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OBJECTIVES

Biotechnology has emerged as a separate discipline due to the major developments achieved in the life sciences during the past few decades. It covers a broad spectrum of technologies involving the use of living organisms and biological processes in a wide range of industrial, agricultural, and medical applications.

Biotechnological techniques—from fermentation to genetic manipulation—will become increasingly relevant to food and beverage industries, fuel production, chemical and pharmaceutical manufacture, waste management, and many other areas of social, environmental, and economic importance. Consequently, academic as well as industrial institutions will need to keep abreast of the concepts, data, and methodologies evolved by continuing research.

Increased interest in biotechnological developments has generated an abundance of literature. *CRC Critical Reviews in Biotechnology* will provide a forum for critical evaluation of recent and current publications and, periodically, for state-of-the-art reports from various geographic areas around the world. Contributing authors are recognized experts in specific fields of endeavor, and each article is reviewed by an expert referee to ensure accuracy and objectivity of the presentation.

We are confident that *CRC Critical Reviews in Biotechnology*, like all other *CRC Critical Reviews*, will become an important medium for the dissemination and evaluation of scientific knowledge. Its impact will be reflected in the advances made by research and industry in years to come, and ultimately in the benefits these efforts will bring to all mankind.

G. G. Stewart
I. Russell

CRC CRITICAL REVIEWS in BIOTECHNOLOGY

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The review will concentrate on the recent developments in the process engineering and equipment used for large-scale isolation of both intra- and extracellular enzymes produced by microorganisms. In particular the kinetics of production during fermentation, release from the cell, separation methods for cell and cell debris collection, and purification and fractionation methods will be described. The relationship between criteria such as quantity, condition, utilization, and activity of a specific enzyme product and its isolation process will be investigated. The role of genetics and the influence of the recent progress made in genetic engineering will be evaluated in relation to enzyme production and isolation. Current attempts to apply optimization techniques to both individual operations and the whole process will be described. A comparison will be made of the various sources of enzymes based on biological and process engineering parameters such as activity, efficiency, and economics.

EDITORS' INTRODUCTION

The last few decades have witnessed major developments in the biosciences. Developments 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 has been prompted by various motives. In the first place, there is man's constant quest for scientific knowledge. This is particularly marked in the life sciences because of the mysterious nature of life due to its complexity and man's awareness that he is inextricably bound up with the natural life of this planet. This gives a perspective to the life sciences which differs from that of the technical sciences, which are largely concerned with man's own creations. Secondly, there is the desire to put discoveries into practical use. Knowledge of the life sciences can be employed to fight disease, enhance food supplies, and to stimulate the development of industrial applications such as food and beverage preparation and the production of hormones, antibiotics, and enzymes.

Biotechnology is the manifestation of the second motive for research in the life sciences. It involves the use in industry, agriculture, and medicine of living organisms or their components (such as enzymes and antibiotics). It includes the introduction of genetically engineered microorganisms into a variety of industrial, agricultural, and medical processes. However, genetic engineering, although an important component of modern biotechnology, comprises only one technique of biotechnology. The pharmaceutical, chemical, and food and beverage industries, in that order, are most likely to take advantage of advances in molecular genetics. Others that might be affected, although probably not as immediately, are the mining, crude oil recovery, and pollution control industries. Because nearly all the products of biotechnology are manufactured by microorganisms, fermentation is an indispensible element of biotechnology. Fermentation has been around for centuries and is the process used to make beer, wine and vinegar, and to leaven bread. Microbes have been used in fermentation to produce organic chemicals (for example, citric acid) and enzymes (for example, amylases and invertase). Over the years, the scope and efficiency of the fermentation process has been gradually improved and refined.

Biotechnology as a separate discipline is now emerging faster than many people expected. Rapid advances in both cell and molecular biology have allowed a more confident prediction that a given product can be produced by a biological process or organism at a reasonable cost. Genetic manipulation has become a practical and quite general proposition. As a result, a rational rather than empirical approach to biotechnology is now feasible, with organisms tailored to specific needs and process conditions. This advance confers on biotechnology an importance comparable to that of atomic physics, electronics, and, more recently, microelectronics. It has been said that biotechnology will "launch an industry as characteristic of the twenty-first century as those based on physics and chemistry have been of the twentieth century". It will be an industry based in large part on renewable and recyclable materials and thus adapted to the needs of a society in which energy is expensive and scarce. It will be relevant to a wide range of industries including agriculture, food and feedstuffs, chemical, pharmaceutical, energy and water industries, and to such diverse products as bulk chemicals, antibiotics, vaccines, methane gas, ethanol, metals, food additives, single cell protein, effluent treatment and waste recycling. As such, it is a growth area in technology and will require new processes, equipment, and services, entrepreneurs, venture capital and wide communication of ideas and know-how.

