the removal of cyanides from waste water using a packed bed of cyanide-decomposing enzymes immobilised in a polyacrylamide gel [26].

Selection and genetic engineering

The presentation of a recalcitrant substance as the only extra source of carbon (or of other essential nutrients for the growth of micro-organisms) creates conditions which can lead to mutants which decompose that particular substance.
Results depend on spontaneous mutations, and the process can sometimes be accelerated by raising the mutation frequency, for example by ultraviolet radiation. Harder et al. give an excellent account of the possibilities for the selection of micro-organisms in continuous cultures [27].
The growing body of knowledge in the field of genetic engineering makes it likely that the genetic information for the creation of enzyme systems can be combined with or transferred to a more suitable micro-organism. This could increase the conversion rate or the number of substances which one micro-organism can convert. This has been demonstrated by Friello et al. [28], who combined plasmids for the decomposition of different alkanes in a single *Pseudomonas* cell.
There are no known micro-organisms which can decompose all the components of crude oil, so a mixture of many different species has to be used. The drawback with such mixtures is that there are large variations in the growth rates of the various strains, which means that it takes a very long time to decompose mineral oil completely. It would be a far simpler matter if there was one micro-organism which could decompose all the substances quickly and efficiently. It is known that many of the requisite enzyme systems are coded on plasmid-borne genes.
In their attempts to create the optimum micro-organism, Chakrabarty and Brown [29] succeeded in combining different plasmids in a strain of *Pseudomonas aeruginosa* which then rapidly decomposed all linear alkanes with a chain length of 6 to 18 carbon atoms.
One problem with the multi-plasmid strain is that nitrogen is required for growth. Attempts are therefor being made to incorporate into the strain plasmids bearing the genetic information for nitrogen fixation. This would make it possible to decompose hydrocarbons using air as the nitrogen source [29].

Metals

Many living micro-organisms are capable of concentrating metal ions from a solution. The bacterium *Alcaligenes eutrophus*, for example, is extremely effective at transporting nickel ions into the cell [30]. The yeast *Rhodotorula pilimanae* [31] and the mould *Ustilago sphaerogena* [32] do the same with iron ions. Other micro-organisms display the same property with manganese, for example [33]. These specific cases have been studied in detail. Some metal ions are transported into the cell as ions, while others are temporarily combined with a chelating agent produced by the cell. In the instances mentioned above the micro-organism expends energy in drawing the metal into the cell. In other cases, such as the take-up of cobalt, lead and cadmium by bakers' yeast [34], there is no energy expenditure. Once they are in the cell the metals can be reduced to their elementary state. This has been described for mercury [35].
Micro-organisms can attach charges to their cell walls in various ways, thus creating an effective ion exchange system [36]. Norberg et al. [37] have described the application of the system to environmental protection. In 15 minutes their *Zoogloea* accumulated cadmium, cobalt, chrome, copper, mercury, lead, uranyl and zinc ions from a solution, in concentrations ranging from 0.10 to 0.45 g of metal per g dry weight of cells. Similar results had already been reported by Friedman and Dugan for copper, cobalt, zinc and iron-III ions [38].
As already mentioned, inactivated micro-organisms or cell membranes are also effective at fixing metals.
By immobilising micro-organisms selected for their specific metal-accumulating properties and using them in packed beds it would be possible to extract metals from industrial waste waters, and even to reclaim them.
Use could also be made of the metal-chelating substances produced by micro-organisms. Such substances include dipicolinic acid (which is produced by many micro-organisms, including spore forming bacteria [39]), ferrichrome (produced by the mould *Ustilago sphaerogena*; [32]), rhodotorulic acid (produced by the yeast *Rhodotorula*), and chelin, a peptide-containing substance (produced by *Bacillus thuringiensis*) which specifically binds molybdate [40]. The alga *Hormidium fluitans* produces a polymer which can combine $2^1/_2$ *times its own weight of copper* [41].
The use of chelating substances necessitates study of the possibility of the efficient removal of these substances from the solution, or of their immobilisation.

Recovery of metals from solid waste

Processes have been developed for extracting metals from copper ore by allowing a suspension containing ore-leaching bacteria, such as *Thiobacillus* and *Sulpholobus* species, to percolate through the ore [42, 43] (see Chapter VIII). One obvious development would be to use waste containing metal rather than ore [44].

Coal desulphurisation

One of the problems associated with coal combustion is the emission of sulphur dioxide. The method currently employed to dispose of this problem is stack gas desulphurisation. Fluidised bed combustion is being developed for smaller generating units, and here the sulphur is combined with chalk/dolomite during the combustion process. This yields a gypsum-like product for which no application has yet been found.
One possible solution would be the removal of sulphides and sulphur from the coal itself.
This can be done microbiologically, and a research proposal was submitted in May 1980 by Poorter and Schuiling of the Geochemistry Department of the Vening Meinesz Laboratory in Utrecht [45].
Basically this process consists of allowing a dilute sulphuric acid solution to percolate through a coal stack and microbially extracting the metals dissolved in the solution. The sulphuric acid solution can be recycled. Excess sulphuric acid can be used for other purposes, after purification if necessary. The micro-organisms involved are *Thiobacillus ferrooxydans* and *Thiobacillus thiooxydans*.
The residues will be contaminated with arsenic, iron and selenium. Further processing has therefore to be developed.

Gases

Biotechnological processes currently play a far less dominant role in the prevention of atmospheric pollution than in the treatment of waste water. Standards for preventing an odour nuisance in sewage treatment have stimulated research in that sector on the applicability of biotechnological processes for the purification of polluted air.
It is, of course, possible to scrub undesirable substances from the gases in the traditional way. The resulting waste water stream can be treated as such.
One variant is to scrub the gas with water containing the micro-organisms for the conversion of the undesirable components. One such method is already being used to reduce the odour in the exhaust gases from iron foundries [46].
Methods are currently being developed in the livestock sector for combatting odour at source. The faeces of cattle are inoculated with micro-organisms which convert the malodorous substances quickly and effectively.
Okta and Ikeda [47] succeeded in deodorising the faeces of pigs, chickens and cows, and activated sludge within a few hours. The waste was mixed with dry waste (rice husks, straw, sawdust) in order to increase porosity, and with the starter culture. The main active micro-organisms were *Actinomyces* species (*Streptomyces griseus, Streptomyces antibioticus* and *Thermoactinomyces sp.*), which metabolise sulphite, hydrogen sulphide, mercaptanes and low-molecular fatty acids, among other substances.
Tanaka and Hayashida [48] isolated from the faeces of pigs, cows and chickens a number of rapidly deodorising micro-organisms, *Coprinus macrorhizus, Candida, Tricosporon* and *Streptomyces* strains. The processes which they are

endeavouring to develop, and which have already been tested succesfully in field trials involving 300 pigs, 60 cows and 7,000 chickens, do not only deodorise, but also yield a suitable additive for animal feed, since the processes ultimately produce biomass, i.e. microbial protein.

4. Socio-economic aspects

Social aspects

Dutch society, with its high population density and its concentrated industrial activity, has been increasingly confronted with the problem of waste production in the past few decades.
Many industrial processes were developed at a time when energy was abundant and when waste was regarded as a minor problem--if that. It was simply discharged or dumped.
Public resistance to further degradation of the environment has grown, as has our understanding of the consequences of pollution. At the same time it has been recognised that discharging and dumping have to be regulated by legislation.

Legislation and related measures

Environmental legislation in the Netherlands is primarily directed towards safeguarding the environment. The result has been the restraint or outright banning of discharging and dumping. Examples of Dutch legislation in this area are the Surface Waters Pollution Act, the Air Pollution Act, the Wastes Act (domestic refuse), and the Chemical Wastes Act. Other legislation being prepared at the moment includes the Soil Conservation Act and the Environmentally Hazardous Substances Act.
Discharging and dumping will not only be restrained by the principle that 'the polluter pays', by outright bans, and by the use of permits which are either temporary or difficult to obtain, but it is also reasonable to hope that there will be a move towards acceptable methods of waste treatment and the avoidance and curtailment of waste production. The standstill principle will be applied in order to prevent any further increase in pollution and to protect clean areas.
Other related measures include the promotion of knowledge about waste treatment and management, a better stock-taking of the wastes, and the establishment of a wastes data bank.
Encouragement should also be given to reliable processing, and this would include the use of biotechnological methods, both as an at-source process and as a terminal stage in waste treatment.

Economic aspects

A process which generates less waste incurs fewer costs for treating that waste. Measures designed to reduce waste production generally cost money, and

many people are all too ready to assume that such measures raise costs, have an adverse effect on the competitive position of industry (internationally as well as nationally), and jeopardise employment. This is not necessarily the case. Measures designed to reduce the volume of waste, both at source and at the terminal, can be economic. In fact, this applies to the consequences of all environmental legislation. After all, the costs of maintaining a habitable environment also have to be paid.
The benefits are as follows:
— damage control, for example by reducing discharging and dumping, and from the public health viewpoint (health service costs and sick leave);
— enhanced benefits (recreation, fish catches);
— employment, and government and industrial expenditure;
— innovative consequences and the conservation of energy and raw materials.
In many cases these benefits cover costs, although of course this can vary according to the industry or industrial sector. The idea that environmental measures have an adverse effect on the economy is outdated. Studies by the Institute for Environmental Studies [49] and by the OECD [50, 51] show that such measures can be beneficial to a country's economy.
Biotechnology should take its place in the environment industry, and to that end there should be a thorough examination of the present and future environmental production sector. Government, scientists and industry will not only have to frame an environmental production programme, but they must jointly draw up a plan of campaign for introducing measures for combatting and treating waste. The government will also be in a position to stimulate new developments in biotechnology by a planned equipment purchasing policy.

Future developments

Thorough studies are required on the application of biotechnology to the wastes problem. Research by universities of technology, TNO and industry will have to be coordinated. The introduction of practical results should be encouraged in order to control the wastes problem and the impact on the environment, to improve operating efficiencies, and to maintain or improve market positions. The government's financial support will be essential here.
Industry must gear itself rapidly for the advent of carbohydrate chemistry (starch chemistry) and coal chemistry. Biotechnology can play an important role on both the production side (prevention) and on the waste side (treatment). New methods of anaerobic treatment, the recovery of metals, biogas production, and the production of SCP from waste could begin to play a significant role, as could energy-conserving processes and improved conventional processes. Biotechnological solutions, particularly for waste removal and prevention, will be of vital importance in the future. Moreover, they will have the imprimatur of social acceptability.

5. Summary and conclusions

A great deal of experience has been gained with methods of waste treatment which have now become more or less traditional. In the past few decades there has been an optimisation of process technology, particularly in the case of aerobic methods for treating solid substances and waste waters. A clearer understanding of microbiological and biochemical processes has resulted in the control of nitrification and denitrification in waste water treatment. Biological phosphate removal is still in its infancy, and much basic research remains to be done. Considerable advances have been made in the suppression and elimination of filamentous micro-organisms in the activated sludge process. From the technological point of view there have been improvements in the efficiency of composting methods (mechanisation) and of aerobic treatment (more compact). Further technological research should be encouraged, particularly on fluid bed methods for waste water treatment.
Anaerobic systems for waste water treatment are far less advanced, and there is considerable room for optimisation. Studies in this area should also cover aerobic post-treatment. Composting, too, could be optimised, but the supply and demand equation is a complicating factor here. If there was a great demand for compost, correspondingly more attention could be focussed on optimising process technology.
The (pre-)treatment of industrial waste waters is another area due for improvement. This class of waste streams often contains specific substances which should be recovered, and here the biotechnological route has a valuable role to play. If recovery proves impossible the substances should be removed in order to reduce the burden on surface waters of recalicitrant compounds.
At the moment it appears that the supply of waste is gradually being reduced, while at the same time the possibilities of putting that waste to good use or of rendering it harmless are increasing. However, in order to make practical application possible a great deal of work will have to be done on the further development of theoretical techniques and of techniques which have been evolved in the laboratory.
The Netherlands has traditionally occupied a leading position in the development of waste treatment processes. If it is to maintain that position it must not only follow new developments closely, but must make its own contribution to the further expansion and refinement of waste technology.

Members of the working group

The working group which produced Chapter VII consisted of the following members:

Mrs L.E. Duvoort-van Engers	Institute for Waste Disposal (SVA), Amersfoort
B.A. Heide	TNO, Delft
H.L.M. Lelieveld	Unilever Research Laboratories, Vlaardingen

Dr G. Lettinga	University of Agriculture, Wageningen
R. de Vletter	CSM Suiker, Amsterdam
Dr W.C. Witvoet	DHV Consulting Engineers, Amersfoort
J.H.F. van Apeldoorn (secretary)	Netherlands Study Centre for Technology Trends, The Hague

Guest contributor:

H. Veldhoen	Member of the Second Chamber of the States-General

References:

1. M. Nicholson; Report on Pollution; framing a reference. The Engineer, 7 may 1970, 32-33.
2. Overzicht Afvalverwijdering, SVA 3000 mei (1979) Amersfoort.
3. Concept indicatief Meerjaren Plan 1980-1984. Ministerie van Verkeer en Waterstaat/Ministerie van Volksgezondheid en Milieuhygiëne.
4. Inleiding in de techniek van de afvalwaterzuivering. Nederlandse Vereniging voor Afvalwaterbehandeling en Waterkwaliteitsbeheer, Rijswijk (1979).
5. A.C.J. Koot, Behandeling van afvalwater. Waltman, Delft.
6. Metcalf en Eddy Inc., Wastewater Engineering. Mc. Graw. Hill, London (1972).
7. J.C. Young, P.L. McCarty. Water Pollution Control Federation *41* R(1969) 160.
8. G. Lettinga, A.F.M. van Velsen, J.W. Hobma, W. de Zeeuw, A. Klapwijk, Biotechnol. and Bioeng. *22*, (4) (1980) 699-734.
9. G. Lettinga, A.F.M. van Velsen, H_2O, *7*, (1974) 281.
10. R.K. Genung, D.L. Million, C.W. Hancher, W.W. Pitt, Proceedings of the first symposium on Biotechnology in Energy Production and Conservation. Gatlinburg, Tennessee, (1978) 10-12 May.
11. O. Tabasaran, Manual on solid waste management. Chapter on composting. WHO Copenhagen (1975).
12. Müll- und Abfallbeseitigung. Müll Handbuch Band 3, Erich Schmidt Verlag, Berlin (1964).
13. L.E. van Engers, Slibcomposteringsinstallaties in Duitsland H_2O (12) (1979) nr. 23.
14. A.L. Compere, W.L. Griffith, Fermentation of waste materials to produce industrial intermediates. In: Dev. Ind. Microbiol. *17* (1976) 247-252.
15. G.A. Coulman, R.W. Stieber, P. Gerhardt, Dialysis continuous process for ammonium-lactate fermentation of whey: Mathematical model and computer simulation. Appl. Environm. Microbiol. *34* (1977) 725-732.
16. R.W. Stieber, G.A. Coulman, P. Gerhardt, Dialysis continuous process for ammonium-lactate fermentations of whey: Experimental tests. Appl. Environm. Microbiol. *34* (1977) 733-739.

17. Y.D. Hang, D.E. Splittstoesser, E.E. Woodams, R.M. Sherman, Citric acid fermentation of brewery waste. Journal of Food Science *42* (1977) 383-384.
18. S. Suzuki, I. Karube, T. Matsunaga, Application of a biochemical fuel cell to wastewaters. Biotechnol. Bioeng. Symp. 8 (1978) 501-511.
19. J. Klein, U. Hackel, P. Schara, F. Washausen, F. Wagner. Polymer entrapment of microbial cells: Phenol degradation by *Candida tropicalis* (1976). Lecture held at the 5th International Fermentation Symposium, Berlin.
20. H.Y. Neujahr, Degradation of phenols by yeast. Process Biochemistry *13* (1978), No. 6, 3-7.
21. A.E. Humphrey, E.K. Pye cited in: Enzyme research: production and uses. Chem. Eng. News. Jan. 3, (1972) 25-26.
22. S. Kinoshita, M. Muranaka, H. Okada, Hydrolyses of ε-aminocaproic acid cyclic dimer by cells entrapped in acrylamide gel. J. ferment. technol. *53* (1975) 223-229.
23. R. Jilek. H. Prochazka, I. Kuhr, J. Fuska, P. Nemek, J. Katzer, Removing uranium compounds from waste mine waters. Czech patent 155, 831 (1974).
24. T. Sakaguchi, A. Nakajima, T. Horikoski, Recovery of uranium from aqueous systems by microalgae (1980). Lecture held at the 6th International Fermentation Symp. London, Ontario, Canada, July 20-25.
25. T.J. Beveridge, R.G.E. Murray, Uptake and retention of metals by cell walls of *Bacillus subtilis* J. bacteriology *127* (1976) 1502-1518.
26. K. Kawai, K. Suga, T. Imanaka, H. Taguchi, Annual meeting of the Agricultural Chemical Society of Japan, 157 (1971) (Cited in Immobilized enzyms, Research and Development Ed. J. Chibata. Tokyo: Kodanska Ltd. London: John Wilig and Sons, p. 253.
27. W. Harder, J.G. Kuenen, A. Matin, Microbial selection in continuous culture. J. applied bacteriology *43* (1977) 1-24.
28. D.A. Friello, J.R. Mybroie, A.M. Chakrabarty, Use of genetically engineered multi-plasmid micro-organisms for rapid degradation of fuel hydrocarbons. In: Proceedings of the 3rd International Biodegradation Symposium. Eds. J.M. Sharpley and A.M. Kaplan, (1976) 205-214 London: Applied Science.
29. A.M. Chakrabarty, J.F. Brown jr., Microbial genetic engineering by natural plasmid transfer. Some representative benefits and biohazards. In: Genetic Engineering. Ed. by A.M. Chakrabarty, Florida: CRC Press (1978) 185-193.
30. R. Tabillion, H. Kaltwasser, Energie-abhängige 63 Ni-Aufnahme bei *Alcaligenes eutrophus* Stamm H1 und H16. Archives of microbiology *113* (1977) 145-151.
31. C.J. Carrano, K.N. Raymond, Coördination chemistry of microbial iron transport compounds: Rhodotorulic acid and iron uptake in *Rhodotorula pitimanas* J. bacteriol. *136* (1978) 69-74.
32. T. Emery, Role of ferrichrome as a ferric ionophore in *Ustilago sphaerogena*. Biochemistry *10* (1971) 1483-1488.
33. S. Silver, P. Jasper, Mangenese transport in micro-organisms. In: Microorganisms and minerals, ed. E.D. Weinberg, (1977) 105-149. New York: Marcel Dekker.

