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Chapter 9

Production of Microbial Enzymes

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281

I. INTRODUCTION

Production and use of microbial enzymes is an ancient art. By experience and empirical methods it has developed to a highly sophisticated state; the preparation of fermented oriental food is an example of this.

The combination of microbiological and biochemical science with modern technology has made possible the development of a large enzyme industry. This industry was founded well over 50 years ago, but the economically important developments have taken place only within the last decade.

A. Recent Developments

The regrowth of the enzyme industry began in the early 1960s with the introduction of glucoamylase in starch hydrolysis. The enzyme method was substituted for the traditional acid hydrolysis and resulted in an increased dextrose yield.

Enzyme-containing detergents have been known since 1913, but their use was limited because the enzymes available were unstable in detergent formulations. Around 1965 a new, stable protease produced by *Bacillus licheniformis* was introduced, which was very successful and found wide application for use in detergents.

In 1968–1969, it was discovered that some workers handling enzyme concentrates were experiencing allergic reactions. This caused a strong public reaction against the use of enzymes. Consequently, enzymes were removed from most detergents used in the United States. An official investigation showed that there was no risk involved for the detergent user. After the introduction of dust-free, encapsulated enzyme products, the risk involved in handling enzyme concentrates was also eliminated. Since that time the use of detergent enzymes has grown steadily.

The substitution of microbial enzymes for calf rennet is an old dream, and numerous microbial proteases have been tested for this purpose. In the period 1965–1970 three microbial rennets were successfully introduced, and they are now being widely used in the rennet market.

Due to the high cost of sucrose and the availability of large amounts of inexpensive dextrose, the development of an enzymatic process which would isomerize glucose to fructose has been very attractive. The first commercial process was in use around 1970, and since then it has grown to become one of the most important enzyme applications. The use of immobilized enzymes for this purpose has made it possible to keep the conversion costs to a low level.

The brewing industry has traditionally used malt as an enzyme source.

The partial substitution of barley for malt and the inclusion of microbial amylase, protease, and β-glucanase has avoided some of the difficulties caused by variation of the malt quality. Furthermore, this change has been economically advantageous. The distilling industry, another traditional malt consumer, has in many countries substituted the microbial enzymes, α-amylase and glucoamylase, for malt.

In the pharmaceutical industry, the use of enzymes has been limited. The most important application is for digestive aids, in which a mixture of pancreatic and microbial enzymes is used. Another important application is the use of penicillin acylase in the manufacture of semisynthetic penicillins.

Apart from these large-scale industrial applications of enzymes, there has been a substantial growth in the preparation of microbial enzymes for diagnostic, scientific, and analytical purposes. These enzymes are usually prepared under laboratory-type conditions and are not dealt with here.

B. Present Position

A rough estimate is that the present world market for industrial microbial enzymes represents a sales value of $150–175 million. The economically most important enzymes are listed in Table I, together with an estimate of the relative value of the enzymes and the amount of enzyme produced per year, calculated as pure enzyme protein.

The major producers of industrial enzymes are listed in Table II. NOVO and Gist-Brocades have by far the largest share of the market.

TABLE I. Production of Industrial Enzymes

Enzyme	Amounts produced per year (tons of pure enzyme protein)	Relative sales value (%)
Bacillus protease	500	40
Glucoamylase	300	14
Bacillus amylase	300	12
Glucose isomerase	50	12
Microbial rennet	10	7
Fungal amylase	10	3
Pectinases	10	10
Fungal protease	10	1
Other	—	1

TABLE II. Companies Producing Microbial Enzymes

Denmark	Grindstedvaerket
	NOVO Industri
France	Sté. Rapidase
Germany	Boehringer Ingelheim
	Hoechst
	Miles-Kalichemie
	Röhm
Holland	Gist Brocades (KNGS)
Japan	Amano
	Daiwa Kasei
	Godo Shusei
	Kyowa Hakko Kogyo
	Meito Sangyo
	Nagase
	Sankyo
	Shin Nihon
Switzerland	Swiss Ferment
United Kingdom	ABM
	Glaxo
	ICI
United States	CPC
	G. B. Fermentation Industries, Inc.
	Miles
	Novo Biochemical Industries
	Pfizer
	Rohm & Haas
	Standard Brands

II. DEVELOPMENT OF NEW ENZYMES

Fewer than 50 microbial enzymes are of industrial importance today, but patents have been applied for on more than a thousand different enzymes. This reflects the increasing interest in developing new enzyme products and shows that it is easier to find a new enzyme than to find a profitable application.

A new enzyme product becomes a commercial success only if a demand exists and if the product possesses properties which satisfy the technical and economic requirements of the process. Most new enzymes fail in at least one of these respects. Demands for new enzymes arise from the development of new processes or from the unsatisfactory performance of known enzymes in established processes.

The properties of enzymes can to some extent be changed by chemical modification of the molecule. The scientific literature on the subject is extensive, but the methods have so far not been used on technical enzymes, presumably because the results have not been economically or technically attractive. The search for new enzymes has, therefore, been a search for new microorganisms. The basic principles which are guidelines in this search are described below.

A. Strain Selection

1. Enzyme Properties and Taxonomy

It is generally held that macromolecules, such as nucleic acids and proteins, are specific for the species and that the phylogenetic development which has given rise to the microbiological variation basically has been caused by variation in these molecules. As a consequence, it is to be expected that enzyme types, such as protease, α-amylase, and lactase, which are found in several species, will have properties which vary as much as the other properties of the organism. One usually finds that closely related organisms have enzymes with closely related properties, while unrelated organisms have enzyme systems which differ widely.

The protease subtilisin Carlsberg from *Bacillus licheniformis* is closely related to the protease subtilisin NOVO from *Bacillus amyloliquefaciens*, just as the species are closely related. Yet, the differences are distinct, and it appears that these enzymes are specific for the species. Unfortunately, many incorrect classifications of microorganisms in the biochemical literature make the establishment of such rules as this difficult.

2. Enzyme Properties and the Environment

Extracellular enzymes must work in the environment of the microorganism. One may therefore rightly assume that they have their optimum activity and stability close to the optimum conditions for growth of the microorganism. Numerous examples of this may be cited.

Proteases from alkalophilic *Bacillus* species have pH optima several units higher than that of the protease from the "neutralophilic" *B. licheniformis*. The α-amylase from the thermophile *Bacillus coagulans* can be used at 10°C higher temperatures than that of the mesophile *B. amyloliquefaciens*. There are known exceptions. The α-amylase of the mesophile *B. licheniformis* is more thermostable than the amylase of *B.*

coagulans. Several *Aspergillus* species, which have growth optima at about pH 4, produce an alkaline protease, with pH optimum about 10, in addition to an acid protease.