The stimulus to biotechnology comes not only from rapidly advancing techniques such as recombinant DNA ("gene splicing"); there has also been a significant change in its potential economic impact. The increase in oil prices since 1973, the associated heightened awareness of the finite nature of resources, and the rapid industrialization and improved living standards of some less developed countries have all led to growing economic and trade pressures for cheaper and more secure supplies of energy and chemical feedstocks, for more efficient production, storage and distribution of food and feedstuffs, conversion of foodstuffs into animal products and for improved agrichemicals and veterinary materials. At the same time, social pressures have increased societies' awareness for improved environmental control and waste management and for more effective and less expensive drugs, vaccines, hormones, antibiotics, etc.

Although biotechnology is of increasing importance to the food and beverage industry, until recently only a handful of food and beverage companies have been directly concerned with genetic engineering and biotechnology as it is understood as a modern science. The Office of Technological Assessment of The U.S. Congress recently estimated that only 13% of the total U.S. effort on applied genetics is being directed to food and beverage applications, as compared to 48% on pharmaceuticals and 26% on chemicals. However, also in the U.S., 8 to 10 million people work in the meat, poultry, dairy, baking and wine industries; in canned, cured and frozen food plants; and in moving food from the farm to the dinner table. In 1979, the payroll was over $3.5 billion for the meat and poultry industries, $2.6 billion for baking, $4.5 billion for brewing, and $1.9 billion for food processing.

Because of the increased interest in biotechnological developments, there have been published a number of new journals and review publications and another such publication could be deemed to be unnecessary. However, the *CRC Critical Reviews in Biotechnology* is designed, as are all Critical Review Journals, to be a format for providing critical evaluations of the newest concepts, methods, and data. In the near future a number of opportunities for developments in biotechnology will be considered. These will include *recombinant DNA, cell fusion, production of (inexpensive) liquid or gaseous fuels, enzyme and immobilized enzyme systems, monoclonal antibodies and immunoglobulins, waste treatment, plant cell culture, single cell protein, chemicals and solvents, pharmaceuticals, pesticides, interferon and vaccines, nitrogen fixation, process engineering, and microbial leaching.*

It is also intended that biotechnological developments in a particular geographical area be reviewed from time to time and this inaugural issue contains such a review of developments in Scandinavia. This issue also contains reviews of developments in the fields of "Single Cell Protein" and "Large-scale Industrial Enzyme Production".

This journal is not intended as a publication medium for original scientific data; it is intended as a means to review in a critical fashion, data already published and to afford a means for scientists and engineers interested in biotechnology to keep abreast of developments in the expanding and rapidly developing field.

G. G. Stewart
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BIOTECHNOLOGICAL DEVELOPMENTS IN SCANDINAVIA

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I. INTRODUCTION

Biotechnology in Scandinavia is clearly confined to certain areas in which research and development has reached a high level. Studies on the traditional biotechnical processes have a long tradition in Denmark where beer is an important export product. In Norway, flavor formation in beer fermentations and various food processing problems have been extensively studied. Important research into problems of yeast and beer production is also carried out in Finland.

Biochemical separation methods have been developed mainly in Sweden, where the immobilization of enyzmes and cells has also been extensively studied. Research into production and utilization of microbial enzymes has in Denmark and Finland lead to industrial production of a number of enzymes.

Recent interest in renewable resources in combination with a long tradition in wood chemistry has directed research in Sweden and Finland towards the study of enzymatic degradation and utilization of lignocellulosic materials.

The area of control engineering and modeling of biotechnical processes has been relatively recently studied in Scandinavia and the practical applications are so far few.

Medical biotechnology is well advanced in Denmark and Sweden but since publications in this field are scarce it is not considered in this review.