34. R. Heldwein, H.W. Tromballa, E. Broda, Aufnahme von Cobalt, Blei und Cadmium durch Bäckerhefe. Z. für Allgemeine Mikrobiologie *17* (1977) 299-308.
35. R.L. Brunker, T.L. Bott, Reduction of mercury to the elemental state by a yeast. Applied Microbiol. *27* (1974) 870-873.
36. R. Neihof, W.H. Echols, Physiochemical studies of microbial cell walls. I. Comparative electrophoretic behaviour of intact cells and isolated cell walls. Biochem. Biophys. acta *318* (1973) 23-32.
37. A. Norberg, S.O. Enfors, N. Molin, Accumulation of heavy metals by *Zoogloea raminigera* (1980). Lecture held at the 6th International Fermentation Symposium London, Ontario, Canada July 20-25.
38. B.A. Friedman, P.R. Dugan, Concentration and accumulation of metallic ions by the bacterium *Zoogloea*. Developments in Industrial Microbiology *9* (1967) 381-388.
39. D.J. Ellar, Spore specific structures and their function. In: Relations between structure and function in the prokaryotic cell. Ed by R.Y. Stanier, H.J. Rogers, and J.B. Ward. Cambride: Cambridge University Press (1978) 295-325.
40. P.A. Ketchum, M. Sommerville Owens, Production of a Molybdemum-coordinating compound by *Bacillus thuringiensis*. J. bacteriology *22* (1975) 412-417.
41. J.C. Madgwick, B.J. Ralph, The metal tolerant alga, *Hormidium fluitans* (Gay) Heering from acid mine drainage waters in Northern Austalia and Papua-New Guinea. In: Conference Bacterial Leaching, ed. W. Schwartz, pp 85-91. GBF Monograph No. 4 Weinheim New York: Verlag Chemie (1977)
42. A.O. Summers, S. Silver, Microbial transformations of metals. Annual Review of Microbiology *32* (1978) 637-672.
43. D.G. Lundgren, M. Silver, Ore leaching by bacteria. Annual Review of Microbiology *34* (1980) 263-283.
44. K. Bosecker, M. Kürsten, Recovery of metallic raw materials by microbial leaching. Process Biochemistry *13* (1978) No. 10 2-4.
45. R.P.E. Poorter, R.D. Schuiling. Concept onderzoekproject 'Microbiologische voorreiniging van steenkool'. Veening Meineszlaboratorium, Utrecht.
46. H. dr. E. Paul, H. Kohler, Geruchtsreduzierung durch biologische Abluftaufbereitingsverfahren. Dechema Monographien vol. *86*/1 (1980) No. 1743-1744, 69-77.
47. Y. Okta, M. Ikeda, Rapid microbial deodorization of animal and agricultural wastes (1980). Paper presented at the 6th Int. Ferm. Symp., London, Ontario, Canada, July 20-25.
48. Y. Tanaka, S. Hayashida, Microbial treatment of livestock feces (1980). Paper presented at the 6th Int. Ferm. Symp., London, Ontario, Canada, July 20-25.
49. Rapport Economische Structuur en Milieu (1978) Instituut voor milieuvraagstukken, VU Amsterdam.
50. OECD studies. Employment and environment (1978).

51. OECD studies. Environmental policies and economic prospects for the 1980's. (1979).

VIII Microbial leaching

by Dr H.C. Volger

One of the essential stages in the conventional process for extracting metals from ores is an acid oxidative treatment for obtaining the metals in a solute form as metallic salts. However, there are economic objections to the use of this method if the metal content of the ore is low.
The growing awareness that mineral resources are finite and in short supply has stimulated a search for ways of extracting metals from low-grade ores using economically acceptable processes. Another eligible source would be the vast quantities of waste produced by earlier ore mining.
In certain cases the use of micro-organisms for extracting metals is an acceptable solution. Extraction is still based on oxidation of the ore, in which the metal is present as a sulphide, into a soluble metallic salt in the form of sulphates. Certain oxide-like ores are also amenable to this sort of treatment [1, 2, 3, 4, 5].
Microbiological metal extraction offers the following advantages.
— Extraction takes place at ordinary pressure and at relatively low temperatures. As a result the energy requirement is very low, and certainly far lower than in pyrometallurgical processes.
— It generates less pollution. Acid oxidation releases large quantities of gaseous sulphur compounds.
— There is a higher degree of metal extraction.

The process is not based on leaching by microbiological oxidation of the ore. It is usually presented as an oxidation of the ore under acid conditions by trivalent soluble ferric salts, which convert the metal sulphides into soluble metal sulphates. In the process the ferric salts are reduced to bivalent ferrous salts. The micro-organisms, using oxygen (air) then reoxidise the ferrous salts into ferric salts. The alternative, a chemical reoxidation of the ferrous salts, is a rather slow process, and the only way of accelerating it to an acceptable rate involves the use of far higher temperatures and a relatively high oxygen pressure. From the commercial standpoint that route is not a viable solution. The micro-organisms commonly used are capable of assimilating carbon dioxide from the air, and therefore need no organic matter.
Ideally the micro-organisms should meet the following requirements:
— autotrophism (no organic matter required);
— stable at temperatures up to 80°C;
— tolerant of high metal concentrations. Some micro-organisms can grow extremely well in the presence of relatively high metallic salt concentrations.

The following micro-organisms are used: *Thiobacillus ferrooxydans, Thiobacillus thiooxydans* (oxidation of sulphur), *Thiobacillus organoparus, Thiobaus acidophylicus, Sulphobacillus thermosulfido-oxydans, Sulpholobus acidocaldarius* [1, 2, 3, 6, 7, 8].
In certain circumstances sulphur is formed, and a mixed culture of micro-organisms is necessary in order to oxidise the sulphur to sulphate.
It should be pointed out that our knowledge of the genetics of the micro-organisms involved is very limited indeed. The future will have to show how far recombinant DNA can succeed in producing more suitable organisms.
Although most of the current applications are for copper and uranium extraction there is no reason, in principle, why micro-organisms should not be employed to extract other metals.
In the United States between 10% and 15% of all copper is extracted via the microbiological route [2]. The technology involved is fairly elementary. A heap of low-grade ore is sprinkled continuously with water. The micro-organisms and the trivalent iron salt (often present in the ore) can dissolve the metal if there is good aeration. The metallic salt solution is collected at the base of the mountain, the metal is removed using the standard method, and the residual solution is then recycled.
The operation takes place on a truly massive scale. A heap of 250,000 tonnes of material (0.4% Cu) has been treated on a daily basis [2, 5, 9], yielding 200 tonnes of copper per day.
The method is also used at the mine. The advantage here is that the ore does not have to be physically mined. There is, however, one drawback. It is not always possible to control the flow pattern of the metallic salt solution, and this can lead to pollution and losses.
One application which merits study is the recovery of metals from metal catalysts which have become inactive, in many cases because of sulphur deposition. The reactivation of the catalyst using micro-organisms or the recovery of the metal itself are future options worth considering.
Microbial leaching is only peripherally important in the Netherlands, where there is no ore to be mined. One possible application would be the recovery of valuable metals. In addition, of course, the know-how could be used by Dutch companies abroad.

References

1. A.E. Torma, Advances in Biochemical Engineering, *6*, (1977) 1-37.
2. C.L. Brierley, CRC Critical Reviews in Microbiology, *6*, (1978) 207-262.
3. O.H. Tuovinen, D.P. Kelly, Zeitschrift für allgemeine Mikrobiologie, *12*, (1972) 311-346.
4. L.E. Murr, A.E. Torma, J.A. Brierley (eds.) Metallurgical Applications of Bacterial Leaching and Related Microbiological Phenomena. Academic Press, London en New York (1978).
5. D.P. Kelly, J.R. Norris and C.L. Brierley, Microbial Technology: current State, Future Prospects: S.T. Bull, D.C. Ellwood, C. Ratlegde (eds.) 263-308

(London, New York, Melbourne: Cambridge University Press) (1979).
6. R.S. Golovacheva, G.I. Karavaiko, Microbiology, *47*, (1977) 658-665.
7. C.L. Brierley and J.A. Brierley, Canadian Journal of Microbiology, *19* (1973) 183-188.
8. T.D. Brock, K.M. Brock, R.T. Belly, R.C. Weiss, Archives for Microbiology *84* (1972) 54-68.
9. K. Bosecker, M. Kürsten, Process Biochemistry *13* (1978) 2-4.

IX Agriculture

1. Introduction

The agricultural sector has been included in this study for two reasons. In the first place, it is a sector which employs a number of biotechnological procedures which are either industrial or which follow the industrial pattern, and which are not dealt with in the other chapters of this study. Secondly, various current or impending developments in agriculture have parallels with certain industrial developments. Their significance can be gauged by the importance of agriculture to Dutch society.
Broadly speaking the importance of Dutch agriculture can be divided into three aspects:
— food supply;
— social and economic factors;
— culture and recreation.

Food supply

Although the high level of production costs in the Netherlands makes it cheaper to import many foods, there is still a significant proportion of the total range which can best be produced domestically (and without subsidy) for reasons of price and quality. This includes dairy produce, meat, eggs, vegetables (including potatoes), and some fruit. One special reason for maintaining adequate domestic production capacity for all basic foods is to counteract the threat of a food shortage or even famine caused by partial or total isolation as a result of any future international conflict.

Social and economic factors

Figures from the Agriculture and Economics Research Institute show that in 1978 there were some 100,000 agricultural concerns employing some 127,000 people, and nearly 25,000 horticultural units with a workforce of nearly 49,000. In terms of man-years this amounted to some 6% of the national total. Non-agricultural exports in that year came to Dfl. 108.2 billion (10^9), and imports to Dfl. 114.4 billion, representing a deficit of Dfl. 6.2 billion. Agricultural exports earned Dfl. 20.3 billion, as against an import figure of Dfl. 10.5 billion, giving a surplus of Dfl. 9.8 billion.
In percentage terms the list was headed by the livestock sector with 41.5%, followed by horticulture with 28.2%, of which a little over half was accounted for by glasshouse fruit and flower production. Arable farming contributed 15.4%,

and intensive farming (fattening calves, pigs, poultry) 14.9%. The overall importance of agriculture to the Dutch economy is made even greater by the large scale of the supply and processing industries. Of the Dfl. 23 billion ex farm, Dfl. 10 billion remained in the Netherlands, for which the consumer paid Dfl. 30 billion.

Culture and recreation

The cultivated landscape in the Netherlands, and much of what is commonly regarded as natural landscape, was created by farming, and it can only be preserved by farming.
Low agricultural profitability would increase the pressure on the quality of the countryside and thus jeopardise its value as a cultural and natural asset. Moreover, the very practice of agriculture is a cultural asset: way of life, mentality, production methods, etc.
Agriculture consists chiefly of growing, tending and harvesting, and it is these elements which have been the focus of agricultural research. Although work on the intrinsic properties of plants, animals and soil (plant improvement, breeding, soil improvement) has yielded important results, it has attracted less attention than cultivation and maintenance.
Within this heterogeneous domain only a few sectors have links with the present project. On the output side of agriculture, biotechnological procedures are employed in product and refuse processing on an industrial scale (beer, cheese, etc.) and on farm scale (silage, biogas, etc.). On the input side there is the production of antibiotics, insecticides etc. Such subjects have been considered in previous chapters and will not be reviewed here again. One line of true agricultural biotechnology not treated elsewhere is discussed in this chapter: the *in vitro* propagation of plant material.

Recent developments in biotechnology and in agriculture show parallels which are of interest to both. The most prominent is the field of genetic engineering or genetic manipulation. In agriculture it can be applied to any sector in which genetic variation is of importance. In practice it is limited to subjects which are accessible to thorough genetic analysis and which do not present basic technical restrictions. Plants are the most promising subjects outside industrial applications. In addition, most of the techniques required for plant improvement (breeding) are the same as those necessary for applying plant cells in the industrial production of specific organic compounds. This confronts the (agricultural) research organisations with the dilemma of a choice between promoting the production of such substances in plants on the farm, or to have them made industrially on the basis of simpler compounds (sugar, starch) provided by agriculture, for less money. Livestock have been well studied genetically, and the basic techniques for genetic manipulation have been developed. Basic technical restrictions have as yet prevented serious attempts at application. Animal cells used for the production of specific proteins are more promising, but are outside the scope of this chapter. Insect pests can in principle be controlled genetically, but neither the control systems nor the

genetic manipulation techniques have been sufficiently developed. The same is true of micro-organisms as diseases, as symbionts of ruminants, as soil components, etc. Only the nitrogen-fixing symbionts of plants are an exception.

This chapter therefore contains a discussion of the following topics:
— the *in vitro* culture of higher plants;
— plant breeding;
— genetic engineering in plant cells;
— the genetic manipulation of symbionts;
— aspects which affect these developments.

2. *In vitro* culture of higher plants [1, 2]

Introduction

The cultivation of higher plants covers the growth and development of plants in pots, glasshouses or out in the open. *In vitro* culture is the growth or development of plants, or parts of plants, in glass, i.e. in culture tubes, bottles or flasks. The *in vitro* culture of higher plants, seeds, embryos, organs, tissues, cells or protoplasts takes place on artificial nutrient media under sterile conditions.
This new method of growing plants was discovered around 1920 when plant breeders were searching for a way to prevent embryo abortion in plants. Their solution was to isolate the embryos *in vitro* just prior to abortion, and culturing them from there. Around 1922 orchid growers began sowing out orchid seeds on nutrient media in bottles instead of in the soil. The most spectacular development of *in vitro* culture has taken place in the last 10 years. A totally new biotechnology was developed in laboratories which made it possible to grow complete plants from isolated protoplasts (cells from which the wall has been removed), cells, tissues and organs. The main object of this form of biotechnology is to create plants which can only be obtained very laboriously and slowly, if at all, by the traditional propagation methods. According to recent estimates, approximately 100 million plants were produced *in vitro* in the United States in 1980. In the Netherlands the figure was around 8 million.
The *in vitro* culture of higher plants has led to the development of a number of specialisms. It became a valuable aid for agriculturalists, horticulturalists, plant breeders, geneticists, molecular biologists and others. The most important practical application is undoubtedly the cloning or vegetative (asexual) propagation of higher plants. The cloning of plants *in vitro* is particularly widespread in horticulture. It is expected that vegetative propagation will increasingly shift from the soil to the culture tube, particularly for horticultural plants. *In vitro* cloning not only enables the breeder to cut back sharply on the glasshouse area given over to cuttings, but also on the area reserved for the parent plants which provide the starting material. So in addition to reducing his capital investment it also cuts down his heating bills. *In vitro* propagation becomes more attractive the higher energy prices rise. *In vitro* research in the

Netherlands will undoubtedly have to be intensified if there is to be mass propagation *in vitro* of a wide range of economically important plants.
In vitro culture is also used in attempts to carry out processes which would otherwise be very difficult, if not impossible, to realise. This applies particularly to manipulations involving protoplasts and cells. Here the following conditions have to be met.

1. Both the nutrient media and the plant material have to be free of bacteria, moulds, viruses, etc. All operations have to be carried out in a sterile room or in a laminar airflow cabinet.
2. The preparation and composition of the nutrient media have to be such that the growth and development of the plants meet the desired goal. In many cases this entails the use of fairly complex media. Phytohormones and regulators (synthetic phytohormones) generally play a vital role.
3. The choice of plant material (age of the plant or organ, cultivation method) is often of major importance.
4. The climate in the culture room (light, temperature, etc.) has to be precisely controlled, and must suit the plant species used and the process under study.

When *in vitro* culture is used for cloning plants the following additional requirements have to be met.

1. Cells and tissues grown *in vitro* must not lose their ability to regenerate (reconstruct) a plant. Loss of regenerative ability often occurs with tissues derived from shrubs and trees.
2. Plants produced *in vitro* should in principle be identical to the parent plant.

The following paragraphs consider a number of specialisms within *in vitro* culture which are already of practical importance, or which are expected to become so in future.

Embryo culture

Embryo culture, which is the oldest form of *in vitro* culture, is the sterile isolation and culture of a mature or immature embryo in order to produce a viable plant. The object of this technique is to prevent embryo abortion in the intact plant by isolating the embryo *in vitro* prior to abortion. Embryo abortion frequently occurs in early-ripening stone fruit species (cherry, peach and apricot), and when there is, in breeders' terms, a crossing barrier. Embryo culture is also used on occasion to shorten the seed-to-seed cycle or in order to obtain haploid individuals, as in the case of barley. Embryo culture is employed on only a limited scale, mainly by plant breeders and geneticists.