The properties of intracellular enzymes are usually not too dependent on environmental conditions. The internal pH of the cells is close to neutrality and appears to be little influenced by external conditions. Thermostable enzymes, however, are usually found in thermophilic organisms. The thermostable lactase of *Bacillus stearothermophilus* is such an example. Thermostability of an isolated enzyme may be lower than the growth optimum of the strain would indicate, because the enzyme is especially stabilized in the intact cell.

3. Enzyme Properties and the Presence of Substrate

Microorganisms are responsible for the majority of the mineralizations of organic material in nature. It is claimed that all organic material can be microbiologically decomposed. Microorganisms which will produce enzymes for the degradation of certain materials are, therefore, usually found where these substances are abundant. For example, producers of lignolytic enzymes, pentosanases, and cellulases are found in forest soil, and uric acid decomposers in chicken pens. Microorganisms capable of metabolizing a new compound are often quickly established in sewage plants and effluent streams.

4. Methods for the Isolation of Enzyme-Producing Organisms

Traditional microbiological methods are most often used for isolation. Especially important is the use of enrichment cultures and selective media. In the ideal system, the substrate for the enzyme serves as the sole source of one or more vital elements. For example, a uric acid–salts medium is used in the isolation of urate oxidase-producing microorganisms.

Agar diffusion tests are used whenever possible for the detection of enzyme activity. The principle is valuable as a qualitative or very rough quantitative test but the results are often misleading, particularly if the enzyme substrate is incorporated in the growth medium for the microorganism.

. In the design of isolation methods, the imagination of the researcher is essential for success. A combination of a good source of microorganisms and a specific isolation method, often nontraditional, will lead to a useful result. An example is the search for alkali-stable proteases, where enrichment and selection on media with 0.1 M sodium sesquicarbonate

and with casein as a protease indicator led to the discovery of a large group of *Bacillus* species that produce alkaline proteases.

5. Safety Measures

Microorganisms may be pathogenic or producers of toxic materials, and this must constantly be borne in mind when working with unknown organisms. Until they are identified, therefore, cultures and products should be handled with the proper precautions. This is especially important for fungal isolates since fungal spores spread easily.

B. Strain Development

1. Desired Properties of Production Strains

The organism should grow on an inexpensive medium and give a constant, high yield of enzyme in a short time. Secondary enzyme activities and the content of metabolites in the fermented broth should be minimal. Furthermore, recovery of the enzyme should be simple and inexpensive and lead to a stable product which can be handled safely and which has an acceptable appearance. Last but not least, the process must be safe to the personnel in the production plant, and the effluents from the plant should not disturb the environment.

The fulfillment of these objectives requires a combined optimization of the strain properties and process parameters. Optimization of strain properties is very attractive because this, as a rule, offers an inexpensive and permanent solution to the problem. An example is the development of a constitutive mutant which eliminates the need for an expensive inducer, a problem which has been relevant in the production of the xylose-induced glucose isomerase.

Health and safety problems can often be alleviated by strain development. The viable counts of the products, for example, can be reduced by using asporogenic mutants of *Bacillus* strains. Objectionable by-products, such as antibiotics, can be eliminated by using mutants.

Wild strains will often have undesirable enzymatic side activities which have to be removed in the purification process. This is the case with the transglucosidase in glucoamylase. A better method is to develop a mutant which does not produce the side activity.

2. Methods for Strain Development

The exciting progress in microbial genetics offers many attractive methods to the industrial researcher. The use of most of these methods,

however, is hampered by the facts that the enzyme-producing organism usually is genetically unknown and that the efforts and especially the time which can be spent on a single organism is limited. The method of choice, therefore, will, as a rule, be straightforward mutagenization and selection. The mutagens used are those commonly applied in microbiology. In the development of selection methods, experience and personal skill are important.

The success of the mutation work is of great economic value to the enzyme industry. The methods used are therefore carefully guarded and very little information about them is published.

3. Strain Maintenance

The highly developed production strains must be protected against degeneration, contamination, and loss of viability. The most convenient way to secure this is to store the strains lyophilized or at the temperature of liquid nitrogen. It is possible to store the strains almost indefinitely without any changes using these methods.

III. THE FERMENTATION PROCESS

Traditionally, microbial enzymes were produced by surface cultures, i.e., cultures of microorganisms in thin layers of liquid or moist, solid media. This technique is still used for a few products, primarily of fungal origin, such as *Aspergillus* amylase, proteases from *Aspergillus* and *Mucor* species, pectinases from *Penicillium* and *Aspergillus* species, and also cellulases. Originally, the cultures grew in trays that were handled manually, but today mechanical systems for cleaning, filling, and emptying the trays are used. Systems where the semisolid culture is tumbled in a rotating drum also have been developed. Control of infections and also uniform control of temperature, humidity, and aeration present difficulties. Few developments of this technique have been published in the last decade.

Submerged culture methods today dominate in the production of enzymes because handling costs and the risk of infection are reduced, and because modern methods of control are more easily adapted to these processes. Yields are also generally higher. The production methods discussed below refer to submerged fermentation.

A. Inoculum Preparation

Highly mutated strains are increasingly used for enzyme production. An important requirement for a propagation technique, therefore, is that

the production capability of the strain be preserved. The technique should also minimize the risk of contamination. Numerous recultivations of laboratory cultures and multiple propagation steps in the factory should be avoided. It is generally possible to develop a method where the seed flask is inoculated directly from a lyophilized culture and where only one seed tank is used.

In a few cases, the enzyme yield or the mycelial form of the culture depends on a specific propagation method (Meyrath et al., 1973); but this is the exception. In general, the process pattern is unaffected by rather wide variations in the propagation technique. Agar media may often be used in the seed flask. These cultures are easier to handle and have a longer period of applicability than shake-flask cultures. Cells or spores from the agar culture are suspended in a sterile solution before being transferred to the seed tank.

The medium in the seed tank often resembles the production medium. Excessive heat sterilization of the medium retards the growth of the inoculum. The volume of the seed tank usually constitutes 3–10% of the volume of the production fermentor.

The propagation time in the seed fermentor varies from 10 to 80 hours, depending on the process. Examples of parameters used as applicability criteria of the seed culture are change of pH, relative mycelial volume, development of carbon dioxide, or an easily detectable enzyme activity.

B. Medium Formulation and Preparation

The medium should provide the energy source for the process. Furthermore, it should include nutrients providing carbon and nitrogen sources and also special growth requirements, such as essential amino acids. Stimulating factors may be added to reduce the lag time or to increase the growth rate.