II. TRADITIONAL BIOTECHNOLOGY

Research into brewing, baker's yeast production, and production of distilled alcoholic beverages has long traditions in Scandinavia. 1983 is the centennial of the introduction of pure cultures of microorganisms in industrial fermentations. This step from old home-brewing to scientifically controlled fermentation was taken by Hansen at the Carlsberg Brewery in Copenhagen. Research at the Carlsberg Laboratory has, since its foundation in 1876, been devoted to biochemistry, microbiology, genetics, and related fundamental sciences but has always included an element of biotechnology.

In Finland biotechnical research was started in the early 1920s by Virtanen. He was working in close cooperation with the dairy industry and studied many aspects of bacterial biochemistry and fermentation.

Brewing research has been strong in all the Scandinavian countries during the whole post-war period. Important topics in brewing research during recent years have included malting barley, yeast, and flavor formation. Not only brewer's yeast but also baker's yeast has been extensively studied. This research has, however, often been of a more fundamental biochemical character.

A. Malting Barley

The research on barley includes barley breeding where special emphasis has been laid

on development of new anthocyanogen free varieties. These varieties give an improved shelf life to beer.[1,2] The studies also include chemical investigation into proanthocyanidins.[3] Furthermore the suitability of six-row barley varieties which traditionally are not used for brewing in Europe has been studied.[4] Changing of the malting and brewing methods renders certain advantages to the use of six-row barleys.

The protein metabolism of germinating barley is of importance for malting and also for mashing of malt.[5] The peptide transport in germinating barley grains[6] and the liberation of amino acids in mashing[7] have been investigated. As a consequence of these studies the general picture of the breakdown of proteins and their role in beer production has been clarified.

Not only the biochemical but also the structural changes during malting have been studied.[8] Using specific histochemical staining methods for β-glucan and for α-amylase the transformations during germination have been elucidated.

The role of fungal contaminations of barley has been investigated. Methods for the detection of fungi on barley and malt have been developed.[9] The formation of toxins by *Fusarium* molds has been studied[10] and the role of fungi in causing gushing of beer has been investigated.[11] Furthermore the concentration and influence of trace elements, especially zinc in malt on brewery fermentation has been studied.[12]

B. Yeast, Fermentation, and Flavor

Yeast plays an important role in flavor formation during the production of beer and other alcoholic beverages.[13] The formation of flavor compounds is influenced by wort composition,[14] i.e., availability of nutrients,[15] and especially by oxygen supply or concentrations of unsaturated fatty acids and sterols.[16,17]

Brewer's yeast needs oxygen for the biosynthesis of unsaturated fatty acids and ergosterol. If the sterol concentration in yeast decreases the metabolism changes which is reflected in a decreased acetoin formation.[18] Esters are another group of important flavor compounds.[19] Ester formation too is linked to yeast growth but it is also dependent on esterase activity.[20] The pH is important; a low pH leads to higher ester concentrations.[21]

An important off flavor of immature beer is diacetyl which is formed by a spontaneous chemical reaction from α-acetolactate produced by yeast during fermentation.[22] In normal secondary fermentation the diacetyl is reduced by yeast. The use of an enzyme preparation, α-acetolactate decarboxylase isolated from bacteria (*Enterobacter aerogenes*), has been proposed as an agent for accelerated maturation of beer.[23]

Yeast research includes both genetic[24,25] and biochemical studies. Recombinant DNA technology has opened new possibilities for the genetic improvement of industrial strains (cf. Section VIII).

Biochemical studies of baker's yeast have been concerned with the role of biotin in yeast metabolism.[26] Biotin is essential for yeast growth and can influence the yield in baker's yeast production. Furthermore the carbohydrate content[27] and the cellular water content[28] of baker's yeast during storage have been studied.

III. BIOCHEMICAL SEPARATION METHODS

There is a long tradition in the development of biochemical separation methods at Uppsala University. These fundamental studies of electrophoresis and chromatographic methods were started by The Svedberg and Arne Tiselius in the beginning of this century and the work has continued through the whole post-war period. In recent years affinity chromatography[29] and aqueous two-phase systems have been developed in a number of laboratories in Sweden.