Germination of orchid seeds

The vast majority of orchids are sown not on soil but on nutrient media. The method came into widespread use after 1920, when it was demonstrated that orchid seeds could germinate asymbiotically on synthetic media.
In nature orchids can only germinate if they live in symbiosis with mycorrhiza.

The seeds are totally dependent on specific fungi which produce substances which promote germination. The chances of entering on symbiosis with a specific fungus are small. Since orchid seeds are also extremely small and fragile, without differentiation and with very little food reserve, their chances of survival on soil are minimal. Consequently, orchid sowing is now done *in vitro* on a large scale throughout the world.

Vegetative propagation of higher plants

The vegetative (asexual) propagation of plants, also known as cloning, is necessary in order to obtain individuals which are identical to the parent plant and which cannot be produced generatively (using seed).However, cloning can also be vitally important for generative propagation (seed production), since some crossing parents can only be propagated vegetatively. Cloning methods have been developed for numerous plants, and involve division, cuttings, tubers, bulbs, grafting, etc.
However, there is also a large group of plants which can only be propagated very slowly by cloning, if at all, and for this group propagation *in vitro* is an important aid. *In vitro* cloning can also be valuable to the plant breeder. It has been shown that many plants can be reproduced vegetatively *in vitro* in one way or another. This provided the proof for the old hypothesis, known as the totipotency theory, that every plant cell is totipotent and is capable of reproducing itself by vegetative propagation.
A number of *in vitro* methods have been developed for the vegetative propation of plants.

1. Allowing dormant axillary buds to sprout.
2. Changing an isolated shoot tip into a structure which starts to produce many shoots. This method has been highly developed for orchids.
3. Exposing a shoot tip to a high dose of cytokinin (a phytohormone), as a result of which shoots repeatedly sprout in the axils.
4. Explant culture. New plants can be formed by isolating pieces of tissue from the plant and giving them the appropriate hormone balance.
5. Callus culture. This involves allowing a piece of tissue (group of cells) to form a callus, propagating this strongly proliferating tissue, and then allowing plants to form from it. Plants originate from calli in two ways: by sprout regeneration in the callus, or by somatic embryogenesis. In the latter route an embryo very similar to the embryo produced by fertilisation differentiates from a cluster of cells.
6. Culturing individual cells and/or protoplasts, which preferably develop immediately into embryos from which plants form.

The retention of genetic stability is one of the main problems in vegetative propagation. The greater the breakdown of the existing organisational structure (points 4, 5 and 6 above), the greater the chance of deviations from the original genotype. *In vitro* cloning using the callus or individual cell methods often demonstrates that a plant which appears to be genetically homogeneous is not so at all. Cell division takes place in abnormal cells which are present in

nature, or abnormal cells are induced by the addition of hormones like cytokinin and auxin to the nutrient medium. The isolation of protoplasts and cells and subjecting them to mass culture for plant production opens up the interesting possibility of obtaining new genotypes (mutants).
In practice use is only made of those methods of vegetative propagation which are absolutely foolproof (points 1-3 above). It is extremely regrettable that the above propagation methods are not really applicable to shrubs and trees. *In vitro* cloning would be equally desirable in arboriculture and forestry.

Production of disease-free plants

Vegetative propagation results in the new clones inheriting virus diseases, and sometimes bacterial and fungal diseases as well. Since virus diseases are very difficult to eradicate, two French scientists devised an *in vitro* method of obtaining disease-free plants. They started out from the fact that the virus concentration in the upper section of the shoot is unevenly distributed, and posited that the shoot meristem (the very tip of the shoot) might well be virus-free. They isolated shoot meristems and succeeded in producing virus-free plants from them. This method, known as meristem culture, was later successfully applied to numerous plant species. In order to simplify the process it was later combined with heat treatment (to inactivate the virus particles). Meristem culture also led to the production of bacterium-free and fungus-free plants.

In vitro fertilisation

Fertilisation sometimes fails to take place in nature, for a variety of reasons. In many cases this can be remedied by *in vitro* fertilisation. If the flower falls off the plant prematurely it can be cultured *in vitro* and fertilised (pistil fertilisation). In other cases (no germination of the pollen on the stigma, poor growth of the pollen tube) the pollen is introduced *in vitro* into the neighbourhood of the ova. A few cases of *in vitro* fertilisation are described in the literature.

Haploids

If unfertilised egg cells or sperm cells grow into individuals, the plants are known as haploids. They have only half the number of chromosomes of a fertilised offspring, since fertilisation unites two sets of chromosomes, restoring the parental number of chromosomes. Haploid plants are particularly important to breeders and geneticists. If the number of chromosomes in a haploid doubles again (either spontaneously or after treatment with an alkaloid called colchicine) the result is a diploid plant (a doubled haploid) which is homozygous. This homozygote (with two complete, identical sets of chromosomes) produces identical gametes after meiosis (reduction division). Homozygotes are an essential element in breeding programmes, mutation breeding and hybrid production.
The *in vitro* culture of anthers and pollen grains makes it possible to generate

haploid tissues and individuals artificially. This has been particularly successfull with members of the *Solanaceae* family (tobacco, tomato, thorn-apple and petunia). The main problem with haploid induction *in vitro* is to restrict division to the haploid cells. If one isolates an anther composed largely of diploid cells, then there is obviously a considerable chance that the weaker haploid cells will be suppressed by the stronger diploid cells. *In vitro* haploid induction is far from simple, and many obstacles will have to be overcome before the technique becomes suitable for large-scale application in plant breeding.

Cell fusion, somatic hybridisation and other genetic manipulations

The isolation and *in vitro* culture of protoplasts (cells without their walls) enables them to fuse and to be used for somatic hybridisation. A somatic hybrid cell or individual combines the genetic information from two individuals. This technique has been used in attempts to create plants which cannot be produced sexually, i.e. by the fusion of gametes. The technique of somatic hybridisation is no longer particularly difficult, and a number of successful hybridisations have been described in the literature. Leaving aside a number of other obstacles, the main problem is the lack of efficient selection procedures for the preferential growth of somatic hybrid cells. Somatic hybridisation and other genetic manipulations (transplantation, transformation and modification) are dealt with at greater length in sections IX.3 and IX.4.

Other applications

1. In recent years increasing use has been made of *in vitro* culture for mutation breeding. If tissues are irradiated and then allowed to regenerate, the resulting plants are often fully mutated. Another attractive option is mutation induction in cell populations, particularly haploid populations, above all when selecting for resistance to disease, salt, toxins, heat and herbicides. Only time will tell whether it is also possible to select for vigour, photosynthesis efficiency, etc.
2. Cell and tissue culture is also used to obtain polyploid plants (plants with more than two sets of chromosomes) with the aid of colchicine.
3. *In vitro* culture can also be used to change chimaeric plants (composed of cells which are not genetically identical) into non-chimaeric plants.
4. One very special case is the biosynthesis of compounds *in vitro*. Valuable substances can be obtained from cell, suspension or callus cultures. The biosynthesis of those substances can be stimulated *in vitro* by feeding with precursors. The substances can then accumulate in the cells and tissues, from which they are then extracted. In other cases the substances are excreted into the medium, from which they must be removed.
5. The maintenance of disease-free plant collections *in vitro* provides a stock of healthy material and valuable starting material for the grower and the breeder. Low temperature storage *in vitro* also saves time, space and energy. The culture tube is also an ideal container for the disease-free transportation of plant material on a small scale.

3. Plant breeding: potential and constraints

The object of plant breeding is to ensure the constant availability of plants which are better adapted to human requirements.
The genetic improvement of plants mainly concerns the following complex features:
— yield (food, animal feed, industrial feedstocks);
— quality (baking and brewing quality, fibre quality, aroma, colour, content of useful and harmful substances, digestibility);
— security of yield (resistance to disease and pests, and to such abiotic factors as frost, heat, drought, low acidity);
— efficient production (including low energy requirement per unit of product supplied, suitability for mechanical harvesting).
The final product of breeding, the improved plant, is known as the cultivar. A cultivar is always a compromise in the sense that it is impossible to breed a cultivar which is ideal for all the properties listed above.

Genetic variation

Genetic variation is the key to progress through breeding. On the one hand the breeder can draw on existing genetic variation, and on the other hand he can create new variation. Existing variation is available in the form of established domestic and foreign cultivars, landraces (heterogeneous, regional populations), and the many species and genera related to cultivated crops which occur in nature concentrated in specific regions. The available variation is collated and evaluated using methods drawn from various disciplines, such as phytopathology and physics (resistances), physiology and biochemistry (quality), cytogenetics (relationship and crossability), flower biology, quantitative genetics and biometrics (polygenic features).
New variation is obtained:
a. from crosses between cultivars;
b. from crosses with related species;
c. by doubling the number of chromosomes;
d. by halving the number of chromosomes;
e. by inducing mutations;
f. from selfings.

a. *Crosses between cultivars*
A cultivar generally has a gene content (genotype) which is highly valuable to man but which is never quite perfect. Attempts are made to remedy its shortcomings by crossing it with a cultivar which does not have those failings. A search is then made among the descendants for the desired new genotype. The drawback with crosses is that highly valuable, balanced genotypes fall apart into gametes with half the number of chromosomes, whereupon gametes from the one cultivar (ova, for example) fuse with gametes from the other. The breeder then has to wait and see whether and with what frequency desired new genotypes occur among the progeny in the first or subsequent generation. It is

worth considering whether it would not be better to selectively mutate certain undesirable genes to desirable genes, leaving the remainder of the genotype intact. However, treatment with mutagens is far too unpredictable for it to be used to mutate certain genes selectively.
If it was decided to use one of the modern methods of genetic engineering on a cultivar, it would have to be possible to integrate the desired genes in the genome of protoplasts of that cultivar and then regenerate the improved cultivar from the modified protoplast. This means that one must also be able to identify and isolate the desired genes, and then transfer and integrate them using a vector.

b. *Crosses with related species* [3].
The aim of crossing a cultivated crop with wild, related species is generally to incorporate in the cultivar specific genes from the wild species (often resistance genes). The conventional method is to cross a cultivar with the related species, and then to backcross the descendants with the cultivar several times in order to eliminate the 'wild genes', while selecting for the desired genes after each backcross.
The use of related species could also provide a solution for the refined genetic engineering mentioned in a. above, but so far there is no experience with this.

c. *Doubling the number chromosomes* [4]
Doubling the number of chromosomes is often employed to eliminate the sterility of species hybrids, or to increase the yield of a diploid crop (such as grasses and trefoils).
Colchicine treatment is the usual method for doubling the numer of chromosomes of a plant, but tissue culture methods have also been developed which geve a far higher yield of doubled plants than colchicine and without too many disturbing side-effects (with tobacco and potatoes, for example).

d. *Halving the number of chromosomes* [5]
Halving the number of chromosomes of diploid and functionally diploid (allopolyploid) crops produces total homozygosity in a single step. The resulting products, the so-called monoploids and amphimonoploids, are completely sterile, but doubling the number of chromosomes usually results in fertile homozygous diploids, referred to in the literature as DHs (doubled haploids), which can be very important in breeding (see below). Halving the number of chromosomes of an autopolyploid (such as the potato) is often done in order to simplify breeding.
There are numerous ways of halving the number of chromosomes, and almost all of them involve obtaining plants directly from female gametes (*in vivo*) or male gametes (*in vitro* anther culture).

e. *Inducing mutations* [6]
Mutations generally occur with a low frequency, and so large populations of plants have to be grown and screened in order to find desirable mutations.
Moreover, chimaerism can often occur, so that only part of the plant is mutated.

The mutagenic treatment of cells or protoplasts *in vitro* makes it possible to select mutants from very large cell populations which then yield non-chimaeric plants after regeneration.

f. *Selfings*
The self-fertilisation of a heterozygous plant yields a heterogeneous (segregating) population. Repeated self-fertilisations result in homozygosity after six of seven generations. Homozygosity can be achieved in one generation using the DH method described in d. above.

Selection methods [7]

Selection methods are ways of selecting desired genotypes from heterogeneous populations.
The breeder looks for outstanding specimens, and since they generally occur with a low frequency, large numbers of plants have to be screened. In view of the limited availability of manpower and trial fields, considerable importance is attached to efficient selection methods.
In vitro selection at the cellular level would be the ideal solution, but it is expected that this microbiological method will only be applicable for very specific features (such as resistance to high acidity and heavy metals, monogenic resistance to disease, certain quality components). It is unlikely that *in vitro* selection will be applied for the really important properties, such as yield, uniform polygenic resistance, etc.

Maintenance and propagation of cultivars

When a potential cultivar has been selected it has to be propagated in order to compare it with existing cultivars for newness and performance. The object is to get the potential cultivar registered as a new cultivar (which confers breeder's rights) and entered in the offical List of Varieties (which, in the case of agricultural crops, gives the right to market seeds and planting material). In the horticulture sector a list of varieties is merely a recommendation.
The General Netherlands Inspection Services and the Government Institute for Research on Varieties of Cultivated Crops (RIVRO) play an important role in ensuring healthy and correct propagation and in testing potential cultivars. The breeder often sub-contracts the propagation of his cultivars to specialised farms (see para. IX.6.1).
A potential cultivar of a vegetatively propagated crop, such as the potato, consists of a single, highly heterozygous genotype (clone). The main problem with maintenance and propagation is keeping the crop healthy and, above all, virus-free. Virus-free vegetative maintenance and propagation can currently be done *in vitro*, and this also applies to various crops which do not propagate vegetatively in nature.
A potential cultivar of a self-fertilising crop, such as barley, wheat and rice, is known as a pure line, i.e. a group of plants with the same, virtually homozygous genotype. A line is obtained and maintained by self-fertilisation (inbred line).

The main problem with maintenance and propagation is uniformity (the purity of the line), which is threatened by outcrossing, the incidence of mutations, and the segregation of heterozygous loci. The latter does not apply to a DH line obtained from monoploids.
A potential cultivar of an outcrosser is known as a family. This is a group of heterozygous plants with traits characteristic of the group which are maintained after generative propagation. The main difficulty with propagation and maintenance is to retain a sufficient degree of heterozygosity along with the required uniformity. The difficulty of combining heterozygosity with uniformity has led to the systematic perfection of methods of breeding hybrid varieties of several crops, mainly cross-pollinating types (maize, sorghum). This breeding method consists of the following steps:
— the production of inbred lines by a series of forced self-fertilisations or via monoploids (DH method);
— testing the inbred lines for combining ability, i.e. the degree of complementarity, which is expressed, among other things, in the marked vigour (heterosis) of their F_1 hybrids;
— the production of seed of the hybrid variety.
There are various problems associated with this method, but they will not be dealt with here.

4. Genetic engineering in plant cells

Introduction

Plant genetics has acquired a new offshoot in the past decade. Rather than working with plants and their gametes, use is now being made of body cells from the organs of the plant. These somatic cells are used in the form of protoplasts. Protoplasts are cells whose walls have been removed, generally enzymatically, leaving them enclosed merely in a membrane. They are 'stripped' cells, as it were, and as such are very similar to animal cells. Viable protoplasts can be obtained from a large number of plant species. Millions of single and isogenetic cells can be isolated, which makes it possible to subject them to techniques and approaches which are known from microbiology and animal cell research. At a cellular level mutants can be induced and selected, and genetic analyses can take place after fusion of the protoplasts. Protoplast fusion is referred to as somatic cell hybridisation. The absence of the cell wall enables the protoplasts to take up a wide range of macromolecules, such as RNA and DNA, as well as microscopic bodies like mitochondria, chloroplasts, nuclei and micro-organisms.
This opens up the possibility of genetic analysis at the cellular level of functions which are localised both in the nucleus and in the cytoplasm. This is known as somatic cell genetics.
Since plant cells are totipotent, meaning that a fertile plant can be regenerated from a single cell, somatic cell genetics could assist in broadening the genetic variability of plant species, in particular by isolating mutants and by the integration of new genes. The latter is known as genetic engineering, and it can

be done using somatic cell hybridisation, DNA transformation, and by the uptake of particles with a genetic content.
The main importance of genetic engineering in plant cells is in gathering information about the molecular biology of plants. This will enable us to learn how the total phenotype of a plant (morphogenesis) is determined by the specific interaction of biological processes which are the result of differentiated gene activity and which form the basis of the integrated organism. Secondly, it is expected that genetic engineering will contribute to plant breeding in due course. For ideal results we will have to know how to obtain plants reproducibly from the genetically modified cells.
This requires building up information about the processes which regulate cell division and cell differentiation. This will entail using plant species which are easily accessible as model systems for experimental purposes. The remainder of this section gives a brief account of the current state of the art in areas which have a bearing on genetic engineering in plants.
Further information will be found in [8, 9, 10, 11].