However, enzyme production may be negligible in a medium designed for good growth. The enzyme production often depends on the presence of an inducer in the medium, or it may be repressed by a component of the medium. Catabolite repression often controls enzyme production (Paigen and Williams, 1970). The strongest repression is seen in media containing glucose. Repression may be avoided by replacing glucose by slowly fermentable carbohydrates or by partly hydrolyzed starch. In a process for pullulanase production, the use of liquefied starch with a dextrose equivalent less than 42 is recommended (Heady, 1971).

In order to increase the productivity, highly concentrated media are used. In a patented process for the production of glucoamylase, concen-

trations of liquefied starch as nigh as 25% on a dry weight basis are used (Smith and Frankiewicz, 1978). Often a concentrated medium will result in catabolite repression, in retardation caused by the high osmotic pressure, or in a culture which is difficult to aerate. These difficulties may be overcome by incremental or continuous feeding of a concentrated medium to the culture (Kalaboklas, 1971; Hulme, 1973). Typical protein and carbohydrate components of industrial media for enzyme production are listed in Table III. Salts are often added as supplementary sources of nitrogen, phosphorus, sulfur, or calcium. Trace metals are normally present in sufficient amounts in the tap water or in the main raw materials. Strong pH buffers, mostly phosphate buffers, are still widely used to reduce pH fluctuations during the fermentation. Proper balance of carbon and nitrogen sources is also important to the pH pattern of the process if pH control is not applied.

Economy is very important in medium formulation. Typically, raw materials account for 60–80% of the variable costs of an enzyme fermentation process. Much development work is directed toward the replacement of costly ingredients with components available in large quantities at low cost. An enzyme fermentation medium normally has a high content of solids, such as ground whole grains or flakes.

Certain raw materials must be avoided in enzyme fermentations because the quality is too variable. Either a food quality is not available or they have an adverse effect on enzyme recovery, product quality, or waste disposal.

Most media are still sterilized batchwise in the fermentor. Continuous sterilization methods, however, are gaining wider application, particularly for the feeding medium. In some cases the feeding medium may be sterilized by filtration. Continuous heat sterilization is performed as a high-temperature/short-time process which results in improved preservation of growth factors and less development of color. Recovery of heat may be an additional advantage. Fouling of heat-exchange surfaces and clogging of pipes by grainy material in the medium should be considered when the sterilization system is designed. Direct steam injection may, in some cases, solve this problem.

TABLE III. Typical Medium Constituents for Enzyme Fermentation

Carbohydrates	Starch hydrolysate, molasses, saccharose, corn, barley, wheat
Proteins	Soybean meal, cotton seed meal, peanut meal, corn steep liquor, yeast hydrolysate, whey, gluten

C. Process Conditions and Equipment

Since microbial enzymes are relatively low-volume products, it has been difficult to justify the development and construction of specialized equipment for submerged fermentation. Equipment and techniques are most often adapted from antibiotics fermentations. Tall cylindrical fermentors of stainless steel with capacities of 10–100 tons and furnished with strong mechanical agitators and air spargers are typical. The advantage of this traditional setup is flexibility. It is easy to switch between products.

A schematic diagram of a typical enzyme fermentation process is shown in Fig. 1. The diagram indicates the extent of auxiliary equipment and control systems required for the process. As in many other fermentation processes, control of an enzyme fermentation is hampered by the fact that neither growth nor product formation may be determined rapidly enough to be of value for control. Primary physical variables, such as temperature, air flow, and pressure, are controlled within narrow limits. Other measurable variables, such as pH, oxygen tension, or oxygen consumption, are often also applied in process control. Foaming is normally controlled by automatic oil addition.
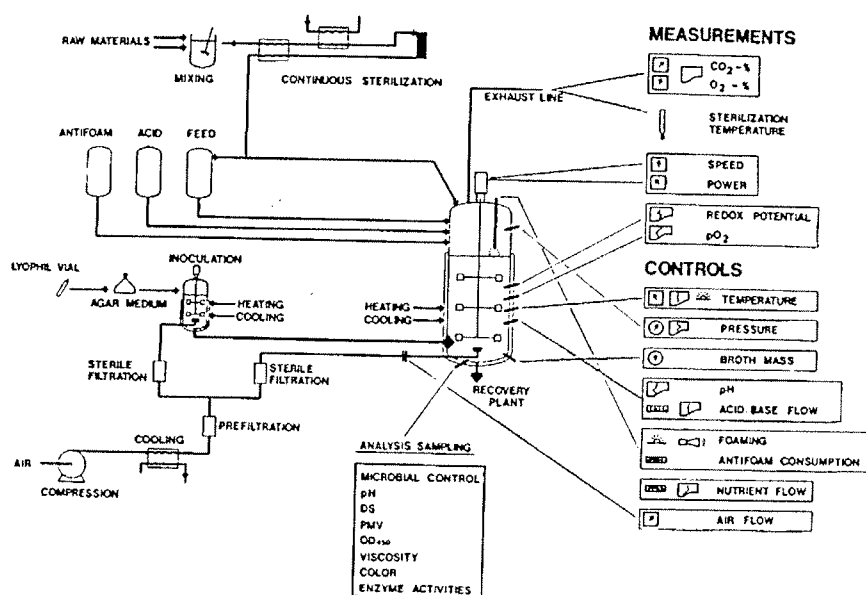

FIGURE 1. Schematic diagram of a typical enzyme fermentation process. Examples of useful measurements and controls are indicated.

Enzyme fermentations are especially vulnerable to microbial contamination. Rich media with a neutral pH value are typical, and the protection afforded by antibiotic activity is normally lacking. Infection generally means loss of a batch. Strict attention must be given to the contamination risk in the design and construction of fermentor, pipes, and auxiliary equipment. A fully welded system is recommended. Ports and valves should be steam-sealed, and all transfers of cultures and media should be done by compressed sterile air. A positive pressure must be maintained in the aseptic system. Mechanical agitator seals are normally used.

As a rule, extracellular enzymes are produced by batch processes which last from 30 to 150 hours. The optimal harvesting time falls between the point of maximum productivity and the point of maximum enzyme activity. Relative costs of raw materials, utilities, and recovery, as well as utilization of the plant capacity, determine the optimum. Often the process is terminated before or carried on beyond the optimum in order to obtain a broth with properties which facilitate product recovery. Finally, the scheduling in the plant restricts the choice of fermentation time. The optimal harvesting time is often signaled by a characteristic change in pH, oxygen tension, or some other parameter which is easy to determine.

