Production, Isolation, and Economics of Extracellular Enzymes

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I. INTRODUCTION

Microorganisms synthesize numerous enzymes which all have their function in growth, metabolism, and autolysis. Most enzymes operate inside the cell in a protected and highly structured environment, but some enzymes are secreted from the cell. These extracellular enzymes are unique among microbial products, designed as they are to work for the benefit of the microorganism at a distance. Their function is to make food materials available to the microorganism by hydrolysis of high-molecular weight-compounds; hence they are all hydrolases.

Since the extracellular enzymes work in the medium surrounding the microorganism, outside the protecting cell membrane they must possess good stability to variations in the chemical and physical properties of this environment. Furthermore, the large volume in which the enzymes react makes it necessary for the microorganism to produce them in large quantities. These attributes make the extracellular enzymes suitable for industial utilization.

Extracellular enzymes have been a popular research area for biotechnologists; thousands of publications describing enzyme fermentation, properties, and applications have been issued in the last few years. For the nonspecialist it is difficult to evaluate what is of industrial relevance and how the enzyme industry operates.

It is the purpose of this chapter to present factual information about those extracellular enzymes that are now of commercial importance. Industrial production methods, enzyme properties, and application methods will be described, and an estimate of the economic importance of the enzyme industry will be given.

The information has been collected from scientific journals, patents, and brochures issued by the enzyme manufacturers. The information is often confusing or contradictory, and details about production methods are usually not disclosed. A critical evaluation has been attempted, but in order not to make the text unduly long, detailed discussions of doubtful points have, as a rule, been avoided.

II. HISTORY

Cultivation of microorganisms which produce extracellular enzymes is a simple way of obtaining hydrolytic enzymes in large quantities. In the Orient this method has been used since prehistoric times in the preparation of a variety of fermented foods and beverages. The principal enzymes were amylolytic and proteolytic, and the organisms used were predominantly of the genera *Aspergillus* and *Mucor*. Semisolid fermentation of moist rice was used, and production was on a small

scale with refinements based on generations of experience. These methods are still used for the manufacture of enzymes, but production has been concentrated on fewer, large producers, and the methods used in some factories are based on the latest scientific developments and extensive automation.

Commercial microbial enzymes were introduced to the West around 1890 when the Japanese scientist Takamine settled in the United States and started an enzyme factory based on the Japanese technology. The principal product was Takadiastase, a mixture of amylolytic and proteolytic enzymes prepared by cultivation of *Aspergillus oryzae*. Takamine improved the traditional process significantly by the replacement of rice with wheat bran.

Bacterial enzymes were developed in France in 1913 by Boidin and Effront, who found that the hay bacillus, *Bacillus subtilis*, produces an extremely heat-stable α-amylase when grown in still cultures on a liquid medium made by extraction of malt or grain. The enzyme was primarily used in the textile industry for removal of the starch size that protects the warp in the manufacture of cotton fabric. The protease also formed in this process was of minor interest but found some application in the tanning industry.

Around 1930, it was discovered that pectinases could be used with advantage in the preparation of fruit products. Crude enzyme mixtures were prepared in the same way as Takadiastase by strains of the *Aspergillus niger* group, e.g., *Aspergillus wentii*.

In the following years, several other hydrolytic enzymes were developed and sold on a commercial scale, e.g., pentosanase, cellulase, and lipase, but the technology was not satisfactory. The enzyme products were crude, of low activity, and rather expensive, thus, new applications progressed slowly and resulted in many disappointments.

After World War II, the fermentation industry underwent a rapid development as efficient methods for submerged cultivation in the production of antibiotics were discovered. These methods were soon adopted in the production of enzymes but did not result in extensive economic expansions in the industry.

Around 1960, glucoamylase was introduced as a means of hydrolyzing starch for the manufacture of dextrose. The enzyme had been known for a long time, but the usual preparations of *A. niger* gave incomplete hydrolysis. The discovery that removal of transglycosylase from the preparation allowed an almost 100% hydrolysis of starch to dextrose paved the way to industrial application. Today, enzyme hydrolysis has completely substituted acid hydrolysis in the manufacture of dextrose.