Protoplasts

The isolation of protoplasts is no longer an insuperable problem in the case of many plant species [8]. There is, however, one proviso, and that is that the protoplasts must be capable of forming a cell wall and then dividing. Ideally it should also be possible to regenerate plants from the callus cultures resulting from repeated division. Some 40 species have been regenerated successfully, 30 of them belonging to the nightshade family (*Solanaceae*). Tobacco, petunia and thorn-apple, which are members of this family, are ideal model plants for the development of a protoplast technology and for somatic cell genetics in general. The outlook is less promising for the monocotyledons (single seed leaf plants), to which all the cereals belong, and for the legumes or papilionaceous flower plants, such as the pea and the bean. The protoplasts of most cereal plants do not achieve division. Only in the case of rice and maize has it been possible to obtain cultures from protoplasts isolated from plant organs. Reproducibility, however, is still poor. Cultures of wheat, rice, barley, maize, sorghum and millet have been obtained from protoplasts isolated from existing cultures, which were themselves derived from explants and embryos. Nevertheless, it is confidently expected that a procedure will be found for regenerating cereal plants (which supply 80% of human and animal food requirements) from protoplasts. This optimism is based on the recent success in regenerating millet (*Pennisetum americanum*) from protoplasts. In the case of legume protoplasts it is generally possible to obtain cultures, but no plants. One exception to this is clover. Significant advances have been made with a number of other food crops, drug plants and horticultural plants. Table 1 lists the economically valuable plants which can be regenerated from protoplasts.

These plants have already moved from the laboratory to the open field. Recent advances have meant that the potato, which is an important Dutch export, can now be used as a good model plant. Encouraging results have also been

Table 1. Economically valuable plants which can be regenerated from protoplasts

crops	drug plants	horticultural plants
Asparagus officinalis	*Atropa belladonna*	*Petunia hybrida*
Brassica napus	*Datura innoxia*	*Petunia parodii*
Citrus sinensis	*Datura metel*	
Daucus carota	*Datura meteloides*	
Lycopersicon esculentum	*Hyoscyamus muticus*	
Nicotiana tabacum		
Solanum tuberosum		

obtained with cassava, which is an extremely important source of human and animal food in many developing nations. Shoots have been obtained from calli produced from protoplasts.

In order to regenerate plants it has been discovered that the tissue culture phase has to be kept as short as possible. It is therefore best to isolate the protoplasts from young organs obtained from *in vitro* culture of shoots or immature embryos. This is because the cells in a tissue culture are usually genetically unstable, and in many cases they rapidly lose their morphogenetic potential. This loss can often be remedied by supplying the protoplasts with phytohormones and allowing them to grow into small cell aggregates rather than into large calli. Morphogenesis can then be stimulated by culturing the cell aggregate further in a medium with a lower hormone concentration, or with no hormones at all. However, even when plants of valuable crops have been obtained from protoplasts, regeneration has often been sporadic and uncontrollable.

Theoretically, protoplasts are an ideal starting material for the selection of mutants [8, 9]. Biochemical selection can be applied to a large population of individuals as an alternative to analytical screening, thus saving time and space. Instead of making thousands of analyses to find varieties, cultures of single cells can be placed under positive selection conditions for regulatory mutants which produce an altered amount of a particular compound. If the selection strategy is properly planned, and if it is known how to obtain crop protoplasts which regenerate well, the selection of mutants at the cellular level would be a very welcome development. Unfortunately, our knowledge of the regulation of the synthesis of interesting products is still fragmentary, and this will have to be remedied if we are to devise goal-oriented selection systems. The most obvious applications are positive selection pressure for monogenic resistance to herbicides, fungicides, insecticides and pathogen toxins. Tobacco plants displaying increased resistance to *Pseudomonas tabaci* have been obtained from tissues which had a lower sensitivity to methionine-sulphoxamine, which is regarded as an analogue of the *P. tabaci* toxin.

Herbicide-resistant cell lines have been selected for various plant species, but in most cases it was impossible to regenerate plants from these cultures. However, Picloram-resistant plants have been obtained from Picloramresistant cultures of *Nicotiana tabacum*, and selection in tissue cultures yielded

N. tabacum plants which were less sensitive to the fungicide Carboxin. Genetic information on these resistances is still lacking.
Cell lines of various plant species have been obtained which have an increased tolerance for such abiotic factors as cold, excessive salt concentrations, and toxic metals like mercury and aluminium. Plants with an increased salt resistance have been obtained from a salt-resistant cell line of *Nicotiana sylvestris*.
Another important discovery noted by various researchers is that regeneration from protoplasts can result in a whole series of varieties. Potato plants have been produced which have a modified resistance level to the pathogens *Alternaria solani* and *Phytophthora infestans*. In addition, plants have been found with an altered pattern of plant geometry, tuber initiation, tuber phase, earliness, tuber depth, seed number and tuber shape.
Finally, it should be noted that protoplasts play an important role in the study of virus infection and replication, and of the translation of the RNA of plant viruses. Virus infection and replication in protoplasts from resistant and non-resistant plants are currently being investigated, and this could yield data on the action of virus-resistant genes.

Somatic cell hybridisation (protoplast fusion)

Plant cells contain three known types of genophores (self-replicating organelles containing genetic information).

1. The nucleus, which contains the chromosomal genes (the karyome).
2. The plastids, such as chloroplasts, each of which has several copies of circular DNA in which some of the cytoplasmic inherited genes are located. These are known as plastidomes.
3. The mitochondria, each of which contains a large circular DNA in addition to several smaller ones. The remaining cytoplasmic inherited genes are located here, where they form the chondriome. There are more and more genetic and biochemical indications that male sterility in various plant species has its basis in mitochondrial genes. This is known as cytoplasmic male sterility (cms). This sterility can be remedied by restorer genes, which are located in the karyome.

In sexual crossing between plants, the karyomes of both parents are usually transmitted to the next generation. However, with a few exceptions only the maternal plant's plastidome and chondriome are transmited to the next generation. In general, therefore, sexual reproduction results in only a limited number of combinations of genetic information. Protoplast technology could provide the breakthrough here [8, 9]. Protoplast fusion makes it possible to obtain symmetrical combinations of complete genomes (karyome, plastidome and chondriome) between and in species, genera and families of flowering plants. If there is no fusion of nuclei in the fusion product, segregation can leave the nucleus of one parent in the cytoplasm of the other, thus forming a cytoplasmic hybrid, or cybrid. If fusion involves two different chloroplast populations there is rapid segregation, with only one population or the other remaining. Some of these asymmetrical combinations could be of use to plant

breeders. For instance, it has proved possible to produce male sterility in tobacco and petunia, which is important for breeding hybrid varieties.
Chromosome elimination is to be expected, particularly when fusing the protoplasts of distantly related species. This has resulted in new genetic variability in somatic hybrids. It has been observed in plants which were regenerated from somatic hybrids of *Solanum* + *Lycopersicon* (potato + tomato), *Brassica* + *Arabidopsis* (rape + thale cress), and *Daucus* + *Aegopodium* (carrot + ground elder). Fertile plants have not yet been obtained from these hybrids, but the results show that it is possible to combine sexually incompatible genomes in a single plant, even in several important crop plants. Table 2 lists a number of somatic hybrid plants.

Table 2. Various somatic hybrid plants

Nicotiana glauca + *Nicotiana langsdorfii*
N. tabacum normal + *N. tabacum* tumour
N. tabacum + *N. sylvestris*
N. tabacum + *N. knightiana*
Petunia hybrida + *Petunia parodii*
Daucus carota + *Daucus capillifolius*
D. carota + *Aegopodium podagraria*
Datura innoxia + *Datura discolor*
D. innoxia + *D. stramonium*
D. innoxia + *Atropa belladonna*
Solanum tuberosum + *Lycopersicon esculentum*
Brassica campestris + *Arabidopsis thaliana*

In some cases it has been observed that the chromosomes of one of the parents were not present in the somatic hybrids, but that certain genes from that parent must have been present. Those genes were probably incorporated in chromosomes from the other parent, as has been noted in animal cell hybrids. There are indications that the somatic hybrids obtained from distantly related species can regenerate into plants provided there has been a sufficient degree of chromosome elimination.
The fact that flowering plants have been obtained from *Arabidopsis* + *Brassica (Arabidobrassica)* with only a few *Brassica* genes or chromosomes suggests that in due course it will also be possible to obtain fertile plants from hybrids of this sort. Work on animal cell hybrids has shown that conditions can be established in which interesting genes can be selectively retained or eliminated. If it is found that this also applies to somatic plant cell hybrids then a form of chromosome/gene transfer will have been established which will have an important practical application in plant breeding. Polygenic inherited properties could be improved by somatic cell hybridisation. Pathogen resistance which is determined polygenically is far more important than the monogenic resistance referred to above.
The main potential of somatic hybridisation for plant breeding lies in intraspecific and interspecific fusions. It appears that this technique can be used for incorporating polygenic features from wild varieties in cultivars. For example,

protoplasts from two sexually incompatible petunia species have been fused. The *P. parodii* + *P. parviflora* hybrids are currently being used successfully in a breeding programme designed to introduce the 'hanging basket' behaviour of *P. parviflora* in the cultivated petunia. Protoplast fusion in the potato offers a suitable way of obtaining a stable variety. Since protoplast fusion involves the combination of the parental chromosomes without prior halving, there is growing interest in the use of haploids in somatic cell hybridisations. In the case of *Datura*, an important species of drug plant, amphidiploid somatic hybrids have been obtained from *D. innoxia* + *D. stramonium* which produce 20% more scopolamine (an alkaloid with important medical applications) than the best parent, and they are already being planted out in the fields.
It appears that somatic cell hybridisation can also be used in order to regenerate plants from calli which cannot be stimulated to morphogenesis *in vitro*, or only with difficulty.
Intergeneric fusion between *Brassica* and *Arabidopsis* has led to the production of complete flowering plants from the somatic hybrids. Occasionally, though, roots form from callus cultures of *Brassica*, but no shoots, while *Arabidopsis* calli showed a total lack of morphogenetic activity. The same phenomenon has been observed in both somatic and sexual hybrids from different *Nicotiana* species. Hybridisation evidently leads to a restoration of the morphogenetic potential of the cell. This is of prime importance for the regeneration of plants from cells in which new genes have been integrated by DNA transformation. Should those cells not regenerate into plants, possibly because the tissue culture phase lasted too long, the solution might be to fuse them with a related species which regenerates well [8].
There are various techniques of protoplast fusion [8, 9]. The commonest fusion aids are polyethylene glycol (PEG) and calcium ions at a high pH. Combinations of the two are also used, as well as the combination of PEG with dimethyl sulphoxide (DMSO). Virtually every type of protoplast can be fused by these methods, which are of course adapted to suit the system in question. They also enable the protoplasts to take up organelles, subprotoplasts, micro-organisms, RNA and DNA.

Uptake of organelles and micro-organisms

It is believed that organelles such as mitochondria and chloroplasts evolved from bacteria and blue-green algae. Moreover, there is often a fairly close symbiosis in nature between plant cells and micro-organisms. Symbiotic nitrogen fixation is a very important process, which is why attempts have been made to encourage protoplasts to take up certain nitrogen-fixing micro-organisms in order to effect intracellular symbiosis. Plants from those protoplasts might then be capable of fixing nitrogen. Success was achieved in having protoplasts of the fungus *Phizopogan* take up the free-living, nitrogen-fixing organism *Azotobacter vinelandii*. The absorbed bacteria retained their metabolic activity and survived for two years in the fungus. Blue-green algae, yeasts and rhizobia have also been taken up by plant protoplasts. Rhizobia form

root nodules on leguminous plants. In the cells of those nodules the bacteria change into bacteroids, and in that form they fix nitrogen. There has been success in maintaining nitrogen fixation in somatic cell hybrids formed from cells with bacteroids and normal cells. At present this approach is regarded as having little practical value, and the same applies to the uptake of isolated organelles by protoplasts. The problems encountered are twofold. It appears that organelles lose their viability when isolated, although their synthesis capacity *in vitro* is good. The organelles also degenerate during uptake, due to fusion of their membrane with that of the protoplast.
It is widely believed that the best way of transferring organelles from one species to another would be to fuse protoplasts among themselves, or to fuse protoplasts with subprotoplasts.
Subprotoplasts can be divided into cytoplasts, karyoplasts and miniplasts, the latter being protoplasts with a reduced amount of cytoplasm. Cytoplasts contain cytoplasm only, and no nucleus, whil karyoplasts are largely nucleus.
Another way of encouraging organelle uptake is by means of liposomes. Liposomes are enclosed vesicles which are formed spontaneously by dispersing artifical phospholipid membranes in water. Molecules or particles present in the aqueous solution are thus enclosed. Good results have been obtained in animal cells for the uptake in liposomes of isolated chromosomes, virus RNA and virus DNA.

DNA *transformation*

DNA transformation is one of the most effective ways of integrating one or a stretch of genes in a cell. The following requirements have to be met:
— it must be possible to transform the protoplasts with a sufficiently high frequency;
— there have to be DNA vectors which function in both a bacterium and in a plant cell;
— the new, integrated genes must come to expression in proteins, and preferably in regenerated plants as well;
— in order to ensure stable transmission the new genes should preferably be integrated in the plant genome;
— it must be possible to obtain fertile plants from these genetically modified cells.

The current candidates as vectors for plant cells are cauliflower mosaic virus (CaMV) and the tumour-inducing (Ti) plasmid of *Agrobacterium tumefaciens* [8,9,10]. In future we can expect to have vectors derived from gemini viruses and isolated plant replicators. It is difficult to isolate gemini viruses in sufficient numbers, and as yet very little is known about them. It seems that it will be possible to isolate plant replicators via yeast, but here too our knowledge is sketchy.
The CaMV DNA is not integrated in the nucleus DNA of the host. This means that new genes (which are taken up using the virus DNA as vector) cannot be transmitted with seed. It is, however, known that the virus can spread to all cells of the plant, and so this would also apply to the integrated new genes. A

prerequisite, though, is that the infectivity of the virus remains intact. This would enable new genes to be maintained in a cultivar which had been propagated vegetatively. However, it is uncertain whether infectivity will persist after the insertion of new genes. The first experiments along these lines ended in failure. Various restriction maps of the CaMV DNA are now known, and the complete base sequence has been established. A DNA area has been localised which probably contains the information for the dissemination of the virus by aphids. This area might be suitable for the insertion of genes without affecting the infective potential. Success has already been achieved with unmodified CaMV DNA in infecting leaves and protoplasts, with subsequent formation of the virus.

To date the most promising vector for genetic engineering in plants appears to be the Ti plasmid of *Agrobacterium tumefaciens*. This plasmid carries genes enabling this soil bacterium, which belongs to the *Rhizobiaceae*, to cause tumours in injured plants. Agrobacteria are capable of inducing tumours in almost all dicotyledons (two seed leaf plants). The have a limited host range, However, monocotyledons (single seed leaf plants) such as cereals are unreceptive. The bacterium does not penetrate during the initiation of the tumour cell. It attaches itself to the cell wall and transmits Ti plasmid DNA to the cell. Part of the plasmid DNA is integrated stably in the nucleus DNA of the host, and in this way it comes to expression in the plant cell. On the Ti DNA are the genes which cause unlimited cell division and tumour cell growth *in vitro*. There is also a gene on the Ti DNA for a tumour-specific enzyme. Depending on the type of Ti plasmid, this enzyme is either lysophine dehydrogenase (LpDH) or nopaline dehydrogenase (NpDH). These enzymes catalyse a group of compounds known as opines. The opines accumulate in the tumour cell and can only be consumed by the agrobacteria in the tumour, which use them as a source of carbon and nitrogen. These compounds also induce conjugation (union) between different agrobacteria and between agrobacteria and other bacteria species. The system is suitable for integrating new genes whose products can be useful to man. They may be food proteins or valuable compounds, proteinaceous or otherwise. It has proved possible to regenerate shoots from certain tumour cells of tobacco varieties, but the shoots do not form roots. After grafting onto a healthy lower stem of a young tobacco plant the tumour shoot develops into a flowering cutting. It should be noted that regenerated tumour tissues retain their morphogenetic potential for more than 30 years.

There is also a good *in vitro* transformation system for *Agrobacterium tumefaciens* and pure Ti plasmid [8]. It has been shown that leaf protoplasts of various *Solanaceae* can be transformed with a high frequency, using both the bacterium itself and the Ti plasmid. Vectors have been constructed for DNA work which can be used in both *E. coli* and *A. tumefaciens*. This makes it possible to use agrobacteria which have been supplied with new genes in Ti plasmid DNA for the direct integration of those genes in the plant genome. Moreover, the tissue culture phase for the modified plant cells can be kept short. Regeneration to shoots occurs at an early stage in a reasonably high percentage of tobacco leaf protoplasts which have been transformed with the

bacteria.
Integrated new genes also have to come to expression in the plant, and one problem which may be encountered here is differentiated gene expression in different organs. Very little information is available on the regulation of gene expression in plants. However, a number of plant genes have been isolated using the recombinant DNA technique, and their base sequence established. Isolated genes of this sort can be used to study how and when they come to expression. This will lead in the foreseeable future to the establishment of the base sequences that regulate expression. It will then be possible to determine the extent to which those sequences are species-specific. It will also be possible to alter the regulatory sequences of the genes *in vitro* in order to reduce or reinforce expression. This can be studied by using a vector to integrate the manipulated gene in the plant genome.
Results with crown gall cells have shown that constitutive expression of a new gene in protein can occur in all the organs of a plant. Attempts could be made to increase protein production by manipulating these sequences. This is currently being investigated.