Continuous culture techniques have been applied in several studies of enzyme production on a laboratory scale. Jensen (1972) and Fabian (1969), investigating *Bacillus* proteases, and Mitra and Wilke (1975), investigating *Trichoderma* cellulase, point out the advantages of a two-stage system. The first stage is operated under optimal conditions for growth, while conditions in the second stage are optimal for enzyme production. On an industrial scale, continuous methods have been reported in use only for the production of glucose isomerase (Diers, 1976). One reason for the limited application of continuous culture by the industry is the instability of the production strains (Heineken and O'Connor, 1972).

While continuous methods are rarely applied, the batch process is often extended, and the enzyme production favored by continuous feeding of carbohydrate or protein. One feeding strategy is to maintain a low reducing sugar level (Kalaboklas, 1971). The feed rate may also follow an empirical program or be controlled by pH, redox potential, oxygen consumption, or some other measured or calculated variable.

Most enzyme fermentations have a high oxygen demand, requiring aeration and agitation rates similar to those used in antibiotic fermentations. Very viscous media with non-Newtonian behavior are often employed; in other cases heavy mycelial growth complicating the oxygen transfer develops. In the glucoamylase process the productivity is limited by the oxygen transfer rate (Aunstrup, 1978). However, high oxygen tension may, in certain processes, inhibit enzyme formation. Diers (1976)

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reports that glucose isomerase production by an atypical variety of *Bacillus coagulans* is optimal during oxygen limitation and simultaneous addition of glucose at such a rate that the concentration in the broth is infinitesimal.

During process development much attention must be given to the properties of the broth in product recovery and to the quality of the final product. Since enzymes used for technical purposes are marketed as rather crude protein solutions or precipitates, the quality is strongly influenced by the fermentation method. Choice of raw materials, sterilization method, foam-control method, aeration and agitation rates, and fermentation time affect not only the product recovery yield and cost but also color, smell, stability, powder properties, and similar quality parameters.

A general kinetic model of enzyme synthesis has not been developed, but the regulation of the formation of many specific enzymes has been studied. A few enzymes used commercially are formed during exponential growth, but most are formed in the postexponential growth phase. As mentioned in the preceding sections, many factors other than growth control enzyme formation. A stoichiometric relationship for the process cannot be expressed. The yield of useful enzyme protein may reach 1–5% of the initial medium dry substance. The cell yield in a typical enzyme fermentation may be 2–10% on a similar basis. Residual nutrients and metabolites usually constitute 5–10% of the broth at the end of a fermentation.

IV. RECOVERY AND FINISHING

Methods for the recovery of enzymes for use commercially are simple unit operations such as centrifugation, filtration, vacuum evaporation, and precipitation of proteins. The complications in the processes arise from the character of the fermented broth. The broth has a variable, unspecified composition, a high content of colloidal material, and often a high viscosity.

The most significant development in recent years has taken place in the finishing processes for solid enzymes. Ten years ago, commercial products were dusty powders, but today the majority of solid enzymes are supplied as dustless granulates or as immobilized enzymes.

A. Cooling and Pretreatment of the Broth

After termination of the fermentation, the broth is rapidly cooled from the fermentation temperature of 30°–50°C to about 5°C. The culture broth is an excellent medium for a wide variety of microorganisms. Thorough

cooling of the broth and strict hygienic measures in the plant are the most important methods to control infections during the recovery process. The addition of preservatives is normally unacceptable because many enzyme products are used in food processing. Also, many preservatives may effect the biological treatment of the wastewater from the recovery plant.

The refrigerated broth is normally pretreated before the separation processes—filtration or centrifugation. The character of pretreatment depends on the type of broth and on the equipment used for recovery.

Broth fermented by filamentous organisms can often be filtered or centrifuged directly after adjustment of pH to the stability optimum of the enzyme.

Broth from bacterial fermentations is more difficult to process. Fermentations on rich media and fermentations that leave large amounts of colloidal particles in the broth present the largest problem to the recovery plant. In these cases pretreatment with a coagulating or flocculating agent is needed. Inorganic salts, e.g., calcium phosphate precipitated in the broth from soluble phosphate and calcium salts, may be used to enclose cells and colloids in a precipitate. More efficient is a flocculation using synthetic polyelectrolytes which are available in several types, ranging from strongly cationic over nonionic to anionic. Each type can be used alone or in combination with another type or with inorganic chemicals. The synthetic polyelectrolytes are large molecules often based on polymerized ethyleneimine, acrylates, or acrylamide.

A special pretreatment step prior to a filtration may, for instance, be the addition of 2–4% diatomaceous earth to the broth as a body feed.

B. Separation of Solids

Most enzymes of industrial interest are extracellular, i.e., excreted from the cells into the liquid. The first task in recovery of an extracellular enzyme is to remove cells and other suspended material from the broth.

During the past decade there have been few developments in equipment used for this purpose. The main alternatives are still vacuum drum filters and centrifuges. The disk-type centrifuge with a self-cleaning bowl is the one mainly used. The use of vacuum drum filters precoated with diatomaceous earth or expanded perlite are particularly widespread in the industry.

C. Purification

The purification of the enzyme after the initial separation step may be more or less extensive, depending on the intended use of the product.

Figure 2 shows examples of purification steps leading to commercial-grade enzyme products.

After a polishing filtration, the solution is concentrated by vacuum evaporation at low temperature or by ultrafiltration. Ultrafiltration, although a relatively new technique, has been developed to a degree which makes it suitable for large-scale application. Compared with vacuum evaporation, ultrafiltration has the advantage that it removes smaller molecules, e.g., molecules of a MW below 10,000, from the concentrate. Consequently it is often possible to make a more concentrated enzyme solution by ultrafiltration than by vacuum evaporation. One drawback, however, is that the membranes are easily clogged by precipitates which may form during the concentration of the solution.

The concentrated enzyme solution can be clarified by a polishing filtration, and the remaining germs can be removed by a sterile filtration on cellulose-containing filter pads.

Stabilizers or preservatives may be added before or after the sterile filtration. Typical stabilizers are calcium salts, proteins, starch hydrolyzates, and sugar alcohols. Microbial stability of a liquid product may be secured by addition of 18–20% NaCl or by the use of a food-grade preservative, such as benzoate, parabens, or sorbate.

There is a preference of liquid rather than solid enzyme products, because the liquid products are cheaper to produce and are also safer and more convenient to apply. In some cases, however, only a solid preparation can be used. Solid enzyme concentrates can be obtained by precipitation or by direct spray-drying of the enzyme solution. It is often impossible to spray-dry a solution concentrated by evaporation, but it can usually be done on an ultrafiltered solution.