In affinity chromatography, biospecific sorption reactions are utilized for selectively binding compounds to an insoluble material. Lectins are carbohydrate binding proteins

which cause agglutination of erythrocytes and other cells. Lectins are present in many plants and especially in beans and peas. Concanavalin A from Jack bean is readily available and has been used for the fractionation of blood group substances, serum proteins, and many other glycoproteins. In affinity chromatography the lectin is bound to an insoluble matrix. Thus concanavalin A was coupled to agarose gel beads and this sorbent was then used for chromatrography of serum proteins.[30] The method has also been used for the purification of viruses and cells. All enveloped viruses contain glycoproteins and thus they can be fractionated by chromatography on immobilized lectins.[31] The same techniques can be applied to the purification of viral surface glycoproteins.[32]

The method has also been used for purification of lectins from various seeds. A galactose specific lectin was purified from Sunn Hemp seed (*Crotalaria juncea*) by affinity chromatography on galactose coupled to agarose.[33] This kind of technique works well for lectins which are strongly bound to simple carbohydrates. Other lectins impose very specific stereochemical restrictions on the oligosaccharide structure to which they bind. These structures are believed to be present only on the cell surface. The preparation of a biospecific sorbent for these lectins is thus rather difficult. This problem has been overcome by using erythrocyte membrane residue or stroma entrapped by glutaraldehyde treatment as sorbent for lectins from beans (*Phaseolus vulgaris* and *Phaseolus coccineus*).[34] Whole yeast cells immobilized by entrapment in polyacrylamide have recently been used for the purification of lectins from *Lens culinaris*.[35]

The affinity of proteins for heavy metal ions has also been used as a basis for affinity chromatography.[36] Metal ions which can form coordination compounds with histidine and cysteine residues in proteins can be bound to agarose and used for purification of proteins. This method has been used for the purification of lactoferrin from human milk,[37] enzymes,[38] and nucleotides.[39]

A special biospecific sorption method has been used in the purification of cellulases.[40] The enzymes were sorbed on the substrate amorphous cellulose and thereafter release by letting them degrade the substrate. During an incubation for 20 hr at 50° C the cellulose was dissolved and the enyzmes released.

High-performance liquid chromatography (HPLC) is another quickly developing branch of chromatographic separation methods. Bio-affinity supports have been used also in HPLC.[41]

Using AMP coupled through covalent linkages to glycerylpropyl-silica particles isoenzymes of lactate dehydrogenase were separated. The elution was performed with a NADH solution and the total time was less than 10 min. The technique was also applied to immunosorbents. Serum albumins were separated on a column with immobilized antiserum albumin.

The various separation methods developed have found extensive use in biochemical research and many of them have also been scaled-up and are used in industrial-scale separations.

IV. ENZYME PRODUCTION

The industrial production of enzymes is highly developed in Scandinavia. This is especially the case in Denmark but also in Finland a growing enzyme industry is emerging. It is typical for this industry that research on production processes is not published, while research on the use of enzymes is published in order to encourage potential customers. Consequently there are few publications on enzyme production coming from the industry but universities and research institutes tend to publish their work. It is nevertheless clear that these publications also reflect the industrial interests. The enzymes studies are starch degrading enzymes, glucose isomerase, β-galactosidase,

proteases, and rennin, which all are in industrial production.[42] Furthermore, peptidases, cellulolytic enzymes, and xylanases have been studied.

A. Development of Microbial Strains

Selection of the microbial strain and genetic improvement of it is important in the development of industrial production. In many cases the thermostability of the enzyme is of primary importance.[42] Thus for α-amylase production *Bacillus subtilis* largely replaced malt α-amylase because of its higher temperature optimum. For the same reason the *B. amyloliquefaciens* enzyme has been replaced by the more thermostable *B. licheniformis* α-amylase. In the development of the *B. licheniformis* strain, a mutation program consisting of many steps lead to a ten-fold increase in α-amylase production. In this way the same level of enzyme production was reached as that of the *B. amyloliquefaciens* enzyme.

Strain development has also been used to improve other properties. Thus the *B. coagulans* used for glucose isomerase production originally was inducible requiring xylose as inducer. Through mutations the present production strain which produces the isomerase constitutively was obtained.[43] The use of an expensive inducer is thus not necessary.

Strain development by mutational techniques requires large amounts of work and a good screening method is essential. In the case of *Trichoderma reesei* cellulases[44] and *Aspergillus niger* β-galactosidase[45] such techniques have been published. In both cases a three- to fourfold increase in enzyme production was reached. It seems that the increase in enzyme production level obtained through relatively large mutation programmes normally is three to tenfold. The level of enzyme production reached gives an enzyme concentration of 1 to 3 g/ℓ for bacteria[42] and 1 to 10 g/ℓ for molds[44,45] (Table 1).