At the same time it was discovered that the alkaline protease from

Bacillus licheniformis, Subtilisin Carlsberg, was well suited for incorporation into detergents. Because the enzyme detergents were well accepted by consumers enzymes were very quickly added to almost all detergents, and this application became the largest outlet for microbial enzymes. After a setback in 1970 caused by some factory workers' allergic reactions to enzyme dust, dust-free preparations were developed and detergent enzymes have again become an important application of hydrolytic enzymes.

Substitution of microbial enzymes for calf rennet is an old dream of the industrial microbiologist. Numerous microbial enzymes have been tested for this purpose, but only three have wide application, viz., the proteases from *Mucor pusillus*, *Mucor miehei*, and *Endothia parasitica*. These enzymes were introduced in the 1960s and are now extensively used. When applied properly, they will produce excellent cheese; furthermore, they have the advantage over calf rennet that they are less expensive and their supply is unlimited.

In the last decade, development has been concentrated on enzymes with improved properties. An interesting example is the extremely heat-stable amylase from *B. licheniformis*, the first enzyme used at a temperature above 100°C (115°C). Furthermore, highly alkali-stable proteases from alkalophilic *Bacillus* species have been introduced. These enzymes have optimum activities up to pH 12 and are used with advantage in detergents and dehairing processes.

At present, industry is awaiting the next breakthrough, but it is not easy to see where it will happen. Extensive work is being done in a number of areas, e.g., in waste hydrolysis, especially of cellulose. Although cellulose is easily hydrolyzed by extracellular enzymes, an economical process is not available. Moreover, the natural product lignocellulose requires a chemical or enzymatic pretreatment which is not yet economical.

In conclusion it can be said that, although a large number of extracellular enzymes are known and may become of technical interest, there is no immediate large, new application in sight. From the technical literature one receives the impression that future developments will center on intracellular enzymes or immobilized microbial cells.

III. THE ENZYME INDUSTRY

A. Extracellular Microbial Enzymes for Industrial Use and the Producing Companies

The major enzyme producers are listed in Table I, and the most common enzyme products are found in Table II. A number of enzyme

TABLE I
COMPANIES PRODUCING EXTRACELLULAR ENZYMES FOR INDUSTRIAL USE

Denmark	1. Grindstedvaerket A/S, Brabrand
	2. Novo Industri A/S, Bagsvaerd
France	3. Soc. Rapidase, Seclin (subsidiary of No. 8)
Germany	4. Miles Kali-Chemie GmbH, Nienburg a.d. Weser (subsidiary of No. 20).
	5. Röhm GmbH, Darmstadt
Great Britain	6. ABM, Stockport, Cheshire
	7. Glaxo, Greenford, Middlesex
Holland	8. Gist Brocades NV, Delft
Japan	9. Amano, Nagoya
	10. Daiwa Kasei, Osaka
	11. Meiji Seika, Tokyo
	12. Meito Sangyo, Nagoya
	13. Nagase, Osaka
	14. Sankyo, Tokyo
	15. Shin Nippon, Tokyo
	16. Yakult Biochemicals, Nishinomiya
Switzerland	17. Swiss Ferment AG (subsidiary of No. 2)
	18. Schubert AG (subsidiary of No. 2)
United States	19. GB Fermentation Industries, Kingstree, South Carolina (subsidiary of No. 8)
	20. Miles, Elkhart, Indiana
	21. Novo Biochemical Industries, Frank Linton, North Carolina
	22. Pfizer, New York, New York
	23. Rohm and Haas, Philadelphia, Pennsylvania

suppliers without basic production and many enzyme products are not listed in the tables.

Because of a lack of information, no East-European enzyme producers have been included. It is known, however, that there is substantial enzyme production in the Soviet Union and some production in the German Democratic Republic, Czechoslovakia, Rumania, Hungary, and Bulgaria. Several large companies in Europe and the United States produce amylolytic enzymes for their own use. They are also excluded from the table. Novo and Gist Brocades are by far the largest producers in terms of turnover. Together, they account for the major part of sales of industrial enzymes.