A higher degree of purity is obtained when the enzyme is precipitated with acetone, alcohols, or organic salts, such as ammonium sulfate or sodium sulfate. Fractional precipitation yields a higher purity than a one-step precipitation. In large-scale operations salts are preferable to organic solvents to eliminate the possibility of explosion. The precipitate may be dried by freeze-drying, vacuum-drying, or spray-drying depending on the heat stability of the enzyme.

D. Intracellular Enzymes

Recovery of intracellular enzymes is more difficult. The yield of enzyme on a total broth basis is often less than for extracellular enzymes because the cell mass is limiting.

To harvest the cells, centrifugation is usually preferred to filtration to avoid mixing the cells with diatomaceous earth. In industry the cells are often used without extraction of the enzyme, thereby saving activity and
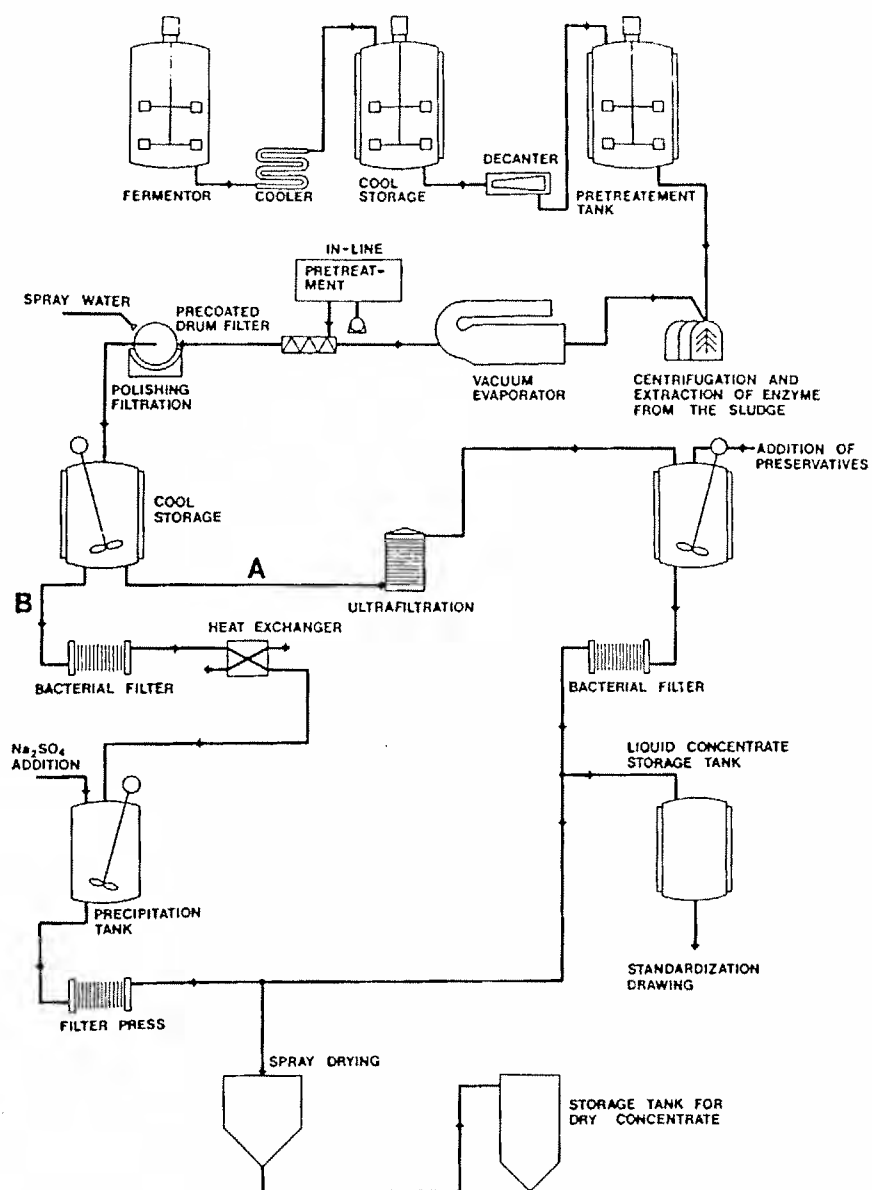
FIGURE 2. Examples of enzyme recovery processes.

expensive purification steps. If the enzymes must be extracted, autolysis may be used, but on an industrial scale physical methods are preferred, e.g., disruption of the cells by means of a homogenizer or a bead mill (Melling and Phillips, 1975). When the cells have been broken, the enzyme can be purified as are extracellular enzymes, but the process is usually more difficult due to the content of cell debris and nucleic acids from the broken cells.

E. Finishing of Solid Enzymes

A few years ago, all solid enzymes were sold as dusty powders with a small particle size. A significant proportion of the particles were smaller than 10 μm. This type of product requires very careful handling in order to avoid exposure to enzyme dust. Problems in handling and admixture of the powders, especially in the detergent industry, led to the development of methods to convert the enzyme concentrate to dustless granulates. An additional advantage of the granulates is often improved storage stability.

In the field of detergent enzyme granulation several methods have been developed, but on an industrial scale only a few are applied. A common method is to embed the enzyme into spheres of a waxy material consisting of a nonionic surfactant by means of a spray-cooling or prilling process. The "marumerizer" process is another important method by which the enzyme is mixed with a filler, a binder, and water; then extruded; and subsequently formed into spheres in a so-called marumerizer. After drying the spheres are coated with a layer of waxy material (see Fig. 3). Enzyme granules produced by these methods are rigid and have a very low dust level. The particles have an average diameter of about 500 μm, and practically no particles are smaller than 200 μm. Furthermore, the granules may be coated with an inert film. Today most enzymes used in the detergent industry are granulated.

In other applications, where the enzymes normally are used in industrial processes, the handling properties are less critical, and simple powders are still used. One reason is that the additives used in the granulates for the detergent industry are unacceptable in many other applications. A significant improvement in handling properties may be obtained, however, by a relatively simple granulation step, such as a fluid bed agglomeration.