B. Fermentation

For the production of enzymes the composition of the medium, possible inducers, pH, and temperature are the critical factors. These are seldom published by industrial enzyme producers and thus the literature in this field is scarce. Some information, has however, been published on the production of β-glucanase,[46] peptidases,[47] cellulases,[48] α-amylase,[49] and β-galactosidase.[50]

In the production of extracellular enzymes the regulatory control systems of the microorganism are essential. These have been studied in *Bacillus* species. The production of β-glucanase by *B. subtilis* occurs in the stationary phase when certain nutrients already are exhausted. Feeding of starch at this stage markedly increased enzyme production.[46] In this way repression caused by high initial carbohydrate concentrations was avoided.

Production of α-amylase by *B. licheniformis* was studied in continuous culture.[49] When succinic acid was fed to the steady-state culture an increase in α-amylase activity was observed. It was concluded that the increase was caused by either induction or derepression. Some information is available on the control of fermentation processes but this will be treated in a separate section (IX).

C. Down-Stream Processing

If little information is published on the microbiological process of enzyme production, still less is found in the literature on the treatment of culture liquids after the fermentation process. In some processes little treatment is needed and the liquid can be used after removal of the microorganisms and concentration. In many cases, however, purification and stabilization is an essential part of the production process. This part of the process can form a major part of the total costs. Thus the lack of literature in this area is understandable. A special form of treatment is immobilization of whole cells or

Table 1
PRODUCTION OF ENZYMES BY PRODUCTION STRAINS OF DIFFERENT MICROORGANISMS

Organism	Enzyme	Concentration in culture liquid: Total protein (g/ℓ)	Concentration in culture liquid: Enzyme (g/ℓ)	Ref.
Bacillus amyloliquefaciens	α-Amylase	—	3	42
B. amyloliquefaciens	Neutral protease	—	1.3	42
B. amyloliquefaciens	Alkaline protease	—	1.5	42
B. licheniformis	Alkaline protease	—	2—3	42
Aspergillus niger	β-Galactosidase	0.7	—	45
A. niger	β-Glucosidase	3.6	0.1	95
Trichoderma reesei	β-Glucosidase	11	0.1	95
T. reesei	Cellobiohydrolase	10—11	6	44, 95
T. reesei	Endoglucanase	10—11	1	44, 95

separated enzymes. This step has been subject to extensive studies at universities and numerous publications are available. Immobilization is treated in Section VI.

V. UTILIZATION OF ENZYMES

Enzyme producers are generally interested in supplying their customers with information on the use of enzymes. A prerequisite for this is active research in the field and consequently the publications on enzyme utilization are numerous. The enzymes studied through the years in Scandinavia are proteolytic enzymes, the enzymes used for production of syrups from starchy materials, and the cellulolytic enzymes.

A. Proteolytic Enzymes

The proteolytic enzymes constitute the economically most important group. The production and utilization of them has also been studied for many years at the Carlsberg Laboratory, by enzyme producers and by research laboratories connected to the food industry. The work at the Carlsberg Laboratory has mainly been concerned with fundamental biochemistry of proteolytic enzymes. Subtilisin was previously studied but in recent years carboxypeptidase Y has been carefully characterized. The studies include isolation of carboxypeptidase Y from yeast by affinity chromatography.[51] The amino acid sequence is currently being analyzed.[52] Of potential biotechnical interest are the studies on transpeptidation activity of carboxypeptidase Y and its use in peptide synthesis.[53,54] It is envisaged that for the synthesis of peptides of certain unique amino acid sequences transpeptidations may be used. Furthermore, carboxypeptidase can be used to modify the C-terminal end of peptides. This technique has been applied to modifying porcine insulin.[55] One product obtained was human insulin. Unfortunately the purification of the product is rather complicated.

The serine proteases are well-established industrial enzymes.[56] *Bacillus licheniformis* forms a serine protease called Subtilisin Carlsberg which is used in detergents. Other species of the genus *Bacillus* also produce alkaline proteinases with an even higher optimum pH. These enzymes may prove useful in detergents because of their good activity and stability in washing conditions (pH 9 to 10, 50° C). They may also be valuable in enzymatic dehairing of hides.