B. Market Situation

World consumption of industrial extracellular microbial enzymes is difficult to evaluate, but an estimate expressed as the amount of pure active enzyme protein produced per year has been made (Aunstrup, 1977). Figure 1 shows the estimated world production in 1976.

TABLE II
COMMERCIAL EXTRACELLULAR ENZYMES, TRADE NAMES, AND SOURCES[a]

α-Amylase	*Aspergillus oryzae* Fungamyl (2); MKC clarase (4); Veron (5); Amylozyme (6); Mylex (7); Fermex, Mylase, Mycolase (8); Biodiastase (9); Asperzyme, Sanzyme, Takadiastase (14); Clarase, Dextrinase (20); Mycozyme (22); Diastase, Rhozyme (23) *Bacillus amyloliquefaciens* (*B. subtilis*) BAN, Aquazym (2); Rapidase (3); Optiamyl, Optimash, Optisize (4); Bacterase, Nervanase (6); Bactamyl, Maxamyl (8); Biokleistase, Kleistase, Diasmen (10); Biotex, Spitase (13); HT amylase, Tenase (20) *Bacillus licheniformis* Termamyl (2); Thermoase (10)
β-Glucanase	*Aspergillus* sp. Glucanase GV (1); β-glucanase 2000 D (6) *Bacillus amyloliquefaciens* Cereflo (2)
Cellulase	*Aspergillus* sp. Cellulase C (5); Cellase (8); Cellulase AP (9); Celluzyme (13) *Trichoderma reesei* (*T. viride*) Cellulase (2); Meicelase (11); Cellulase onozuka (16) Other sources MKC Cellulase (4); Cellulase P (5)
Dextranase	*Penicillin* sp. Dextranase (2)
Glucoamylase	*Aspergillus niger* (var.) AMG, SAN (2); Optidex, Optisprit (4); Ambazyme (6); Agidex (7); Amigase, Maxydrase (8); Diazyme (29) *Rhizopus* sp. Gluzyme (9); Sumyzyme (15)
Hemicellulase	*Aspergillus niger* Galactomannanase (2); MKC Hemicellulase (4)
Lactase	*Aspergillus niger* Lactase LP (8)
Lipase	*Aspergillus* sp. Lipase AP (9); Lipase B (23) *Candida cylindraccae* Lipase MY (14) *Mucor miehei* Piccantase (8) *Mucor* sp. Lipase AMP (9) *Rhizopus* sp. SNS (3); Saiken (13)
Mutanase	*Trichoderma* sp. Mutanase (2)

TABLE II (*Continued*)

Pectinase	*Aspergillus* sp. Pectolase GV (1); MKC Pectinase, Opticlar (4); Meliovin, Pectinol D, Rohament R (5); Klerzyme (8); Pectinex (17); Ultrazym (18); Spark L (20); Pectinil (23)
	Rhizopus sp. Macerozyme (16)
Protease	*Aspergillus niger* Proctase (11); Pamprosin (16)
	Aspergillus oryzae Veron P (5); Panazyme (6); Prozyme, Biozyme A (9); Sanzyme (14); Sumyzyme AP (15); Fungal Protease (20); Rhozyme (23)
	Rhizopus sp. Newlase (9)
	Bacillus amyloliquefaciens (*B. subtilis*) Neutrase (2); Rapidermase (3); Proteinase 18 (6); Protin (10); Bioprase, Nagase (16); Rhozyme (23)
	Bacillus licheniformis Alcalase (2); Optimase (4); Maxatase P (8)
	Bacillus thermoproteolyticus Thermoase, Thermolysin (10)
	Bacillus sp. *alkalophilic* Esperase, Savinase (2); Highly alkaline protease (8)
Protease, microbial rennet	*Endothia parasitica* Surecurd, Suparen (22)
	Mucor miehei Rennilase (2); Fromase (8); Marzyme (20); Morcurd (22)
	Mucor pusillus Emporase, Meito rennet, Noury lab (12)
Pullulanase	*Klebsiella aerogenes* Pulluzyme (6)

[a] Companies are identified by numbers referring to Table I.