5. Genetic manipulation of symbionts

Nitrogen and phosphorus fertilisers are essential for high agricultural output. The high energy costs of manufacturing fixed nitrogen make this product prohibitively expensive, certainly for Third World countries. The excessive use of artificial fertiliser also creates severe salination of the soil and pollution of surface waters.
Phosphorus fertiliser will also become scarce in the future due to the shortage of natural sources. In nature, mycorrhizas often provide the plant with phosphorus salts which it would otherwise have to obtain from the soil. Mycorrhizas are fungi which live in close association with the root system of the plant, and they are often essential to the good development of plants in nature. Important natural nitrogen fixers are to be found among the blue-green algae, actinomycetes and bacteria. The most important factor in rice-fields is the symbiosis between the aquatic fern *Azolla* and the blue-green *Anabaena azollae*. In the case of the alder and the sea buckthorn, which live on very poor soil, a great deal of nitrogen fixation takes place by symbiosis with actinomycetes. These endophytes can now also be cultured *in vitro*, and are thus accessible for research purposes.
In agriculture the most important nitrogen-fixing organisms are *Rhizobium* and *Azospirillum*. Certain lignin-decomposing bacteria, which also fix nitrogen, have recently been identified, and it seems that they will be of great importance in forestry. Rhizobia are responsible for the very specific formation of root nodules on leguminous plants. They are present in the cells of the nodules as bacteroids, and nitrogen fixation occurs. *Azospirillum* is a soil bacterium which is found in alle climatic regions of the world. It is a free-living, nitrogen-fixing organism. When associated with the root system of grasses there is a marked increase in the production of ammonium ions. The association is brought about by the bacteria penetrating the vascular system of the roots. It has also been

discovered that association is better in wild cereal species than in cultivated types. The efficiency of nitrogen fixation by *Azospirillum* is certainly as great as that of *Rhizobium*. Improvement of the nitrogen-fixing capacity and expansion of the host range of good nitrogen-fixing organisms are the main genetic engineering goals for these organisms. This requires a thorough molecular genetic and biochemical understanding of the process of symbiotic nitrogen fixation. W know most about the fast-growing rhizobia: *Rhizobium trifolii*, *R. leguminosarum*, *R. phaseoli* and *R. meliloti*. It has been established for both *R. trifolii* and *R. leguminosarum* that the genes for host specificity, root nodule formation and nitrogen fixation (nif genes) are situated on a large plasmid [12,13]. This Sym plasmid can be transmitted from one species to another. This resulted in rhizobia which not only effected symbiotic nitrogen fixation with their own host, but also with the host of the plasmid donor. The discovery of the Sym plasmid has made it possible to identify the genes bearing the information for the various steps in symbiotic nitrogen fixation. The DNA areas with the genes for the separate stages can be isolated for detailed study using the recombinant DNA technique. They can also be transmitted to good nitrogen-fixing rhizobia whose host range is to be expanded.

There are also plans to incorporate nif genes in mycorrhizas and in the Ti plasmid of *A. tumefaciens*. In the latter case it is hoped to create an organism with the host range of *Agrobacterium* and the nitrogen-fixing capacity of *Rhizobium*. Initial experiments have been carried out in order to discover whether the Sym plasmid of *Rhizobium* can sustain itself in *Agrobacterium* and come to expression. This has proved to be the case with the Sym plasmid of *R. trifolii*. The *Agrobacterium* with this plasmid saw capable of creating root nodules to a limited extent on the host of the donor, which was clover. However, the agrobacteria in the root nodule cells did not have the bacteroid form. Moreover, no leghaemoglobin was synthesised in the nodules, and nitrogen fixation did not take place. This might indicate that the Sym plasmid functions regulating these processes only come to expression if bacteroids are formed. It has also been shown that *R. trifolii* with a Ti plasmid induces both root nodules and tumours. The reduction of nitrogen to ammonium ions is catalysed by a complex enzyme known as nitrogenase [12].

A great deal of energy is required for the enzymatic reduction of nitrogen. Bacteroids generate this energy by oxidising carbon compounds supplied by the plant's photosynthesis. Strong photosynthesis is therefore essential for optimum symbiotic nitrogen fixation.

Much of the energy consumed in nitrogen fixation is lost as a result of high hydrogen production during the reaction. It has been calculated for *Klebsiella pneumonia*, a free-living, nitrogen-fixing organism, that one-third of the metabolic energy required for nitrogen fixation is dissipated by the generation of hydrogen. However, there are species of bacteria, among them certain *Rhizobium* strains, which are capable of using hydrogen as an energy source.

6. Aspects affecting future developments

6.1 Legal protection of procedures and products

Patents and breeders' rights

Under the Netherlands Patents Act of 1979 it is possible to patent both a new procedure and a new product, as well as a new application of an established procedure or product.
There are a number of objections to the use of the patent principle to protect genetically engineered plants or new varieties There are legal objections which are based on the fact that plants reproduce naturally and not as a result of human intervention or machinery. The patent principle could conceivably be applied to new breeding methods, but the use of a patent would inhibit innovation in conventional breeding methods and would thus be a major obstacle to progress through plant breeding. Strictly speaking, varieties could also be brought under the Patents Act, but its protection would lapse as soon as they had undergone spontaneous reproduction. The patent principle is therefore unsuitable and undesirable for breeding methods and for the products of plant breeding, such as new varieties of cultivated crops. The solution which has been found (in Europe, at least) to the problem of protecting products of plant breeding could also provide a basis for devising proper legal protection for products of modern genetic engineering techniques. The following paragraphs therefore examine various aspects of the statutory regulation of breeders' rights.

Why breeders' rights?

The breeding of a new variety, which can take 10 to 15 years, requires numerous investments in the form of labour, glasshouses, laboratories and trial fields. When a breeder produces a variety he has created something new for the benefit of the community. He therefore has the right to be recompensed for his work. The level of his compensation should be in proportion to the use which is made of his product.

Statutory regulations

In some countries (such as the United States) the breeding of new varieties is done mainly by public institutions, so the community automatically bears the costs. In the Netherlands and other European countries the breeding of varieties is in the hands of private stations This made it necessary to legislate on the breeder's property and rights. This was first done in the Netherlands in 1941 in the Plant Breeders' Decree. This decree was not based on the patent principle, which does not apply to living material, nor on the merchandise marks right, which does not offer solid protection for the rights of breeders.
In 1961 an international convention for the Protection of New Varieties of Plants was signed by five EEC countries (including the Netherlands), and it was later

adopted by several other countries. This Paris Convention meant that the 1941 Plant Breeders' Decree had to be adapted, and this took the form of the Seeds and Planting Materials Act of 1966.

Conditions for acquiring breeders' rights

Breeders' rights are granted to a breeding product if it is:
a. sufficiently distinguuishable from existing varieties,
b. sufficiently homogeneous,
c. stable, i.e. retains its characteristic features after propagation,
d. new, i.e. has not previously been put on the market.
If a variety meets these conditions it is entered in the Netherlands Register of Varieties, which legally establishes a breeder's right to a variety. Admission to the register is decided by the Board for Plant Breeders' Rights, which is guided in its decision by the results of registration research carried out by the Government Institute for Research on Varieties of Cultivated Crops (RIVRO) at Wageningen. Breeders' rights conferred by registration are valid for 20 or 25 years, depending on the nature of the crop.

Conditions for acquiiring the right to market the seed and/or planting material of a variety

Although a registered variety is the legal property of the breeder, it can only be marketed if it has been included in the Netherlands List of Varieties. This list has the force of law for almost all agricultural crops, but is only a recommendation in the case of horticultural crops. Listing only takes place if the RIVRO research has shown that the variety has a sufficiently high agricultural value. This ensures that only good varieties come onto the market. The Government Committee for the Registration of Varieties decides whether or not a variety should be listed.
The List of Varieties classifies the varieties on the basis of their value and specific purpose. It gives an objective description of the merits and weak points of the variety, so that the grower can make an informed choice. The list also serves as a source of information for the breeder when choosing the parent material for his breeding programme. Registered varieties can be used at will for the breeding of new varieties. The breeder is legally bound to ensure that the market is adequately supplied with reproductive and propagating material from his variety, and on reasonable terms.
Horticultural crops have not been mentioned because the regulations here are as complex as the range covered by this sector.

Control and inspection of the production of seed and planting material

The maintenance and propagation of registered varieties which are also on the List of Varieties is crucial. If it is not done properly and with the necessary precautions, a variety can undergo change, contain admixtures (such as weed seeds with seed crops), become diseased (virus diseases in potato planting

materials, for example). Control is essential if these impurities are to be prevented.
This control and inspection is carried out by the General Netherlands Inspection Services (NAK). The NAK is responsible for agricultural crops, the NAK-G for vegetable crops, the NAK-B for tree crops, and the NAK-S for ornamental plants. These bodies apply standards and conditions which have to be met by the variety and by the products to be marketed (seed and planting material). The seed and planting material are divided into classes, and the appropriate certificates are issued. Prices, of course, are determined by this classification. This is not always the case with horticultural crops. One paragraph in the Seeds and Planting Materials Act which is particularly important to the breeder stipulates that the Inspection Services must furnish the breeder with a statement of the acreage on which reproductive and propagating material from his variety is being grown. The breeder's financial compensation is determined by the size of this acreage.

The closed system for agricultural crops

Only material which has been approved by the NAK may be marketed. In other words, inspection is required by law. If we then add that:
1. the Inspection Services only inspect reproductive and propagating material from listed varieties,
2. only good and registered varieties are listed, and
3. only new, distinguishable, stable and homogeneous varieties are registered,

then it will be clear that the system of marketing seed and planting material in the Netherlands is a closed system providing protection for all parties involved: breeders, traders, and consumers of seed and planting material.

6.2 Research and teaching

Research

In vitro research on plants has generated a great deal of scientific interest in the Netherlands, and a considerable amount of practical experience has been built up, much of it in the area of molecular biology. Consequently, there is a sound basis for genetic engineering in plants, and this is being pursued at a number of institutions. The Dutch universities, in particular, are well up with international developments. Although the various departments of the University of Agriculture at Wageningen are of a high scientific standard, genetic engineering in plants has not yet emerged from the planning stage. The same is true at the various agricultural institutes throughout the country, with the single exception of the Institute for the Application of Atomic Energy in Agriculture (ITAL), where a research group with experience of *in vitro* techniques (at the cellular level) is exploring new approaches, some of them at the molecular level.
The most advanced institute is the Department of Biochemistry of the University of Leiden, where the emphasis is on gene transfer unsing the Ti plasmid, and on the genetic manipulation of *Rhizobium*. A second group is the

Department of Molecular Genetics at Amsterdam's Free University, which is concentrating on genetic engineering in extra-nuclear organelles. Other groups are the Institute of Genetics, Groningen; the Department of Genetics, Leiden; the Department of Molecular Biology, Wageningen; the Department of Horticulture, Wageningen; ITAL, Wageningen. All of these groups have experience of *in vitro* techniques with plants. *In vitro* propagation on a practical scale is being applied by the Laboratory for Tissue Culture at Roelofarendsveen, by a number of private concerns, and by government institutions (experimental stations, institutes, NAK-S). Similar projects are being carried out at various phytopathology institutes and experimental stations with the object of obtaining disease-free material.
If the field is to be developed properly the institutions involved must have a command of the basic but often complex techniques required. It might be satisfactory to have a few highly specialised procedures carried out at a limited number of institutes for the benefit of other bodies. This, though, means that it will be essential for the agencies of the Ministry of Agriculture and the universities to work together as closely as possible.
Other techniques have to be available at all the institutes involved, and this will often require investment in equipment and personnel. The personnel will often need to be retrained, and this will almost certainly encounter resistance. The same will apply when programmes have to be altered in order to place a greater emphasis on genetic engineering. Existing programmes will have to be retailored, and this could create severe problems, given the existing structure of decentralised, democratised decision-making.
As a result of the above factors, the general lack of manoeuvring room, and legislative restrictions on research, it is not inconceivable that a number of highly gifted researchers might elect to go and work elsewhere.

Cooperative arrangements will have to be strengthened in order to arrive at an efficient allocation of tasks within this heterogeneous branch of complex research, and in order to make facilities available to all the various groups. Some of the coordination could be done by the working groups of the Foundation for Biological Research in the Netherlands (BION). It would be worth making an attempt to bring both agricultural and university research under the aegis of the National Agricultural Research Council (NRLO). However, this would only be worthwhile if agricultural research could make a more substantial contribution, both quantitatively and qualitatively. Agricultural research is vital to this sort of joint approach, and it is therefore essential to stimulate genetic engineering in this area.
There also has to be closer collaboration on industrial biotechnology within the agricultural sector, and here too a place could be found within the coordinating activities of the NRLO.
If all the available expertise is to be marshalled, a contact group should be formed to lay down the practical basis for collaboration. Unfortunately, an EEC programme for cooperation on the research and development of enzyme technology and genetic engineering in micro-organisms and plants has been relegated to the longer term for political reasons. This form of international

collaboration on the basis of project studies and discussions between the participants would have been particularly beneficial to the Netherlands, which is still trailing somewhat in these areas.

Teaching

There is no special course in genetic engineering in the Netherlands, but such courses are being considered at the University of Agriculture and elsewhere. Nevertheless, various laboratories and institutes (Genetics, Biochemistry, Molecular Biology, etc.) at a number of universities are providing an adequate level of training in practically all the individual disciplines. It should be possible to establish an integrated curriculum on this basis. The Department of Horticulture at Wageningen provides a lecture course and practical course on *in vitro* culture of higher plants on a broad basis.
It is not felt that it would be a good idea to elevate genetic engineering to the status of a separate study course or secondary subject, mainly because it is far too much a part of a broader canvas.

6.3 Environment

There are no indications that genetically engineered plants in agriculture will affect the environment any more adversely than other plants. On the contrary, it is theoretically possible that plants might be developed which would impose less of a burden on the environment for an unaltered production capacity, which could even fix nitrogen adequately, and which would require less artificial fertiliser, insecticide, heat, etc. A great deal has already been achieved in this area using current breeding techniques, and the options have not yet been exhausted. In principle, genetic engineering could add an extra dimension. The *in vitro* propagation of plant material reduces almost all of these potential burdens on the environment. In addition, there are various totally new possibilities for the industrial, biotechnological processing of agricultural products and for the production of important substances specifically for the agricultural sector.

6.4 Employment

If it turns out that genetic engineering can make an important contribution to agriculture there would be every reason to make an extra investment in personnel for the labour-intensive research effort which will be required. That research, though, should be strongly goal-oriented. In the present period of increasing unemployment among university-trained and assistant scientific staff, consideration could be given to involving them in this essential but possibly temporary major expansion of the research effort.
Research and development for application abroad is an obvious option for the Netherlands, with its important quota of invisible exports. This might include specialist laboratories for carrying out commissions on a contract basis, and the production of seed and planting material (and possibly of material

propagated *in vitro*) for the international market.
The future of employment in agriculture and horticulture will depend largely on the success of genetic engineering in plant breeding and on the progress of biotechnological routes in other areas. There is little hard information on this as yet.
It should be pointed out that the improvement of traditional crops might improve the grower's income, but it should not stimulate hopes about employment.
The situation would change if it proved possible to develop new crops with a new (or dramatically improved old) product, since this would open up an entirely new market. It is here, in theory, that there are good prospects for genetic engineering.
In order to maintain employment in agriculture and allied areas it is important to concentrate on developing modified crops following the traditional routes, and not on the alternative of production from plant cells *in vitro*. Industry will undoubtedly be tempted to choose the second route, since it would then retain the entire production process in its own hands, which would make it easier to control. This would relegate agriculture to the position of a supplier of the energy-providing substrate in the form of sugars or starch.

6.5 The international position of Dutch agriculture

The quality of Dutch agricultural products is such that they command a relatively good price. That price, though, is under great pressure as a result of the over-production of a number of important products, not only in the Netherlands but in the EEC as a whole. The improvement in productivity and quality which might be brought about by genetic engineering would be unlikely to have a positive effect on this situation. The position might be slightly better in cases where variations in the product can enlarge the market, such as the growing of ornamental plants. Here too, though, there are other approaches which could be adopted.
Genetic engineering is unlikely to have much impact on the cultivation of the common crops, apart from certain improvements in quality (cereals, for example), security of yield (resistance to disease, various tolerances).
New markets could definitely be found for new crops producing pharmaceutical or other industrial raw materials. There is considerable interest in this, chiefly from industry, both within the Netherlands and abroad. Clearly, the Netherlands cannot permit itself to lag behind in this area. If it did so there would be a very real threat to the international position of Dutch agriculture, and to the supply and processing sectors.

7. Summary and conclusions

The past 10 years have seen an extremely rapid development of *in vitro* methods for culturing higher plants, and as a result a totally new form of biotechnology has grown up [11]. Scientists have succeeded in generating complete plants of many crops from protoplasts, cells, tissues, organs, etc. *In vitro* propagation

made its appearance, particularly in the horticulture sector, when it was realised that it offered a far faster and larger-scale method of cloning plants than the *in vivo* route. It is expected that this way of propagating horticultural crops will come into increasing use, partly because many crops in this sector are currently being cloned in expensive glasshouses which have to be heated. Rising energy prices will gradually move vegetative propagation away from the glasshouse (maxiglass) to the culture tube (miniglass). There is also growing interest in *in vitro* cloning in other sectors, and practical applications include the oil palm, Eucalyptus and Sequoia.

Quite apart from plant cloning and genetic engineering with protoplasts, there are numerous other potential applications of the *in vitro* culture of higher plants. They include the production of disease-free plants, haploid induction, and the biosynthesis of compounds *in vitro* [11, 12].