F. Immobilization of Enzymes

Methods for the immobilization of enzymes have been known for several years, but the technique has gained industrial importance only
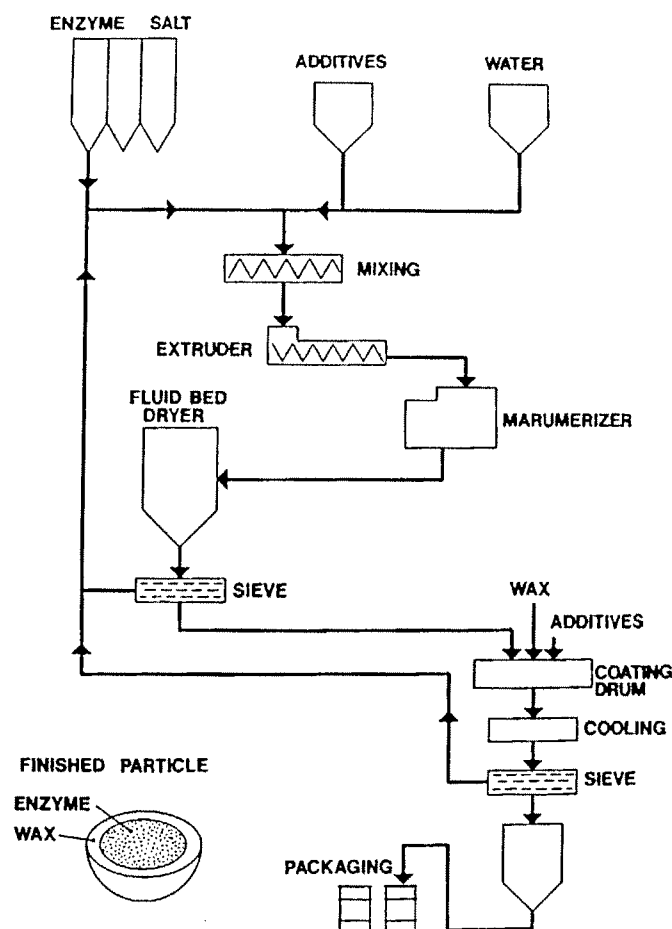
ENZYME SALT

ADDITIVES   WATER

MIXING

EXTRUDER

FLUID BED
DRYER

MARUMERIZER

SIEVE

WAX

ADDITIVES

COATING
DRUM

COOLING

FINISHED PARTICLE

SIEVE

ENZYME
WAX

PACKAGING

FIGURE 3. Typical flow sheet for the preparation of a dust-free enzyme product.

within the last few years. By far the most significant immobilized enzyme product at present is glucose isomerase, an intracellular enzyme produced by various microorganisms. Several manufacturers use the enzyme-containing cells for immobilization without further purification.

Numerous immobilization methods have been published (Barker and Kay, 1975). In some methods of industrial importance, glutaraldehyde is used to cross-link the enzyme or the supporting protein in which the cells are suspended. According to a method described by Van Velsen (1971), the enzymes or the cells are suspended in an aqueous solution of a gelling protein. The protein is suspended in an organic water-immiscible

liquid to produce enzyme-containing droplets. The gelling protein is forced to gel. Subsequently, the particles are cross-linked with glutaraldehyde. Amotz et al. (1976) describe a method in which the cells are harvested, concentrated, and homogenized. Glutaraldehyde is added, and the resulting gel is shaped into granules which are dried.

Another attractive immobilization method is the use of polyelectrolytes to flocculate the enzyme-containing cells. After filtration the filter cake is shaped to particles of the desired size and dried (Long, 1974). Other methods are based on the fixation of the extracted enzyme through chemical reaction or adsorption to solid supports, such as ion exchangers, glass beads, or cellulose fibers.

The particles of an immobilized enzyme must be porous, uniform in size, and physically strong in order to perform satisfactorily in a column over a long period of time. Glucose isomerase, for example, is usually used more than 1000 hours at temperatures between 60° and 65°C.

V. REGULATIONS AND SPECIFICATIONS

Because microbial enzymes are natural products, and in view of their traditional use for food purposes in the orient, they were regarded as inherently harmless substances until about 15 years ago. The increasing knowledge of mycotoxins and the allergenic properties of enzymes has made everybody concerned more cautious. Microbial enzyme products must now meet strict specifications with regard to toxicity and other safety aspects.

A. Toxicity

As a rule, enzymes are completely nontoxic. The only recorded exception is phospholipase C, which is formed by several pathogenic bacteria. Toxic materials may, however, be present in the raw materials, and theoretically they may be formed during the manufacturing process by the metabolism of the enzyme-generating organism or contaminants. Since most enzyme products undergo a relatively crude purification process, they contain all components of the fermentation broth in small or large quantities. It is necessary, therefore, to ensure that no toxic products are formed at any stage of the process. This is done by prescribing food- or feed-grade raw materials throughout the process, by regularly testing for microbial contamination, and by a thorough investigation of the enzyme-generating organism and of the enzyme product.

This investigation also includes a complete survey of the scientific

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literature on the organism and closely related organisms, followed by extensive feeding studies on several species of animals. The feeding studies are usually made with a mixture of several batches, e.g., ten batches of enzyme concentrate prepared according to the actual production process. The amounts used are chosen to ensure the highest possible safety factor. Usually the limit is set by the amount of solids that the animals are able to ingest. In some cases a high content of salts, such as NaCl, Na$_2$SO$_4$, and phosphates, will set an unacceptably low limit, necessitating desalting of the enzyme preparation before it is fed to the animals.

The preparations will always be tested for aflatoxin. They will be tested also for other mycotoxins if their presence is theoretically possible.

B. Allergenicity

Like all proteins, enzymes are antigenic and may thus cause allergy. The antigenicity varies from one enzyme to another, but no general rules can be given. The antigenicity of the enzyme as such cannot be changed, but the formulation can be made so that exposure of the user of the enzyme is minimized. The most important factor is the level of dust formed by handling the enzyme. To keep it low, the enzyme may be encapsulated in inert material. An encapsulated product may contain less than 0.5 μg of pure enzyme dust per kilogram as determined according to Harris and Rose (1972). Whenever possible, liquid products are preferred because they can easily be handled without exposing personnel to enzyme dust.

C. Microbial Safety

The enzyme-generating strain must be a harmless saprophyte. This is ensured by a literature survey and by animal pathogenicity tests, in which the organism must be administered both orally and by injection. In addition, the organism, as a rule, will be completely removed from the preparations, a precaution that serves the additional purpose of preventing competitors from obtaining the strain.

During the fermentation process contamination tests are regularly performed, and contaminated batches discarded. During recovery and handling of the finished product the hygienic standard must be as high as in the food industry. Nevertheless a certain degree of contamination is unavoidable. Regular controls of the microbial standard of the finished product are therefore necessary.

D. Regulations

Responsibility for the safety of an enzyme product remains with the manufacturer. In most countries, though, a new enzyme product for use in food requires approval by the appropriate authorities. In the United States, approval of the Federal Drug Administration is required. National and international bodies have worked out recommendations and specifications for a number of known enzyme products (Food Chemicals Codex, 1975; World Health Organization, 1972, 1975).