World-wide sales of microbial enzymes amount to about 150 million U.S. dollars per year at present (1976). Table III shows the distribution on various enzyme types. It should be noticed that enzymes for medical and analytical applications are not included. From distribution of sales on industries (Table IV) it appears that the starch and detergent industries are predominant; furthermore, the latter is the only nonfood application of importance.

C. Enzyme Costs

Enzymes are always sold on an activity basis, but enzyme prices are difficult to compare because each manufacturer has his own method of analysis. Furthermore, the properties of enzymes from different

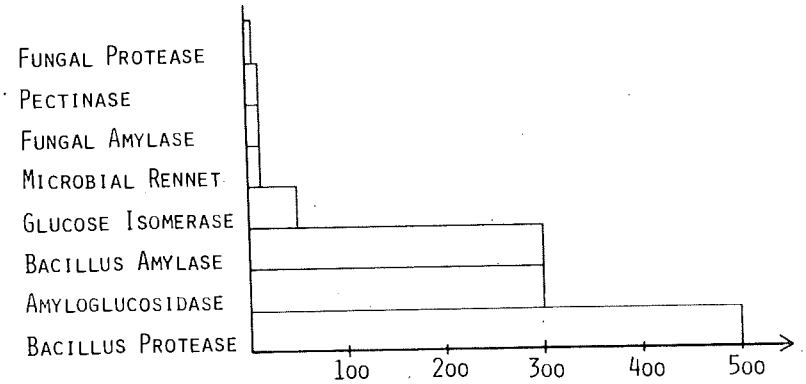

Fig. 1. Microbial enzyme production in tons of pure enzyme protein.

TABLE III
WORLDWIDE SALES OF ENZYMES[a]

Enzyme	Distribution (%)
Bacillus protease	35
Amyloglucosidase	14
Bacillus amylase	10
Glucose isomerase	14
Microbial rennet	5
Fungal amylase	4
Pectinase	10
Fungal protease	4
Others	4

[a] Distribution of enzyme types.

TABLE IV
DISTRIBUTION OF ENZYME SALES IN INDUSTRIES

Industry application	Distribution (%)
Starch	30
Detergent	35
Dairy	5
Distilling	5
Brewing	4
Fruit, wine	10
Milling, baking	5
Others	6

TABLE V
DIRECT ENZYME COST IN ENZYME PROCESSES: UNITED STATES, 1977

Application	Enzyme	Units	Enzyme cost (U.S. cents)
Washing	Protease	1 kg detergent	2–4
Starch			
liquefaction	Amylase	1 kg starch	0.2–0.5
saccharification	Glucoamylase	1 kg starch	0.4–0.8
isomerization	Glucose isomerase	1 kg starch	1.0–1.5
Cheese manufacture	Microbial rennet	1 liter milk	0.1
Alcohol manufacture	Amylase	1 liter alcohol	0.2–0.5
	Glucoamylase		0.7–1.4
Brewing	Amylase	1 liter beer	0.1
	Protease		
Baking	Amylase	1 kg flour	0.01
	Protease		
Juice	Pectinase	1 liter juice	0.1–0.2
Wine	Pectinase	1 liter wine	0.1–0.2
Dehairing	Protease	1 m^2 hide	1–5

sources often vary. This means that a comparison based on an analytical determination of the activity may be misleading because it is usually done under conditions which deviate from those of the actual application. The only proper way to compare enzymes from different sources is by their performance in the intended application.

The cost of an enzyme needed for a given process depends on the process conditions and, of course, on the factors that usually influence prices, such as customs, local trade patterns, and competition. Table V lists the actual enzyme costs of a number of enzyme applications. The enzyme costs are generally very small compared with the added value obtained by the enzyme process. Quality and reliability are therefore more important than a minimum price of an enzyme product.