It would be over-optimistic to regard the *in vitro* culture of higher plants as a universal panacea. There are many problems which still have to be resolved. Many crops still resist cloning, and no solutions have yet been found for the difficulties with shrubs and trees. Cell and tissue cultures sometimes jeopardise genetic stability. In addition, culturing often causes cells and tissues to lose their ability to regenerate plants. Nevertheless, given the massive research efforts going on in many countries, there is every reason to believe that these problems will gradually be solved.

The somatic cell genetics of plants has got well into its stride in the past five years. It is still, of course, in its infancy, but encouraging advances have already been made. This applies particularly to dicotyledon crops, but the monocotyledons, such as cereals, present a more intractable problem. However, a great deal of work is being done on the monocotyledons worldwide, and breakthroughs will probably be achieved with cereal varieties in the next five years. Although cereals are still the most important food crops from the point of view of quality and yield, they are difficult to grow in many developing nations. Moreover, they require a great deal of nitrogen fertiliser if they are to attain a reasonable level of production. This makes the product more and more expensive, and forces the developing nations to rely increasingly on the western world or the eastern bloc. Modern methods for the improvement of local food crops will come into increasing use, one important reason being that those crops are better suited to the eating habits of the local population. This kind of approach may well make a more valuable and immediate contribution to solving the world food problem than the methods currently being pursued. In view of the Netherlands' international position in agriculture, it might be sensible and economically worthwhile for Dutch scientists to direct applied research to this area. Genetic engineering in plants and symbionts might take on a significant role here in the coming decades. The potato and various leguminous plants appear worthy of study, for use in the developing nations and elsewhere.

As regards the practical side of plant breeding, the aim is generally to incorporate a limited number of desirable new features in an otherwise good cultivar. However, both sexual and somatic hybridisation results in the transmission of both attractive and unattractive genes.

In practice, the combination of the total genetic structures of two species is often attended by many drawbacks. Consequently, the development of *in vitro* genetic engineering methods for integrating a limited number of specific genes from a donor will become an extremely important factor in breeding programmes within the next few years. It is unlikely that traditional plant breeding will ever be superseded by *in vitro* methods. In the longer term, genetic engineering will make it possible to create genetic structures for various crops, structures which would otherwise be impossible or very difficult to obtain. This will also help to speed up certain time-consuming stages in the traditional breeding programmes. It has been established that there are two *in vitro* methods which could make a contribution here.

a. Somatic cell hybridisation aimed at eliminating chromosomes of one of the parents. It appears that this method would make it possible for a few genes or chromosomes from one parent to be taken up in the chromosome complement of the other parent. Asymmetrical genetic combinations can also be obtained. In the case of protoplast fusion, preference would be given to the use of haploids in many instances.
b. DNA transformation using Ti plasmid vectors. At the moment they are only suitable for use with dicotyledons, and not with monocotyledons.

DNA transformation using Ti plasmid vectors currently appears to be the most effective and rapid route for integrating a limited number of genes in plants. Developments in this area have been fast and furious. A number of highly qualified groups are currently working on this system, as are several commercial genetic engineering concerns. The genes which are of practical interest to breeders still have to be identified and isolated, but the procedures for doing so have already been developed.

There is a great deal of interest in resistance genes, and it is to be hoped that they will be the first to be applied in practice, since they would ensure a greater security of yield for agricultural crops. Another area is the improvement of the quantity and quality of proteins in crops which are important to Third World countries (cassava, for instance). It is also felt that leguminous plants will have to play a greater role in the food supply of these countries than they have done hitherto. A number of legumes are extremely important sources of vegetable oil and animal feed. The use of artificial fertiliser for leguminous plants can be kept to a minimum by an optimum use of symbiosis with rhizobia. In the case of soya plants it has been shown that production can be raised by using *R. japonicum* with the requisite genes.

Attempts will be made in the next two decades:

— to make plants fix nitrogen themselves;
— to enhance plants which do not enter into any known symbiotic nitrogen fixation, either by providing them with genes which open up a route to symbiosis with rhizobia, or by furnishing the micro-organisms which associate with those plants with the appropriate nitrogen-fixing genes.

In order to obtain nitrogen-fixing plants it will be necessary to integrate suitable genes which are brought to expression at the right moment. However, the physiology of biological nitrogen fixation is so complex that it is uncertain

whether results can be expected in the short term. Even if it turns out to be possible to make plant cells fix nitrogen, there is still the question of whether the cells can generate the vast amount of energy which is needed to reduce the nitrogen without affecting the development of the plant itself.
Since plant cells, like animal cells, display the phenomenon of splicing for their expression, it might be possible to integrate animal genes in them and use them for the synthesis of valuable animal proteins which have important medical applications. Needless to say, this route would have to be able to compete with the production of those proteins by micro-organisms, but this would be possible if protein production was high and energy costs low. Plants have an enormous synthesis capacity. In the case of proteins this is illustrated by the specific synthesis and storage in protein bodies of reserve or storage proteins. Considerable research is being done on the regulation of expression of the appropriate gene and the mechanism by which protein bodies are formed. The knowledge obtained from this research will be used to produce hormone proteins instead of storage protein. One possible development would be peas which contain insulin. Photosynthesis and symbiotic nitrogen fixation could ensure that energy costs remained low.
Various Japanese patents show that the fermentation of plant cells on a massive scale is already commercially attractive.
Immobilised cells remain strongly active synthesisers for more than 40 days. It appears that immobilised cells will come to play an extremely important role in plant biology for the production of high-grade chemical and pharmaceutical products. This will undoubtedly call for the integration of the requisite genes.

Members of the working group

The working group which produced Chapter IX consisted of the following members:

Prof. J.G.T. Hermsen	University of Agriculture, Wageningen
Prof. R.L.M. Pierik	University of Agriculture, Wageningen
Prof. R.A. Schilperoort	Leiden State University
Prof. J. Sybenga	University of Agriculture, Wageningen
J.H.F. van Apeldoorn (secretary)	Netherlands Study Centre for Technology Trends, The Hague

The following provided additional information or made valuable contributions to discussions:

Dr P. Critchley	Unilever Research, Colworth Laboratory, Sharnbrook (UK
Dr R.L. Lyne	Shell Biosciences Laboratory, Sittingbourne (UK)

References

1. R.L.M. Pierik, Plantenteelt in kweekbuizen. Thieme Zutphen (1975) 1-164.
2. R.L.M. Pierik, *In vitro* culture of higher plants. Bibliography Ponsen en Looijen. Wageningen (1979) 1-149.
3. J.R. Harlan, Genetic resources in wild relatives of crops. Crop Science *16* (1976) 329-333.
4. W.L. Lewis, Polyploidy; biological relevance. Basic Life Sciences 13. Plenium Press, New York and London, (1980) 583.
5. K.J. Kasha, Haploids in higher plants, advances and potential. The University of Guelph, Canada (1974) 421.
6. C. Broertjes, A.M. van Harten, Application of mutation breeding methods in the improvement of vegetatively propagated crops and interpretative literature review. Elsevier Scientific Publishing Company, Amsterdam - Oxford - New York, (1978) 316.
7. R.W. Allard, Principles of plant breeding. John Wiley and Sons Inc. New York - London (1960) 485.
8. L. Ferenczy, G.L. Farkas, Advances in protoplast Research. Pergamon Press, Oxford (1980).
9. F. Sala, B. Parisi, R. Cella, O. Ciferri, Plant Cell Cultures: Results and Perspectives. Elsevier / North Holland, Amsterdam (1980).
10. T.A. Thorpe, Frontiers of Plant Tissue Culture. International Association for Plant Tissue Culture, Calgary (1978).
11. J. Reinert, Y.P.S. Bajaj, Plant Cell, Tissue, and Organ Culture. Springer Verslag, Berlin (1977).
12. W.E. Newton, W.H. Orme-Johnson, Nitrogen Fixation. University Park Press, Baltimore (1980).
13. J.K. Setlow, A. Hollaender, Genetic Engineering, Principles and Methods. vol. I. Plenum Press, New York (1979).

X Review of the study findings, conclusions and recommendations

by J.H.F. van Apeldoorn

1. Introduction

The development of biotechnology is destined to have a major impact on society in the next few decades. Various applications have already been indentified, but 20 or 30 years from now they will probably consitute only a fraction of the available options. Even at this stage it is clear that health care, food supplies, agriculture, fine chemicals, waste treatment, energy and extractive metallurgy will be affected, if not revolutionised, by 'the new biotechnology'. The impact of biotechnology will vary from one sector to another, as will the time-scale of its introduction

The industrial application of biological processes has a very long history. The belief that biotechnology is now on the threshold of a period of explosive development is based on a number of spectacular breakthroughs in molecular biology. That, though, is but one facet of the overall picture. Developments in process technology, microbiology and biochemistry have been more gradual, but they too are already confirming the great promise of biotechnology. Our knowledge in these areas has increased so dramatically that we are now in a position to control micro-organisms, the cells of plants and animals, and their active components, and to employ them in industrial processes. The field thus created is sometimes defined as 'the science of the integrated application of microbiology, biochemistry and process technology'. This definition reflects one of the essential features of biotechnology: it is a multidisciplinary science. It is first worth summarising certain features of biotechnology.

Positive features

Highly specific chemical reactions are possible; complex compounds can be synthesised from simple substrates; reactions can take place at low temperatures and pressures; feedstocks are renewable; now that recombinant DNA techniques have proved practicable, it is possible to manufacture compounds which are not normally made by micro-organisms, and which are difficult, if not impossible, to produce via the chemical synthesis route. Biotechnological processes have environmentally benevolent aspects, and the technology can be relatively clean.

Negative features

Process operation costs are comparable to those in the chemical process industry. They are often high, and so in the case of fine chemicals the use of the

biotechnological route is generally only warranted for products with a high added value. At present most processes are batch processes. The waste streams are generally large, but they are not particularly toxic, and are easily biodegradable. The concentrations of the target product are generally low, necessitating intensive processing, which usually involves a high energy input. Purification and processing are often extremely complex, which pushes up the price of the product. Renewable feedstocks require the availability of considerable acreage for the agricultural production systems, certainly in temperate latitudes.

The Dutch case: strong and weak points

Strong points

Biotechnology is highly knowledge-intensive. The successful development of biotechnological processes requires a good infrastructure of training and research at all levels in microbiology, biochemistry and process technology. The Netherlands amply meets this bill.
The basic sciences are generally well developed [1], but their integration into the discipline of biotechnology is less well advanced. The Netherlands is a small country, so Dutch scientists are in far closer touch with each other than their counterparts in other countries.
The Netherlands has a number of thriving fermentation companies, one of which is highly diversified. Dutch agriculture can produce a limited number of suitable fermentation feedstocks, albeit at a high price.

Weak points

University departments are traditionally chary of the multidisciplinary, problem-oriented approach [2]. Research in the life sciences at Dutch universities is highly fragmented. Collaboration between universities and industry is poor. The adverse business climate inhibits the development of the latent spirit of enterprise [3, 4, 5]. The process technology equipment industry is weak. The process of political decision-making and the decentralised licensing system are major obstacles to the development of genetic engineering.

2. Present and future applications: the impact of biotechnology in the Netherlands.

The food industry

Changes in production methods will take place slowly. This is because the consumer is slow to accept changes in taste, and also because of the lengthy safety clearance procedures. In the short term the main change will be the improvement (optimisation and control) of existing processes.

The emphases will be on:
— improving quality
  . increased shelf life (essential, particularly for maintaining taste)
  . consistency
  . taste
— reducing energy consumption
— reducing waste

There will be an increase in the use of biotechnological processes, particularly for the preparation of food ingredients. Development will take a very long time in the case of end products (complex, composite products).
Biotechnological methods can make many waste streams useful, both within the plant and elsewhere.
The new techniques will have to be applied circumspectly to products where sensory quality is paramount. The consumer's attitude towards new production methods will be an important factor in determining the successful application of biotechnology.
At present it is difficult to gauge the extent to which genetic engineering will be used. If micro-organisms actually form part of the food (yoghurt and cheese, for example), clearance could require expensive safety testing.
Advanced methods will have to be developed for the rapid detection and analysis of minute amounts of foreign micro-organisms or their products.

The pharmaceutical industry

The first applications of the new biotechnological techniques will be in the pharmaceutical industry, where they will be used for manufacturing existing products more efficiently. One example will be the partial or total substitution of enzymatic synthesis for chemical synthesis. Another major option is the development and industrial scale production of drugs which are identical to substances produced by the human body.
The pharmaceutical industry will be the first sector to be affected significantly by genetic engineering.
This will apply to both new products and new processes. The pharmaceutical industry is the sector where the greatest revolution is expected.
Pharmaceutical processes are generally fairly insensitive to scale, but the costs of toxicological research and of the introduction of new products are so high that they are already prohibitive in a few cases. Some form of coordinated effort seems indicated here [6]. Investment in production equipment for bulk chemicals can be high. In the case of specialist pharmaceuticals the investment is not so much in production equipment as in research and development and toxicological research.
A study should be carried out to discover whether it would be possible to shorten the registration procedure for certain drugs. such as those which are identical to human natural products.
Diagnostics will benefit immeasurably from biotechnology.
Biotechnological processes could markedly stimulate the development and

production of veterinary preparations in the short term. There is a large market for veterinary vaccines. New techniques could make the production of foot-and-mouth vaccine both safer and cheaper.

The chemical industry

In the bulk chemicals sector the main accent is on new processes. In the fine chemicals sector it is on new processes and new products.
The biotechnological synthesis of fine and bulk chemicals has the following advantages:
— lower production costs as a result of reduced energy input: the more moderate process conditions permit the use of simpler equipment;
— greater safety as a result of the more moderate process conditions;
— more specific conversions in separate process stages.

General conclusions
— The first contribution of biotechnology could be in the synthesis of fine chemicals. However, the Netherlands lacks market know-how in this area.
— The contributions in the bulk chemicals sector would take place in the slightly longer term.
— The use of biotechnology for large-scale energy production would be possible in some countries (with vast expanses of arable land and a suitable climate), but not in the Netherlands.
— Although scientifically attractive, the biophotolytic production of hydrogen is rather speculative at this stage.

It is important to develop efficient, low-energy separation methods. This should be tackled vigorously. Reactor technology must be developed further.

Waste treatment

Important advances could be made in both aerobic and anaerobic waste water treatment.
Research is required on sludge attachment for both aerobic and anaerobic sludge. In the case of aerobic treatment this would lead to far higher sludge concentrations and a marked improvement in sludge retention. This is an area of great promise.
The development of small, local installations should also be encouraged.
At present, researchers are tending to overlook other problems in this field (composting, the processing of clearly defined waste streams, immobilisation, the removal of heavy metals and sulphur). More attention should be devoted to these topics.
It is impossible to predict whether genetic engineering will make an important contribution to waste treatment.

Agriculture

The application of biotechnology in agriculture is of major importance. New methods will eliminate restrictions on traditional crop breeding, making it possible to breed crops with enhanced properties, such as tolerance to poor soil and climatic conditions, disease resistance, yield, etc. The significance of these developments for world food supplies is incalculable.
However, traditional breeding methods will continue to predominate in the continuing improvement of the quantity and quality of agricultural products important to man. The process of plant breeding could undoubtedly be speeded up in the long term by applying unconventional genetic engineering.
In the long term the development of plants which fix nitrogen from the air might be an important energy-conserving option. Improving the efficiency of the nitrogen-fixing capacity of bacteria living in symbiosis with plants could be achieved in the short term, as could alterations to the host range of micro-organisms living in symbiosis with plants.
The *in vitro* propagation of higher plants using tissue culture techniques has a number of important advantages, such as increasing the propagation rate, saving energy, and making plants disease-free. The biosynthesis of compounds using *in vitro* plant cell cultures might prove to be another important development.
There are also interesting prospects in the field of plant breeding and seed production. In the long term the use of genetic engineering could boost the role of agriculture as a producer of fermentation feedstocks.
These new techniques will make themselves felt over a wide time span. For instance, improvement to existing varieties of plants using test tubes is something that is happening now, whereas the implanting of selected genes into plants to make them produce vital biochemicals is new, and there have been few successful experiments out of the large number attempted.

3. Biotechnology and the basic sciences in the Netherlands

Biotechnology can only be practised successfully if the large number of basic sciences involved are properly integrated in the research effort. A good level of knowledge in those basic sciences will not in itself produce results. Integration is the key.

3.1 Basic research

An adequate amount of basic research is carried out in the Netherlands, but it tends to be done in too isolated a fashion. This situation will have to be rectified, and that can be done by making some alterations to the funding structure. Teamwork can be stimulated by financing research from ZWO funds. The research effort would then have to be directed by SON and ZWO.
Research should be basic and problem-oriented.

Microbiology and genetics

Microbiology is well developed in the Netherlands, and there is a tradition of university and technical microbiology at many locations. In principle, microbiology can be regarded as a strong area [1]. However, there is no national policy for spreading the research activities and establishing priorities.
Greater attention should be devoted to the following research areas:
— the microbiology and genetics of organisms which have important industrial applications, such as moulds and yeasts (up until now the research focus has been mainly on bacteria);
— thermophilic organisms (important for carrying out reactions at relatively high temperatures);
— serviceable mathematical models for the growth rate and product formation rate of micro-organisms;
— interaction with biochemical aspects.

Genetics is a very advanced area. This applies to immune genetics, the genetics of inherited abnormalities, the genetics of animal viruses, and radiation genetics. As yet there is only limited applicability of molecular genetics to agriculture and horticulture, due to the fact that knowledge of the biochemistry and cell biology of plants is generally less advanced than that of animal and microbial systems.