E. Specifications

All technical enzyme preparations are sold on an activity basis. This means that an analytical method must be specified by the manufacturer. Although recommendations for enzyme analysis have been given by the Commission on Enzyme Nomenclature, the different enzyme manufacturers generally use their own method of analysis. Even when activities are given in so-called international units, they should be interpreted with caution, because small differences in reaction conditions may have an important effect on the result. The manufacturer will do his best to reduce the deviation of the analytical method. For most preparations it is possible to give the activity with a standard deviation better than 5%.

Usually the manufacturer will guarantee a certain storage stability under specified conditions. For example, less than 10% loss per 6 months at 25°C for liquid products or less than 10% loss per year for solid preparations. In addition, the gross chemical composition may be given as well as the kind and amount of preservatives added. For special enzymes, such as detergent proteases, the specifications usually include limits for enzyme dust, particle size, bulk density, etc.

Immobilized enzymes present special problems, since performance cannot be judged on activity alone. The activity test must be performed in a column operating over a considerable period of time, and other parameters, such as particle size, particle strength, and color formation, become important.

The customer expects uniform, reliable, and safe enzyme products, and the manufacturer must prepare specifications which ensure that these expectations are met.

VI. SURVEY OF ENZYMES AND APPLICATIONS

A list of the enzymes used commercially is given in Table IV All the enzymes with the exception of two are hydrolases. It can thus be said

TABLE IV. Microbial Enzymes for Industrial Use

Enzyme	Microbial source
α-Amylase	*Aspergillus oryzae*
	Bacillus amyloliquefaciens
	Bacillus licheniformis
β-Amylase	*Bacillus cereus*
	Bacillus megaterium
	Bacillus polymyxa
β-Glucanase	*Aspergillus niger*
	Bacillus amyloliquefaciens
Cellulase	*Aspergillus niger*
	Trichoderma reesei
Dextranase	*Penicillium* sp.
	Trichoderma sp.
Glucoamylase	*Aspergillus awamori*
	Aspergillus niger
	Rhizopus sp.
Glucose isomerase	*Actinoplanes* sp.
	Arthrobacter sp.
	Bacillus sp. (thermophilic)
	Streptomyces sp.
Glucose oxidase	*Aspergillus niger*
Invertase	*Saccharomyces* sp.
Lactase	*Aspergillus niger*
	Kluyveromyces fragilis
	Kluyveromyces lactis
Lipase	*Aspergillus* sp.
	Candida lipolytica
	Geotrichum candidum
	Rhizopus sp.
Pectinase	*Aspergillus niger*
	Aspergillus sp.
Penicillin acylase	*Bacillus megaterium*
	Erwinia carotovorum
	Escherichia coli
Pentosanase	*Aspergillus* sp.
	Bacillus amyloliquefaciens
Protease, acid	*Aspergillus* sp.
Protease, neutral	*Aspergillus oryzae*
	Bacillus amyloliquefaciens
	Bacillus thermoproteolyticus
Protease, alkaline	*Aspergillus oryzae*
	Bacillus amyloliquefaciens
	Bacillus licheniformis
	Bacillus sp. (alkalophilic)
	Streptomyces griseus
Pullulanase	*Klebsiella aerogenes*
Rennet	*Endothia parasitica*
	Mucor miehei
	Mucor pusillus

302

that enzyme technology so far has been concentrated on the relatively simple hydrolytic processes, whereas the more complicated enzymatic reactions have not been possible on a technical scale. Most of the more complicated systems require co-enzymes, and this has deterred their use since an economical way of applying them has not been found. The explanation may be that there is no demand for a large-scale application of such complicated systems.

A. Microbial Sources of Technical Enzymes

The majority of industrially produced enzymes are derived from microorganisms of two genera, *Aspergillus* and *Bacillus*. It is characteristic of these microorganisms that they are common, harmless saprophytes; they grow rapidly, are metabolically very active, and they are highly variable. They secrete a number of extracellular enzymes in relatively large quantities. Usually several enzymes are produced at the same time.

Bacillus amyloliquefaciens produces α-amylase, β-glucanase, and neutral and alkaline protease. *Bacillus licheniformis* normally produces an almost pure serine protease with minimal amounts of α-amylase. However, special strains that produce high amounts of α-amylase together with the protease have been developed. Although the taxonomic distinction between the two species seems pretty clear, many manufacturers and scientists unfortunately still prefer to name both strains *B. subtilis*.

Aspergillus oryzae, when grown in semisolid culture, will produce a large number of enzymes, primarily α-amylase, lactase, glucoamylase, and protease. In submerged culture the α-amylase formation is increased, whereas the formation of other enzymes becomes minimal. *Aspergillus niger* strains are used in the production of glucoamylase, pectinase and protease. Special strains are developed for each of these purposes, but it is characteristic that all strains produce most of the enzymes in varying amounts. The nomenclature of the *Aspergilli* is complicated and unclear. *Aspergillus niger* and *A. oryzae* should here be understood as representing the groups of black and green *Aspergillus* species, respectively.

Members of the family *Mucoraceae* have frequently been used for enzyme production, e.g., the use of *Rhizopus* sp. for the manufacture of α-amylase, glucoamylase, protease, and lipase. These organisms are still important enzyme sources in the manufacture of oriental foods but their use in the production of enzymes for technical use is limited. The

two thermophilic *Mucor* species, *M. miehei* and *M. pusillus*, are important in the manufacture of microbial rennet.

The *Actinomycetales* have become important sources of glucose isomerase which is produced commercially using several *Streptomyces* species and one *Actinoplanes* species. Few other microbial species are used for commercial enzyme production (Table IV).

B. Applications

Enzymes offer an attractive solution to many catalytic problems, especially within the food industry, because of their specificity, nontoxicity, and mild reaction conditions. Only the most important processes are described here. A list of selected publications on the application of enzymes is given at the end of this chapter.

1. Enzymes for the Detergent Industry

Proteinaceous dirt will often precipitate on soiled clothes, or it may coagulate during the normal washing process. Furthermore, proteins make dirt adhere to the textile fibers. Such stains, which are otherwise difficult to remove, can be dissolved easily by addition of proteolytic enzymes to the detergent.

The alkaline serine protease of *B. licheniformis*, otherwise known as subtilisin Carlsberg, is the preferred protease for this purpose. The enzyme is well suited for this application. It will attack many peptide bonds and therefore easily dissolve proteins. It may be used in the presence of most nonionic and anionic detergents at temperatures up to 65°C, and its pH optimum is close to 9, the pH normally used in washing fluids. Furthermore, extensive tests have proved the enzyme harmless to the user.