IV. GENERAL METHODS FOR INDUSTRIAL PRODUCTION OF EXTRACELLULAR ENZYMES

Although enzymes are probably the most complex chemical compounds commercially available in large quantities, the methods used for the production of extracellular enzymes are in principle very simple: a microorganism is cultivated in a suitable medium from which the enzyme is subsequently recovered.

The problems lie in the details of the processes. To be competitive, the manufacturer must develop an economical and reliable production process which also meets today's strict requirements of safety and

hygiene. Appropriate modern equipment in good repair is a prerequisite for satisfactory results. The most important factor, however, is the optimum combination of a selected strain of microorganism and suitable fermentation conditions and recovery methods. This combination is achieved through comprehensive and time-consuming experiments. It is constantly improved and is the most important asset of the enzyme manufacturer.

As each company must develop its own methods without knowing the competitors' work, several approaches to the optimum result are likely. It would be interesting to compare the various methods used today, but this is unfortunately not possible. The information given in this chapter is limited to what has been published in scientific literature and patents.

A. Production Strain

In Table II it is shown that most commercial enzymes are produced by organisms belonging to the two genera *Bacillus* and *Aspergillus*. *Bacillus* species are well suited for enzyme production. Apart from the *B. cereus* group, including *B. anthracis*, they are harmless saprophytes which produce no toxins. They are easy to grow in high density and require no expensive growth factors. All *Bacillus* species form the heat-resistant endospores. Sporulation terminates the metabolism of the cell and is therefore undesirable in an enzyme production process. The spores are also undesirable because they may survive the recovery process and thus result in a high content of viable organisms in the final product. Fortunately, it is easy to prepare mutants that lack the spore-forming property. In many instances these mutants have a concomitant higher productivity of extracellular enzymes (Aunstrup and Outtrup, 1973). Many *Bacillus* species will produce polypeptide antibiotics, e.g., the production of bacitracin by *B. licheniformis*. These substances are usually not tolerated in food products; thus, it becomes necessary to use mutants that do not produce these substances. Extracellular enzyme formation by *Bacillus* has been extensively studied (Priest, 1977).

Aspergillus species may be regarded as the fungal parallel to the *Bacillus* species. The genus is highly variable and widespread, and very few species are regarded as pathogenic (*A. fumigatus*) or toxin-forming (*A. parasiticus*).

The *A. niger* and *A. oryzae* groups are the most frequently used for enzyme production (Table II). Based on morphological differentiation, the groups are divided into a number of species, but the differences between these are small. The *Aspergilli* are usually haploid in their

vegetative phase. This means that they are easily mutable; on the other hand, the mutants are rather unstable.

Since inoculation of fermenters or trays in surface cultivations is made with conidial spores, it is important to maintain the sporulating ability of the production strains. This may often be a problem because the strains with highly improved enzyme productivity generally have reduced ability to produce conidiospores.

Strain improvement is important in industrial laboratories. Since most of the organisms used are genetically unknown, it is difficult to use sophisticated genetic methods. When selecting high-yield mutants, there is often no better way than mutagenization and testing of each mutant in a shake flask culture. Each industrial microbiologist develops his own favorite tricks and advantageous methods, but there are no general rules.

It is the author's experience that microbiologists who have worked successfully with such problems for years often develop a "sixth sense" to detect valuable mutants. This is probably based on highly developed powers of observation combined with long-term experience. Highly mechanized testing systems are therefore not recommended as a substitute for traditional hand work for this type of development.

B. Fermentation

Two methods of fermentation are in general use: semisolid cultivation and submerged cultivation. Although the latter would seem to be more rational and more economical, a number of enzymes of fungal origin are still produced more economically by the former method.

1. Semisolid Cultivation

By this method the microorganism is grown on a particulate, moist substrate, preferably wheat bran with various additives. The method is preferred for the enzymes of Table VI.

The cultivation may be performed in trays with a substrate thickness of 1–10 cm or in rotating drums tumbling the substrate to ensure aeration. Combinations of growth forms may be used. It has been reported that, for instance, the use of a thick layer of substrate in part of the growth cycle (high heap) is an advantage.