Biochemistry

There is also a long, solid tradition of biochemistry in the Nehterlands, but it should be more closely linked to microbiology. There are sufficient research worker to cover the field.
Enzymology, which is an important part of biochemistry, is not very well developed in the Netherlands. The universities display little interest in applied enzyme technology, and university research in this area should be stimulated. The important topics include: enzyme production, disruption, production-scale purification, application in non-aqueous environments, immobilisation, multi-enzyme systems, cofactor regeneration, and the development of enzyme-based sensors.
Another important area is the synthesis of DNA sequences. Here the Netherlands is in a strong position, and this should be maintained by concentrated funding.

Molecular biology

Molecular biology is well developed at Dutch universities. Genetic engineering, however, is not, despite the fact that it is essential for building up basic knowledge. Genetic engineering should be reinforced in the fields of micro-organisms and plant cells. The aim should be to develop a few centres of expertise.
One major problem is the lack of opportunity for practising genetic engineering

in the Netherlands. If this situation is allowed to continue for much longer the outcome will be inevitable: the research facilities required by Dutch industry will be moved abroad permanently.

Process technology

Process technology for the chemicals and food sectors is a long-established and well-developed area. The application of process technology to fermentation has not yet been adequately built up at Dutch universities, and this is also true of industrial research centres to a certain extent. This is a vital area, and one which can be developed relatively quickly.
The use of mathematical models is often cited as a way of optimising processes. Although mathematical model making for microbiological process is of a high level in the Netherlands, it needs further development.
Research should be stimulated in the following key areas:
— bioreactor development;
— the development of downstream processing equipment;
— the development of sensors and control equipment.
The question of scaling up, for both production and processing, is another area requiring considerable research.
This will entail research not only at the laboratory level, but also on semi-technical and production scales. The universities are not really equipped for the latter two tasks, and there is only one commercial firm which has adequate facilities for research on productive fermentation plus processing. The choice is far wider for waste water treatment.
There are four ways of improving the situation, none of them mutually exclusive.

1. Where appropriate, Gist-Brocades could work in harness with developers of bioreactors.
2. The construction of a new, limited fermenter capacity plus processing facility. It could be located at Delft University of Technology in a joint project with TNO or other institutes. The investment for a limited number of small installations might be of the order of Dfl. 20 million.
3. In the case of extensive fermentation and processing facilities costing around Dfl. 100 million, thought could be given to a joint venture on an international scale or to participation in an existing foreign institute. The Netherlands lacks the resources to go ahead alone.
4. Stimulating a Dutch industrial sector which develops bioreactors.

3.2 Applied research

There is little applied research in the sense of a synthesis of the basic sciences to biotechnology. There is no fermentation institute, nor is there an institute for proper enzyme research such as exists in the United States and Japan. This observation applies to the universities and to TNO. Applied research of a good standard is found in some commercial companies.
TNO's attempts to expand applied research are praiseworthy, but they will only

be worthwhile if the results actually benefit industry. Research should only be carried out in the form of specific orders backed by project funding.
There is too little applied research at Dutch universities. The main thrust of this research should be on the know-why of applications, and that should preferably be done by project funding from industry or by a planned sector research programme. Delft University of Technology and Wageningen University of Agriculture are the locations where applied research should be concentrated.

3.3 Research coordination

It is clearly impossible to produce work of an international standard in every field. If we spread our potential (university and industrial) and efforts over too wide an area we will achieve nothing. That danger, though, is very real, precisely because of the wide range and the importance of the various areas of application.
In order to avoid falling into this trap, the universities must get together (occasionally, but not always, bringing industry into the discussions) and select a limited number of suitable areas. They should then aim for the highest quality in those areas, which is an absolute precondition for success.
The large industrial concerns also have to choose where they are going to channel their efforts. Nevertheless, it might be possible in some cases to gain more knowledgde and experience with applications by working jointly with foreign concerns. This is already happening, albeit sporadically, and it will enable us to increase our ability te react swiftly and effectively to future developments.
Teamwork involving scientists, industry and government has yielded remarkable results in various countries (particularly in the United States and, inevitably, Japan).
The structure, behaviour and mentality of the Dutch government is probably inhibiting the progress and the financial and legislative stimulation of research and application in biotechnology, as well as hindering the necessary joint approach. This state of affairs is reinforced by the markedly independent operations of the ministries of Economic Affairs, Agriculture and Fisheries, Public Health and the Environment, Science Policy, and Education and Science.
One cause for concern is that the nature of the discussion and decision-making structure in Dutch establishments of higher education is such that fruitful collaboration between sub-departments, departments, faculties and institutes in this supremely multidisciplinary research field will only emerge slowly, and incompletely at that, unless the government steps in to stimulate and supervise. There are no policy agreements between the universities and universities of technology on teaching and research. Implementation of the ideas on the funding of university research contained in the Policy Memorandum on University Research [2] would undoubtedly improve the position. Funding measures, though, are not enough on their own. It is far more important that each discipline should decide what deserves priority – and thus support – in its

own field. There is still no national policy on this point, no university policy, and sometimes not even a policy at the sub-department level.
At Delft University of Technology various sub-departments and departments have got together to decide on a joint approach, and in that sense one can say that Delft does have a policy. A similar trend is taking place at Wageningen University of Agriculture. This is a very welcome development.
The appropriate government ministries, notably Education and Science, Agriculture and Fisheries, and Science Policy, can indirectly influence the research policy at the universities and at TNO. The first steps in this direction have already been taken. The Ministry of Economic Affairs has approached industry to draw up a list of priority topics, and it has stated that it is prepared, in principle, to provide fiancial support.

3.4 Planned government measures

The aim of the Biotechnology Innovation Programme [7] drawn up by the ministries of Science Policy, Economic Affairs, and Education and Science, is to develop technical and scientific background knowledge and expertise in areas which are important for the consolidation and growth of Dutch industry and the service sector.
The report distinguishes four research sectors:
— basic research;
— research which will benefit the environment;
— research which will benefit agriculture, the livestock sector, and the food industry;
— research for applications in other industrial sectors.
The research groups taking part are expected to fund their own contributions themselves. There is also some limited extra funding available. An inter-ministerial steering group will decide on the allocation of additional funds for the programme as a whole. Coordination is the responsibility of the Biotechnology Programme Committee, consisting of specialists from each of the individual sectors, together with a government observer.

In the context of the NRLO's 1982-1986 five-year plan, a Biotechnology Problem Evaluation Committee studied the prospects for biotechnology in agricultural research. It also considered the technical and organisational aspects of approaching research in this area in an NRLO framework [8]. The committee identified seven areas as being of great importance:
— plant cell and tissue culture for the production of high-grade substances such as alkaloids, steroids, terpenes and special proteins;
— dairy biotechnology;
— carbohydrate biotechnology;
— energy-related biotechnological research;
— the biotechnological production of bio-insecticides;
— the use of animal cells;
— the potential *in vitro* biogenetic synthesis of biomimetics.
The study proposes a structure for the research involved, and that structure ties

in closely with the proposals made by the Ministry of Science Policy for the Biotechnology Innovation Programme.

TNO has drawn up an ambitious research programme which is being discussed with the government and industry. The role of TNO and ZWO in coordinating biotechnology research should be more clearly formulated.

3.5 Teaching and the integration of disciplines

The integration of the main disciplines (process technology, biochemistry and microbiology) should be reflected in teaching and research programmes. In order to be able to practise such a multidisciplinary activity as biotechnology, it is essential that training be given to research workers who have already done research work of a sufficiently high standard and who have learned to work with representatives of other disciplines.
In the interests of a full integration of biotechnology teaching it is recommended that a leading role be given to Delft University of Technology and Wageningen University of Agriculture. Groningen University should have a role up to graduate level. The universities have alredy made agreements between themselves on this subject, but unfortunately the system appears to be on the point of breaking down.
We should be on our guard against excess teaching capacity based on exaggerated expectations of biotechnology. Employment prospects in biotechhnology will be slow in maturing.

3.6 The CIII laboratory

The need for a CIII laboratory in the Netherlands now appears less pressing than it once did. However, the establishment of the CIII laboratory at the TNO Medical Biological Laboratory at Rijswijk is looked upon by the universities, industry and TNO as more than just the first CIII laboratory in the country. It is important not only for medical and biological research, but as a practical demonstration of the level of thinking and experiment in the Netherlands. The CIII laboratory can and must have a symbolic value:
— for researchers and industry, as a sign that attitudes towards recombinant DNA research have changed;
— for society as a whole, as evidence that the Netherlands has a top institute which, partly on the basis of its own research, acts as a watchdog on the safety of research and industrial-scale applications (working jointly with the responsible agencies);
— for TNO, as a means of carrying out innovative research for industry.

It is recommended that the CIII laboratory be multi-purpose:
— with work being done on material other than recombinant DNA;
— with rooms which can be easily converted from CIII to CII if the need for CIII work turns out to be less than was originally envisaged.
Large-scale production with cloned organisms will only be possible if the

hazard classification is low or non-existent. However, the impossibility of industrial-scale production under containment conditions at the CII of CIII levels does not mean that industry would not be interested in research at those levels. It is perfectly possible for an experiment to begin with a hazardous organism (such as a toxin-producing bacterium) and yet end with a safe organism intended for production (such as an *E.coli* which produces an immunologically active but physiologically inactive toxin).

3.7 Exchange of information between industry and science, nationally and internationally

TNO has come forward with a proposal for a comprehensive research programme which is currently being discussed with industry and the relevant government ministry. TNO's main task in that programme would be to establish itself as a centre of expertise in the field of genetic engineering.
TNO's second task would be to provide active support for smaller industral concerns in the field of biotechnology. This will require a study of the potential and requirements of small firms.
At the moment, though, TNO does not have any great expertise in process technology.
It is recommended that a decision be taken on this TNO proposal at an early date.
Given these two new functions for TNO, it is also recommended that the organisation structure should allow industry to influence the direction of some of the research by giving it a say in the allocation of funds.
Process technology research should only be carried out when it has the financial backing of industry. If a centre of expertise for process technology is established at TNO, it should definitely not be the only one. Others should be set up at universities (Delft University of Technology and Wageningen University of Agriculture). This will require extra funding and personnel.

The exchange of information in the basic sciences is rapid and intense. However, the establishment of DNA venture capital companies, which have attracted a number of young, promising scientists to their ranks, has not helped the exchange of knowledge. In the United States, in particular, this is seen as an obstacle to progress. Industries involved in the new biotechnology are reticent about their plans. Yet it cannot be said that there is a poor exchange of information between industry and science, either within the Netherlands or with other countries.
Some Dutch concerns have placed orders with DNA venture capital companies. This is probably a good and fast route for doing some experimenting with the application of modified micro-organisms, particularly in the light of the obstacles placed in the way of recombinant DNA work by the Dutch government. The suggestion [1] that companies of this sort should be allowed to set up in the Netherlands and use Dutch know-how on process technology appears less practicable. Dutch legislation does not encourage such companies to establish themselves here. Anyway, multinational corporations have

ready access to foreign knowledge, and it is still fairly easy to attract foreign researchers to the Netherlands.

It seems that international cooperation is no longer a realistic option, given the deplorable history of the EEC biotechnology programme to date.
The stimulus from the CREST programme for biotechnology would have been very welcome, but it now looks as though that programme will never be implemented.
At the European level, the 'Bio-Society' sub-programme of the FAST programme is now being developed.
'The main aim of the FAST programme is to contribute to the definition of long-term Community research and development objectives and priorities and thus to the formulation of a coherent science and technology policy in the long term' [9].
The central objective of the 'Bio-Society' sub-programme is a coordinated consideration of biotechnology strategy at European level. This consideration of strategy has to extend beyond the scientific and industrial, and embrace consideration of the social and political implications.
The 'Bio-Society' sub-programme comprises a coordinated group of study projects, related seminars, and the activities of an informal network [10].
The studies focus on a time-scale with an indicative horizon of three decades. There are 12 research activities bearing on the strategic development of biotechnology and the life sciences, their applications and impacts; involving 11 centres, including multinational groups and a European Federation. The total cost amounts to 605,000 e.u.a., with Commission support of 60% of agreed projects.

In the Netherlands there appears to be little interest in joint ventures with the new small research companies, the argument being that people prefer to gain the knowledge independently. That can be a very healthy attitude, but it imposes a heavy obligation on the Dutch scientific world and on industry. Medium-sized companies, such as Avebe, could receive a fresh stimulus from the opportunities offered by modified micro-organisms and improved enzymes. That will be the task of TNO and the universities--to provide the right knowledge and to make it accessible.
It is difficult to assess what benefit small companies could derive from biotechnology. A study should be carried out on this topic as a matter of priority. The options for small companies are in the production of foodstuffs and food ingredients, equipment manufacture, and waste treatment. There might also be opportunities in areas where chemical processes could be replaced by simple biotechnological processes.
In the interests of cooperation between the universities on the one hand, and industry and TNO on the other hand, ways will have to be found of safeguarding the interests of both sides. University researchers must learn to accept this, or not get involved at all. It is extremely important that business-like arrangements are made, particularly as regards publication and patenting. This will require adjustment on the part of the universities.

3.8 Patent problems*

For a patent to be granted, the most important requirements are novelty, inventiveness and commercial usefulness. Moreover, the invention must be described in the patent specification so that third parties are able to reproduce it.
For patents to be granted in the field of microbiology, any novel micro-organisms must be lodged at recognised institutes from which they are made available to the public, as otherwise third parties would be unable to reproduce the microbiological processes.
The micro-organism has to be lodged when or before the initial patent application is filed. The public gains access to the micro-organism after the patent has been granted or after publication of the application. Such publication often precedes the granting of the patent by years.
The question as to whether micro-organisms as such are patentable has yet to be definitely settled in many countries. There is little jurisprudence on this point. Moreover, the inventor runs the risk that his patent application will be rejected, while the relevant micro-organism has been made available to the public via those countries where applications are published.
Particularly now that man-made micro-organisms produced by recombinant DNA and other molecular biology techniques are being made available, the following questions arise.
— Are micro-organisms, cell lines, etc., patentable?
— Should the patented micro-organism be lodged?
— When should the micro-organism be made available, and to whom?

In the United States, a micro-organism was patented in 1980 by the Supreme Court, whereas living organisms had previously been considered unpatentable. In Europe, the patenting of micro-organisms as such is also theoretically possible, but it is not yet clear what the scope of protection afforded by such patents would be.
Rulings under Dutch patent legislation are being brought into line with the more recant rulings based on the European Patent Convention.
In the recombinant DNA field in particular, it is possible that this lack of clarity will prevail for a long time to come. Inventors in this new field are thus often faced with the difficult choice of either applying for a patent and so making their micro-organisms available to the public, or keeping their inventions secret and regarding them as know-how. From the point of view of the scientific community, applying for a patent is preferable, as the invention or research is then made public.
Over the coming years, many patent applications will be tested as to their worth; only then will it become clear what can really be patented in the field of micro-organisms and cell lines.

*Additional information was provided by Dr A. de Leeuw and P. Mars

4. The societal context

Industry
From the technical point of view biotechnology holds out great promise, but in many cases the real economic prospects are still unclear. If the new developments in biotechnology are not made accessible to industry, the Netherlands will fall behind other countries in a number of areas, including agriculture, waste treatment, and medical applications.
At the moment we are in a period of technology push, and the market still has to be developed. Whether biotechnological production processes will always be less energy-intensive than conventional processes is still unresolved. Feasibility studies are required on this point. A warning against expecting too much of biotechnology in the short term would appear to be in order.

Agriculture
The agricultural aspects should not be underestimated. This is a strong export sector for the Netherlands, and it has a tradition of extremely effective innovation.
The application of biotechnology in agriculture is extremely important. Dutch agriculture will not be able to make any contribution to energy production. However, it is expected that innovation in horticulture and in seed production will be encouraged. These sectors make a substantial contribution to the balance of payments.

Health care
The developmant of biotechnology is of major importance for health care. Many far-reaching consequences will result from the development of new and more refined drugs and methods of treatment, such as the production of new pharmaceuticals which are identical to substances produced in the human body, better quality drugs with a highly specific action, organ substitution, drug targetting, and additional and more specific diagnostic techniques using enzymes and hybridisation.
It will also be possible to replace chemical synthesis routes with cheaper or better biotechnological production methods.

Raw materials, environment
Biotechnology scores reasonably high in these areas. Once again, though, the benefits will not be felt immediately, although the struggle with the recalcitrant material has already been joined.
In principle, biotechnology offers a way of drastically reducing the waste problem. Although the large-scale use of fermentation processes generates large waste streams, they can generally be dealt with by biotechnology itself.
The Netherlands is not in a position to produce raw materials in sufficient quantities to make any significant contribution to energy supply. On the other hand, there appear to be good prospects for producing feedstocks for the fermentation industry, and part of Dutch agricultural production might be earmarked specifically for this purpose.

Public acceptance

Here biotechnology is at a crossroads.

There are many potentially positive aspects, and if properly presented they could give biotechnology a very good image.

The problem is that biotechnology is often equated with recombinant DNA technology, and that technology, sometimes referred to as 'tinkering with genes' is regarded with suspicion, particularly in the Netherlands.

It is extremely important to make it clear to the public that the link between biotechnology and recombinant DNA work is not that direct. A major part of the new biotechnology consists of the further development of applications which have been with us for decades, if not for thousands of years. Moreover, there are many possible applications of biotechnology which, although requiring higher-risk recombinant DNA work in the research and development stage, are low-risk or risk-free in industrial applications.