An interesting application of proteases is limited hydrolysis in the conversion of food proteins.[57] By controlling the extent of hydrolysis optimal functional and organoleptic properties can be obtained. In this kind of usage *Bacillus* proteases may well replace serine enzymes of pancreatic origin (trypsin, chymotrypsin).

In Norway, where fish industries are important the use of proteolysis in the treatment of fish has been studied[58-60] The studies include research on autolytic degradation of fish muscle and the application of industrial enzymes to the production of protein hydrolysates. The latter process is of importance in the utilization of cheap pelagic species of fish and fish processing waste. The enzymes studied have mainly been pancreatic enzymes but here the variety of microbial enzymes would allow development of more advantageous processes.

The biggest user of acid proteases is the cheese industry. The traditional calf rennin has been partly replaced by microbial enzyme products formed by *Mucor miehei, M. pusillus,* or *Endothia parasitica*. One limitation in the use of microbial rennins is their high thermostability which leads to residual milk-clotting activity after pasteurization. The possibilities of reducing thermostability by chemically modifying *M. miehei* rennin have been studied.[61] It was found that oxidizing agents were useful in this respect.

B. Enzymes Hydrolyzing Carbohydrates

The second important group of hydrolases acts on carbohydrates such as starch, lactose, cellulose, and hemicellulose. The conversion of starch to glucose is accomplished by the combined use of α-amylase and amyloglucosidase. The glucose can be further isomerased to form fructose in the production of high fructose syrups. All the necessary enzymes are produced industrially in Scandinavia (Table 2) and their use has been extensively studied.[62]

The use of β-galactosidase from *Kluyveromyces fragilis* and *Bacillus* sp. in the treatment of whey has been investigated,[63] but the enzyme from *Aspergillus niger* seems to be more suited for this purpose.[64]

The application of cellulolytic enzymes to complete hydrolysis of cellulosic materials is treated in Section VII. There are, however, special usages for cellulolytic enzymes which may prove advantageous already before the total hydrolysis becomes economically feasible. In the production of grain alcohol the yield can be improved by adding *Trichoderma reesei* cellulases.[65] The enzymes presumably have a double action: by breaking down cellular structures they facilitate the saccharification of starch and they also directly produce glucose from grain β-glucans.

In malting, the addition of *T. reesei* cellulases has been shown to facilitate the penetration of gibberellic acid into the barley grains.[66] Thus the malting time can be shortened and the yield improved.

Cellulases can also be used to enhance the sugar content of silage.[67] This both stimulates lactic acid fermentation and improves digestability of the silage.

The filtrability of wort and beer can be impaired by soluble β-glucans originating from barley. In such cases an addition of β-glucanases produced by *A. niger* or *B. subtilis* improves the filtrability.[68,69]

VI. IMMOBILIZED ENZYMES AND CELLS

There are often important economical reasons to recycle enzymes used in industrial processes. This has been achieved by the development of immobilized enzyme preparations. At the same time, the development of continuous enzymatic processes has become possible. It is obvious that the great economical advantages as well as the theoretical possibilities of studying enzymes in model systems resembling living cells have attracted scientists engaged in fundamental and applied research.

Table 2
ENZYMES USED IN MODIFICATION OF CARBOHYDRATES INDUSTRIALLY PRODUCED IN SCANDINAVIA

Enzymes	Organism
α-Amylase	*Bacillus amyloliquefaciens, B. licheniformis, Aspergillus oryzae*
Amyloglucosidase	*A. niger, A. awamori*
Debranching enzyme	*Klebsiella aerogenes*
Glucose isomerase	*B. coagulans, Streptomyces* sp.
β-Galactosidase	*Kluyveromyces marxianus, A. niger*
Cellulases	*Trichoderma reesei*

A. Development of Techniques

Immobilization techniques have been studied and developed in several Scandinavian universities. The techniques and supports have been reviewed by Mosbach, who is one of the most active scientists in this field.[70] Recent developments in immobilization techniques include reversible covalent immobilization by thioldisulfide interchange.[71] The advantage of this method is that the column can be regenerated *in situ* by reductive uncoupling of the inactive protein and attachment of a new portion of thiolated enzyme. Furthermore a method for immobilizing enzymes to hydroxyl group carrying supports using reactive sulfonyl chlorides has been presented.[72] This method is useful in the immobilization of pH-sensitive ligands and proteins. This is due to the fact that the ligands are bound directly to the carbon atoms of the support (e.g., agarose, cellulose, or hydroxyethyl methacrylate). No side reactions leading to protein inactivation occur.