The medium is prepared by mixing bran with water and additives and steam sterilizing the mixture in an autoclave equipped with a stirrer. The sterilized medium is transferred to the trays under aseptic conditions. Inoculation is made with spores in the autoclave after cooling, or in the cultivation equipment. Aeration is obtained by blowing

TABLE VI
ENZYMES PRODUCED IN SEMISOLID FERMENTATION

Enzyme	Organism
Glucoamylase	*Rhizopus* sp.
Pectinase	*A. niger*
Protease	*A. oryzae*
	A. niger
α-Amylase	*A. oryzae*
Lactase	*A. oryzae*
Rennet	*M. pusillus*

humidified air over the culture. To avoid overheating of fast-growing cultures, it may be necessary to use cooling surfaces. All handling of the substrate is mechanized as much as possible in a modern factory, and fully automated continuously operating factories are reported to exist. Unfortunately, no detailed descriptions of such factories are available.

Sterilization of the semisolid medium is difficult, and aseptic handling of the sterilized medium is almost impossible; thus, it is difficult to avoid minor contaminations. Fortunately, growth spreads slowly in the trays, and the massive inoculation decreases the ability of infections to overgrow the production organism. Nevertheless, infections are a major problem in this type of fermentation. Another problem is to prevent large numbers of spores of the production organism from spreading throughout the factory. Simultaneous production of different enzymes in the same factory is therefore difficult owing to cross contamination. Refinements like those used in the submerged fermentation technique, such as pH control or supplementation with medium components during the fermentation, are also complicated in semisolid fermentation.

The advantages of this system are the high aeration rate obtainable together with the low water concentration present. These advantages, and perhaps the fact that the mycelium is allowed to grow exposed to air, result in the formation of a broader variety of enzymes than would be possible in submerged culture.

2. *Submerged Cultivation*

The equipment and methods used in enzyme production are similar to the methods used in the preparation of antibiotics. Only the media and some fermentation conditions vary.

The fermentation medium should be inexpensive and support good growth of the microorganism. Usually, proteinaceous feed materials are used, combined with starch-containing materials such as grain and corn or carbohydrate-containing materials such as lactose, sucrose, or starch hydrolysates. Salts such as phosphates, magnesium salts, and ammonium salts or nitrate are added. Table VII gives some examples of media for enzyme production.

TABLE VII
TYPICAL FERMENTATION MEDIA

Submerged culture (composition in grams per liter)
Bacillus protease:
1. Starch hydrolysate 50, soybean meal 20, casein 20, Na_2HPO_4 3.3 (Churchill *et al.*, 1973).
2. Starch hydrolysate 150, lactose 4.3, cottonseed meal 30, brewers yeast 7.2, soy protein 3.65, K_2HPO_4 4.3, $MgSO_4$, H_2O 1.25, trace metals (Feldman, 1971).
3. Ground barley 100, soybean meal 30, pH adjusted to 9–10 with Na_2CO_3 (Aunstrup *et al.*, 1973).
Bacillus amylase:
4. Corn starch 40, ground corn (hominy) 100, corn steep liquor 65 (Smythe *et al.*, 1950).
5. Potato starch 100, ground barley 50, soybean meal 20, sodium caseinate 10, $Na_2HPO_4 \cdot 12H_2O$ 9 (Outtrup *et al.*, 1972).
Fungal protease:
6. Corn starch 30, corn steep liquor 5, soybean meal 10, casein 12, gelatin 5, distillers dried soluble 5, KH_2PO_4 2.4, $NaNo_3$ 1, NH_4Cl 1, $FeSO_4$ 0.01 (Lehmann *et al.*, 1977).
Fungal amylase:
7. Corn starch 24, corn steep liquor 36, NaH_2PO_4 47, $CaCl_2$ 1, KCl 0.2, $MgCl_2 \cdot 6H_2O$ 0.2 (Yamada and Tomoda, 1966).
Glucoamylase:
8. Corn starch 150, corn steep liquor 20, pH adjusted with gaseous NH_3 (Dworschak and Nelson, 1973).
Semisolid culture (composition in parts)
Lactase:
9. Wheat bran 100, 0.2 *N* HCl (containing traces of Zn, Fe, and Cu) 60 (Cayle, 1971).
Lipase:
10. Wheat bran 3, soybean meal 1, water 3 (Smythe and Drake, 1949).