A number of concrete measures must also be taken, such as the early introduction of legislation, the establishment of a supervisory structure, the creation of an authoritative national centre, and (equally important) the formulation of a research programme on the safety of recombinant DNA techniques.

There needs to be an improvement in the position of the *ad hoc* committee responsible for drawing up guidelines on recombinant DNA experiments and for checking that those guidelines are observed. The powers and responsibilities of this committee should be established by statute.

Another, more wide-ranging DNA committee, which is responsible for studying the social and ethical aspects of genetic engineering, should complete its discussions as soon as possible in order to put an end to uncertainty in this area. Those discussions should also cover the industrial use of engineered micro-organisms and their products.

5. Conclusions and recommendations

General

— The many important developments in biotechnology hold out the prospect of a large number of potential applications. They are not, however, a universal panacea for all our problems. In many respects the situation in the Netherlands favours the further development of biotechnology. There are, however, certain obstacles to be overcome.

— The high general level of university education, the good level of knowledge in the relevant scientific disciplines, and the availability of industrial expertise are such that the future prospects of biotechnology in the Netherlands can only be described as favourable. The fermentation industry is by and large a capital-intensive industry, and as a result it cannot be built up so easily in non-industrialised countries. In this respect, too, biotechnology has a definite place in Dutch industrial policy.

The advantages do not mean that biotechnology will automatically prosper; but they do make its stimulation worthwile.

— New biotechnological applications and the improvement of existing applications are of great importance for the Netherlands, particularly in the following three areas:
  - the industrial-scale application of fermentation processes and enzymes in the food, pharmaceutical and chemical industries;
  - the treatment of waste and waste water;
  - agricultural applications.

— One word of warning: the markets appear far more attractive than they may in fact turn out to be.
  Feasibility studies must precede the practical application of theoretical options. We must never fall into the trap of assuming that biotechnology has the answer to all problems. The environment, raw materials and energy are such wide fields that the contribution from biotechnology in the short term can only be modest. Results can be expected earlier in the field of health care. One danger threatening the development of biotechnology in the next five years is a spate of high-flown expectations which can never be realised within such a short time span.

Industry

— The first applications of genetic engineering will be in the field of pharmaceuticals. Substances which were previously manufactured by the chemical route or were isolated from organisms (assuming they could be manufactured at all) can now be produced using fermentation processes. It will be possible to make drugs identical to human natural products or substances isolated from other organisms using biotechnological methods. In the long term this could revolutionise the pharmaceutical industry.

— The heavy financial risks attached to the development of drugs for human application generally exceed the resources of the relatively small pharmaceutical companies in the Netherlands. If this situation remains unchanged it is recommended that the emphasis should be on improving existing processes rather than developing new substances. In the past it has been shown that the Netherlands has considerable competence in this area.

— The financial risk would be reduced considerably if there was a shorter registration procedure for certain drugs. It is therefore recommended that a study be carried out to see whether it would be possible to introduce a shorter registration procedure for drugs identical to human natural products. This would entail a harmonisation of the government regulation policy at an international level.

— The prospects for veterinary applications appear extremely promising.

There is a large demand for veterinary vaccines, and the registration procedure is far shorter and less expensive than for human drugs. The use of genetic engineering will probably make the production of foot-and-mouth vaccine safer and considerably cheaper in the next five years. Bacterial vaccines could also be improved considerably by the use of genetic engineering.

— The possibility of expanding the fine chemicals industry should be examined. Biotechnology opens up new possibilities, including a reduction in the number of process stages and the application of hybrid catalysis. There are definite opportunities in this area as a result of Dutch experience in the fermentation industry and chemical process technology. A knowledge of the market should be acquired.

— Large-scale energy production from biomass is impossible in the Netherlands. It would make too great a demand on the available acreage, while the energy return is negative, or at best marginally positive. The Netherlands could develop process know-how, which could then be exported. This would have to be backed by practical expertise. The development of bioreactors for this field and, above all, of separation methods, is of great importance.

— The development of bioreactors could be an important task for the metal-working industry. There are incentives for this. The process industry and equipment manufacturers are already receiving the special attention of the Ministry of Economic Affairs. It is recommended that a feasibility study be carried out on the prospects in the field of biotechnology. The areas of particular interest are: sensors, containmnet, transport control, and membranes. There are also opportunities here for small companies. The government could provide stimuli by placing specific orders.

— The fermentation industry is by and large a capital-intensive sector which uses advanced knowledge, and as such it cannot easily be built up in non-industrialised countries. However, we should not underestimate the advances which developing nations could make in the longer term. Some of these activities require only a modest investment, and there are developing nations which probably have far more fermentation feedstocks than the Netherlands. We should be thinking of exporting know-how based on our domestic market, not forgetting the possibility of competition from the Third World 10 years or so from now.

— There is still a great deal that can be done in the Netherlands in the field of the anaerobic and aerobic treatment of waste water, as well as in converting waste into useful products. Despite the large effort being devoted to these areas, the end is not yet in sight.
Ways should be found of making better use of our advanced knowledge in this field. The export of installations and the delivery of turnkey projects to other countries should be encouraged.

Agriculture

— The process of plant breeding, which plays an important role in the improvement of agricultural crops, could be speeded up considerably by the use of tissue culture and genetic engineering.

— The development of genetic engineering in agriculture is vital if Duch agriculture is to retain its competitive position. With the exception of a few areas where results can be expected more quickly, such as altering the host range of micro-organisms living in symbiosis with plants, the development of genetic engineering in plants will occupy several decades. In addition to increasing production efficiency, there are opportunities in the fields of plant breeding and seed production and product marketing, i.e. there is an export potential to Third World countries and elsewhere.

Universities and research institutes

— Although the original considerations may no longer apply, the CIII laboratory must definitely be built at TNO, and it must be properly staffed.

— The requisite expertise must be built up around the CIII laboratory, particularly in the field of medicine. The government should support the research financially for a considerable length of time.

— Effective forms of cooperation should be developed between the universities on the one hand, and industry and TNO on the other hand. The structure should accomodate the objectives of both partners. Hard-and-fast agreements on industrial property are particularly necessary.

— It would be wise to restrict the practice of fully integrated biotechnology to a few university institutes. Two centres, at Delft University of Technology and Wageningen University of Agriculture, would certainly be sufficient. Research should be basic and problem-oriented. At Delft the exphasis should be on process technology, and at Wageningen on the life sciences. Elsewhere it will be sufficient to carry out research in the basic disciplines in areas relevant to biotechnology without necessarily becoming a *centre d'excellence*.

Government

— In order to prevent any major duplication or fragmentation of the research effort, the appropriate ministries (Economic Affairs, Agriculture and Fisheries, Public Health and the Environment, Education and Science, and Science Policy) and the research organisations involved (universities, ZWO, TNO) must be prepared to participate in a coordinating structure.

— Industry abroad has a dangerous lead over Dutch industry in genetic

engineering. This applies to both research and application. The domestic guidelines on genetic engineering should therefore be brought into line with those obtaining in the rest of Europe.

— If the new developments in biotechnology are not made accesible to industry we will find ourselves at a punitive and unwarranted disadvantage in a number of areas, such as chemistry, agriculture, waste treatment, and medical applications.

— The maximum permissible production scale for modified micro-organisms is far too low. In many cases production will be uneconomic unless this level is raised dramatically.

— The development of biotechnological production processes can be hampered by high prices for raw materials, by legislative restrictions on marketing compounds which compete with agricultural products, etc. It is therefore recommended that the potential of biotechnology be examined against the background of the EEC levy policy.

Consultation group

Chapter X was compiled on the basis of a series of three written and personal interviews with each of the members of the consultation group. This was followed by a joint meeting of all the members, in the course of which the final form of this chapter was agreed upon.
The following were the members of the consultation group:

Dr J.A.M. van Boxsel	Ministry of Science Policy, The Hague
Prof. S. Bruin	University of Agriculture, Wageningen
P. van Eerde	Heineken, Zoeterwoude
Dr J. de Flines	Gist-Brocades, Delft
J.W. Hillege	Ministry of Economic Affairs, The Hague
R. Keuning	Unilever Research Laboratories, Vlaardingen
Prof. N.W.F. Kossen	Delft University of Technology
Prof. A. Rörsch	Central Organisation for Applied Scientific Research in the Netherlands (TNO), The Hague
Prof. A.H. Stouthamer	Free University, Amsterdam
Dr H.J. Vink	formerly of Philips Research Laboratories, Eindhoven

Interview assistant

G. Blokland

References

1. R.R. van der Meer, Biotechnologie en Innovatie, Publikatie van de Voorlichtingsdienst Wetenschapsbeleid, Staatsuitgeverij Den Haag (1980)
2. Beleidsnota universitair onderzoek, Staatsuitgeverij, Den Haag (1979)
3 De Innovatienota, Staatsuitgeverij, Den Haag (1979)
4 H.K. Boswijk, J.G. Wissema, W.C.L. Zegveld, De Innovatienota; een aanvulling. Stichting Toekomstbeeld der Techniek, Den Haag (1980)
5. Plaats en toekomst van de Nederlandse industrie, WRR rapport, Staatsuitgeverij, Den Haag (1980)
6. H.K. Boswijk, R.G.F. de Groot, De Industrie In Nederland: Verkenning van knelpunten en mogelijkheden, Stichting Toekomstbeeld der Techniek, Den Haag (1978)
7. Innovatieprogramma biotechnologie, Schets van een innovatiegericht onderzoeksprogramma op het gebied van de biotechnologie, Voorlichtingsdienst Wetenschapsbeleid, Den Haag (1980)
8. Biotechnologie in het landbouwkundig onderzoek, NRLO studierapport, Den Haag (1981)
9. FAST Research activities, Directorate-General for Research, Science and Education. EUR 7102 (1980)
10. FAST sub-programme bio-society Research activities, Directorate-General for Research, Science and Education EUR 7105 (1980)

Abbreviations

ACTH	Adreno-cortico-tropic hormone
AF	Anaerobic filter
ATP	Adenosine triphosphate
BION	Foundation for Biological Research in the Netherlands
BOD	Biological oxygen demand
CBS	Central Statistical Office
COD	Chemical oxygen demand
CREST	Scientific and Technical Research Comittee
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EEC	European Economic Community
EMBL	European Molecular Biology Laboratory
e.u.a.	European Unit of Account
FAST	Forecasting and Assessment in the field of Science and Technology
HFCS	High-fructose corn syrup
ICI	Imperial Chemical Industries Ltd.
IFT	Institute of Food Technologists
ITAL	Institute for the Application of Atomic Energy in Agriculture
MSG	Monosodium glutamate
NAK	General Netherlands Inspection Services
NIH	National Institution of Health
NRLO	National Agricultural Research Council
OECD	Organisation for Economic Cooperation and Development
PEG	Polyethylene glycol
RIV	Netherlands Public Health Institute
RIVRO	Government Institute for Research on Varieties of Cultivated Crops
RNA	Ribonucleic acid
SCP	Single Cell Protein
SON	Foundation for Chemical Research in the Netherlands
STT	Netherlands Study Centre for Technology Trends
Ti	Tumour-inducing
TNO	Organisation for Applied Scientific Research in the Netherlands
UASB	Upflow anaerobic sludge blanket
UHT	Ultra-high temperature
ZWO	Netherlands Organisation for the Advancement of Pure Research

Publications of the Netherlands Study Centre for Technology Trends (STT)

(* - available in English, the remainder in Dutch only)

1. The future shape of technology; J. Smit, 1968 (out of print)

2. Technology in perspective: a telescope view of telecommunication; Prof. R.M.M. Oberman, 1968 (out of print)

3. Means of transport; Prof. J.L.A. Cuperus, Prof. J.H. Krietemeijer, G. Veldhuyzen, F. Oudendal, Prof. G.J. van der Burgt, Prof. H. Wittenberg, 1968

4. How to set up a medium-term planning policy; P.H. Bosboom, 1969

5. The transfer procedure in transport; Prof. J.L.A. Cuperus, Prof. L.H. Klaassen, R.J.H. Fortuyn, M.G. de Bruin, A. Blankert, T. van der Meer, J.A. van de Kamp, Prof. E.H. van de Poll, G.C. Meeuse, A.M. Lels, M. van den Bos, E. van Donkelaar, 1969

6. The impact of low-cost electrical energy on technological developments in the Netherlands; Dr. P.J. van Duin, 1971

7.* Electrical energy needs and environmental problems, now and in the future; J.H. Bakker, Prof. J.J. Went, Dr. K.L. Keller, A.J. Elshout, H. van Duuren, J.L. Koolen, P.E. Joosting, Dr. J.C. ten Houten, J.A.G. Davids, Prof. J.A. Goedkoop, M. Muysken, 1971

8. Man and his environment: priorities and choice; L. Schepers, Dr. W.J. Beek, Prof. D.J. Kuenen, Prof. J. van Genderen, Dr. L.J. Revallier, Dr. H. Hoog, 1971

9. Nutrition in The Netherlands, now and in the future; Prof. M.J.L. Dols, J. de Veer, Dr. C. Engel, Prof. J. Boldingh, Prof. H. Doorenbos, W.C. Bus, H. Glazenburg, Prof. A.G.M. van Melsen, 1971

10.* Barge Carriers: some technical, economic and legal aspects; W. Cordia, G.J.W. de Vries, N. Wijnolst, 1972

11. Transmission systems for electrical energy in The Netherlands; Prof. J.J. Went, A. Govers, M.C. Lelie, Prof. H. Wiggerts, 1972

12. Electricity in our future energy supply: options and consequences; Dr. H. Hoog, P.J. Wemelsfelder, Prof. D.G.H. Latzko, Dr. D.J. Kroon, Prof. J.J. Broeze, 1972

13. Communication City 1985: electronic communication with home and work; Prof. J.L. Bordewijk et al., D. van den Berg, Dr. W. Horn, 1973

14. Technology and preventive medical examination; Dr. M.J. Hartgerink, Prof. H.H.W. Hogerzeil, Prof. P. Eykhoff, Prof. J.C.M. Hattinga Verschure, Prof. H.J.J. Leenen, Dr. P. Gootjes, Prof. A.H. Wiebenga, D.H. Bekkering, 1973

15. Technological forecasting: goals and methods; A. van der Lee, T.M.A. Bemelmans, Dr. W.J. Beek, 1973

16. Man and his environment: controlled growth; Steering Group and Working Groups for Environmental Studies, 1973

17. Man and his environment: towards clean air; Steering Group and Working Groups for Environmental Studies, 1973

18. Man and his environment: cycles of matter; Steering Group and Working Groups for Environmental Studies, 1973

19.* Energy Conservation: ways and means; edited by J.A. Over and A.C. Sjoerdsma, 1974

20. Food for everybody, place and role of the EEC; Prof. J. Tinbergen, Prof. J. de Hoogh, Dr. J.R. Jensma, Prof. J. de Veer, I.B. Warmenhoven, Dr. A.W.G. Koppejan, K.K. Vervelde, Dr. W.J. Beek, 1976

21. New approaches of urban transportation problems; Steering Group and Working Groups for Urban Transportation Studies, edited by J. Overeem, 1976

22. Materials for our society; edited by J.A. Over, 1976

23. Industry in The Netherlands: A survey of problems and options, edited by H.K. Boswijk and R.G.F. de Groot, 1978

24. Future of industry in The Netherlands; Prof. P. de Wolff, R.F.M. Lubbers, Dr. H. Kramers, Prof. J. in 't Veld, G.A. Wagner, 1978

25. Data processing and the medical profession; edited by R.G.F. de Groot, 1979

26. Forest and timber: their importance for our future; edited by T.K. de Haas, J.H.F. van Apeldoorn and A.C. Sjoerdsma, 1979

27. Coal for our future; edited by A.C. Sjoerdsma, 1980

28. Distribution and information processing; edited by R.G.F. de Groot, 1980

29. Home and Technology: Yesterday's experience, ideas for tomorrow; edited by J. Overeem, 1981

30.* Biotechnology: a Dutch Perspective; edited by J.H.F. van Apeldoorn, 1981

Other publications:
Innovation, a new direction; H.K. Boswijk, Dr. J.G. Wissema and Prof. W.C.L. Zegveld, 1980

These publications can be ordered from:

Netherlands Study Centre for Technology Trends (STT)
P.O. Box 30424
2500 GK THE HAGUE
Tel. no. (0)70 - 64 68 00

or from the publisher:

Delft University Press
Mijnbouwplein 11
2628 RT Delft
Tel. no. (0)15 - 78 32 54

Biotechnology: a Dutch Perspective assesses the future potential of biotechnology in the Netherlands.
It has been published in English because it is felt that the Dutch case could be of relevance to other industrialised nations.
Although the report is aimed primarily at policy planners and decision makers in industry and government, it is also a useful source of information for researchers in fields allied to biotechnology, as well as providing a thorough introduction to the subject for the general public.
There are already clear signs that life in the Netherlands is soon going to be affected significantly by biotechnology. The sectors where the greatest impact will be felt are health care, the food processing industry, agriculture, fine chemicals and waste treatment. The study evaluates the potential applications of biotechnology in each of these sectors, identifies the bottlenecks which could delay implementation, and assesses the consequences of the introduction of biotechnological processes.
The final chapter presents conclusions and recommendations for industry, the academic world, and government.

Delft University Press