A few other proteases are used in detergents. The serine protease of *B. amyloliquefaciens* has found some application, presumably because the preparations usually have a substantial content of α-amylase. For some applications this may be an advantage. Proteases with improved stability and protein solubilizing properties under washing conditions have been found in alkalophilic bacilli (Aunstrup *et al.*, 1972). These enzymes are particularly advantageous in detergents with low phosphate content.

The success of the detergent proteases has inspired numerous attempts to use enzymes for removal of other stubborn stains, such as fruit colors and lipids, especially in low-temperature washing. So far no satisfactory solution to these problems has been found.

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2. Enzymes for the Starch Industry

Until around 1960, dextrose and glucose syrups were prepared from starch exclusively by acid hydrolysis. The process was corrosive, and the dextrose yield low. It was, therefore, a great step forward when pure glucoamylase in combination with bacterial α-amylase made possible a complete enzymatic hydrolysis of starch to dextrose. Today several enzymatic processes are used in the industry. A schematic summary is given in Table V. Most of the processes are applied in combination, starting with a liquefaction followed by a more or less extensive saccharification depending on the desired end product.

Prior to liquefaction the starch should be gelatinized by heat treatment. For the widely used maize starch, temperatures above 100°C are normally applied in order to secure complete gelatinization. It is consequently an advantage to use the extremely heat-stable amylase of *B. licheniformis* which can function at temperatures up to 110°C. Normally high concentrations (30–50% dry substance) are used in the saccharification process. The equilibrium concentrations of the saccharides formed by resynthesis limit the maximum degree of hydrolysis obtainable, e.g., 97% dextrose at 30% dry matter. Since the activity of glucoamylase toward branching points (α-1,6 bonds) is low, it may be an advantage to use a debranching enzyme, such as pullulanase, early in the hydrolysis process. In the preparation of fructose syrup, a fructose concentration of minimum 42% (on solids) is normally sought. Since this is close to the equilibrium concentration (around 50%), it is important that the initial glucose syrup be as pure as possible, i.e., that the hydrolysis be complete. Glucose isomerase is used in immobilized form, and a major share of the fructose syrup production is made by continuous column processes (Hilmer Nielsen *et al.*, 1976).

Since the processes described here are directly dependent on the enzyme activities, it is important that the enzymes be pure, i.e., free from undesired enzyme activities, such as transglucosidase or protease, and that the activity be accurately standardized. Liquid preparations of amylase and glucoamylase are used, whereas the immobilized glucose isomerase comes in various forms, from frozen cells to dried granules. For column operation, mechanical strength, flow properties, and active lifetime are important parameters.

3. Enzymes for the Dairy Industry

In the dairy industry microbial rennet has to a large extent replaced the expensive rennet from calves. The microbial rennets are acid aspartate

TABLE V. Examples of Enzymatic Processes in the Starch Industry

Process	Enzyme	Source	Reaction conditions		Product
			pH	Temperature °C	
Liquefaction	α-Amylase	B. amyloliquefaciens	5.5–7	90	Maltodextrins DE 10–20
		B. licheniformis	5.5–9	110	
Debranching	Pullulanase	K. aerogenes	6–7	50–60	Intermediate process in the manufacture of dextrose
Saccharification	α-Amylase	A. oryzae	5–7	50–55	High-maltose syrup, high-DE syrup,
Saccharification	Glucoamylase	A. niger (Rhizopus sp.)	4–5	55–60	High-DE syrup, cryst. dextrose
Isomerization	Glucose isomerase	(Streptomyces sp.), B. coagulans, Actinoplanes sp.	6.5–8.5	60–65	Fructose syrup

306

proteases. The reaction mechanism with casein is closely related to that of calf rennet. However, minor differences in the enzyme properties, such as dependency on Ca^{2+}, temperature, and pH, necessitate small adjustments of the cheese-making procedure according to the enzyme used. When the proper procedures are applied, the quality of cheese prepared with microbial rennet equals that of cheese prepared with traditional rennet.

Lactase for the hydrolysis of lactose in whey or milk is gaining increasing importance. Lactase from *Kluyveromyces fragilis* or *Kluyveromyces lactis* is usually used, although the properties of these enzymes are not ideal because of a narrow pH optimum and a low maximum temperature.

Lipase for flavor development in special cheeses (romano, provolone) is an interesting application, and microbial esterases for this purpose are presently being developed.

4. Other Applications

In the textile industry, α-amylase has been used for many years to remove starch sizes. This application is still an important outlet for bacterial α-amylase. An improved, rapid, high-temperature process is possible with the thermostable amylase from *B. licheniformis*.

Bacterial α-amylase is also used in the brewing industry for the liquefaction of brewing adjuncts. The substitution of barley for malt requires three enzymes, α-amylase, β-glucanase, and protease. All are produced by *B. amyloliquefaciens*. It is noteworthy that only the neutral protease is of importance because the alkaline protease is inhibited by an inhibitor in barley. β-Glucanase is often used alone to alleviate filtration problems due to poor malt quality or precipitation of glucans during the fermentation.

Substitution of amylase and glucoamylase for malt in the distilling industry makes high-temperature liquefaction possible and renders the process more reproducible.

The use of pectic enzymes in the wine and juice industry improves the yield and the quality of the products. Empirical methods and experience are especially important in the production and use of pectinase products. They contain the enzymes pectinase, pectin esterase, transeliminase, and polygalacturonidase. In addition, several nonpectolytic enzymes, such as protease, pentosanase, and glucoamylase, are present in small quantities. The combined action of several of these enzymes often decides its application.

Alkaline protease from alkalophilic bacteria may be used for dehairing of hides. The process functions well and is considerably more pleasant, both to workers and to the environment, than the usual sulfide process.

The method is little used, partly because it requires strict control of the process conditions, and partly for economic reasons.

VII. CONCLUSION

The industrial use of enzymes is insignificant and the processes are unimportant when compared with the overwhelming magnitude and importance of the enzymatic processes performed in nature. Some of these processes, such as lignocellulose hydrolysis, are relatively simple and seem to be technologically possible within a few years. Others, such as nitrogen fixation, are extremely complex, and their industrial realization is unthinkable with present technology.

Solution to such important problems as these will require systems of a higher degree of complexity than are so far known in the enzyme technology. The development will include multienzyme systems, free or immobilized, and in most cases coupled to cofactors. The possibilities are legion but the research effort necessary is some orders of magnitude larger than that for the simple problems so far attempted by the industry.

There is no doubt that industrial evolution will favor the use of enzymes. Their specificity, mild reaction conditions, and nontoxicity make them ideal catalysts in a world which becomes more and more conscious of pollution and energy waste.

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