A rather elegant method for entrapment of enzymes in cellulose fibers has been reported.[73] In this method α-cellulose is first solubilized in a mixture of *N*-ethylpyridiniumchloride and dimethylformamide. The enzyme is dissolved in this solution and cellulose fibers containing the entrapped enzyme are regenerated by pouring the solution into water.

The use of two-phase aqueous systems as biochemical separation methods have been studied for a long time in Sweden. This system has also been applied as a kind of immobilization technique.[74] The phase system consists of a dextran solution and a polyethylene glycol solution. A model system was used to saccharify cellulose with cellulolytic enzymes. The advantage in this case was that a continuous process could be used with an insoluble substrate. This would, of course, not be possible with entrapped enzymes or enzymes rendered inaccessible for an insoluble particulate substrate by binding to a rigid support.

B. Industrial Applications of Immobilized Enzymes

Although the research publications on immobilized enzymes are numerous the industrial applications are still few. The production and use of immobilized glucose isomerase, penicillin acylase, and β-galactosidase have been studied in Scandinavia and industrial processes have been developed. Immobilized glucose isomerase has been a big industrial success. One such preparation consists of whole *Bacillus coagulans* cells immobilized by cross-linking with glutaraldehyde.[75] The properties of the enzyme are altered by the immobilization (Table 3). The most important difference is that when the immobilized enzyme is used cobalt ions are not needed as activator. This effect is due to

Table 3
COMPARISON OF PROPERTIES OF SOLUBLE AND IMMOBILIZED *BACILLUS COAGULANS* GLUCOSE ISOMERASE

	Soluble	Immobilized
pH optimum	7.5	8.5
Temperature optimum	85°C	85°C
Activators		
Co^{2+} *M*	10^{-3}	—
Mg^{2+} *M*	0.1	4×10^{-3}

the shift in the optimum pH to an alkaline range. In batch processes an alkaline pH can not be used because of color and by-product formation. This makes cobalt salts necessary in the batch process, which increases refining costs.

The other immobilized enzyme which has entered large-scale industrial use is penicillin acylase. This enzyme is used to produce 6-aminopenicillanic acid (6-APA) by deacylation of either penicillin-G or penicillin-V. The deacylation can be carried out using a chemical or an enzymatic method. The enzymatic method is more favorable and more than 50% of all 6-APA is today produced using immobilized enzymes.[76] The methods used have not been published, but usually the enzymes used are penicillin-G acylases. In one case a study of penicillin-V acylase has been published.[77] This enzyme, which was produced by a Gram-negative aerobic coccus, was membrane bound. The procedure developed was based on direct immobilization of the bacterium with a branched polyethyleneimine and glutaraldehyde. The immobilized enzyme preparation seems to be effective but its industrial use has not been reported.

A third immobilized enzyme which is being used in practical scale is β-galactosidase. The enzyme is obtained from *Aspergillus niger* and it is immobilized by adsorption on a phenol-formaldehyde resin followed by cross-linking using glutaraldehyde.[64] The immobilized enzyme is used successfully for the treatment of whey before the use as fodder for pigs. Due to the good stability of the enzyme preparation the use of it has proved economically advantageous.

Another enzyme of technical interest is β-glucanase. Barley β-glucan can cause filtration difficulties in the brewing industry. The degradation of barley β-glucan by an immobilized fungal β-glucanase preparation has been studied.[78] The activity of the enzyme was modified by the immobilization method and the characteristics of the immobilized enzyme were very different from that of the enzyme in solution. The use of immobilized β-glucanase to improve filtrability of beer has been suggested.[79] According to this investigation the viscosity of wort could be reduced from 1.7 cP to 1.4 cP which resulted in a considerable improvement of filtrability. A laboratory-scale column of a commercial β-glucanase preparation immobilized on phenol-formaldehyde resin was run for 2 months without loss of activity.

Beer is in many breweries stabilized by treating it with proteolytic enzymes, usually papain. A continuous process has been developed in which immobilized fungal acid protease is used for this purpose.[80] The stabilization is achieved in 2 min at 0°C and the conditions are thus acceptable for beer treatment in the industry.

C. Immobilized Living Cells

Many enzymes need cofactors for their activity. The cofactors are expensive and the regeneration of them in industrial operations is difficult or impossible. This has directed