The medium composition should be balanced so that the pH does not exceed the accepted limits during fermentation. This can be achieved by the addition of a buffer system such as phosphates or $CaCO_3$ to the medium. Another method is to add substances whose metabolism gives rise to a change in pH, such as salts of organic acids and nitrates that will raise the pH, or ammonium salts that will tend to lower the pH.

Glucose represses the formation of some enzymes, e.g., α-amylase. In these instances, it is important that the glucose concentration of the medium be close to zero at all times. This may be achieved by using slowly decomposable carbohydrates such as starch or lactose or by adding the glucose slowly during fermentation. The advantage of the latter method is that it allows the addition of large quantities of carbohydrates to the medium without producing a large concentration of dry substances which might tend to inhibit or retard growth.

Some enzymes, e.g., pectinase and lactase, require inducers for high-yield formation. These inducers—normally the enzyme substrate or their hydrolysates if the substrate is a polymer—must be added to the medium. If the inducer is expensive or unpleasant to work with, it is usually preferable to develop a mutant of the production strain that does not require the inducer.

The composition of the medium should not only be based on the requirement of the fermentation process, but the subsequent purification step must also be taken into consideration. It is important, therefore, that the composition of the medium be such that at the end of fermentation, total dry substance content and viscosity are low, the cell mass easily separable, and the content of free carbohydrate and amino acids in the liquid at a minimum.

Strict aseptic conditions are necessary in order to obtain a high enzyme yield and to ensure that no toxic substances or harmful microorganisms are introduced into the product. Aseptic conditions are particularly difficult to maintain in many enzyme fermentations performed at close to neutral pH and in rich media without any protecting antibiotic activity. This means that the equipment used must be of high standard and good steam sterilization of the medium is necessary.

All enzyme fermentations are aerobic, but in many instances they take place under oxygen-limiting conditions, e.g., amyloglucosidase (Aunstrup, 1977). In some of these cases the oxygen limitation appears to be an advantage, and an increased aeration rate will reduce the yield.

The composition of the broth at the end of fermentation depends on the initial medium composition. In percentage of initial dry matter

content of the medium, a typical composition is enzyme protein 1–5, residual nutrient and metabolites 5–10, cell mass 2–10.

C. Recovery and Finishing

The purpose of the recovery process is to prepare a finished product of satisfactory purity and stability in as few steps as possible and with a minimum loss of enzyme activity.

1. Pretreatment

An important point in the recovery process is to prevent introduction of toxic materials or harmful microorganisms into the product. When the broth leaves the fermenter, aseptic conditions can no longer be maintained; and, as the broth is a good substrate for a variety of microorganisms, precautions to avoid contamination must be taken. This is done by cooling the broth to about 5°C as quickly as possible, e.g., in a counter-current heat exchanger, and by using high-quality standard dairy equipment throughout the process. Furthermore, scrupulous cleanliness is maintained in the recovery plant. The use of chemical preservatives is normally not acceptable at this stage, first, because the high cell density requires a high dosage for the substance to become active, and second, because most of the effective preservatives are undesirable or prohibited in the final enzyme preparation if it is to be used as a food additive. The fermentation broth contains the enzymes in soluble form mixed with solids from the medium components and bacterial cells, fungal mycelium, or their decomposition products.

The solids are removed by filtration or centrifugation. They are often of colloidal nature and difficult to remove directly, in which case coagulating or flocculating agents may be added. An old device, which is often useful, is to precipitate calcium sulfate or calcium phosphate in the broth. Usually, it is more efficient to employ some of the flocculating agents which have been developed in recent years, e.g., for water treatment. They are polyelectrolytes such as polyamines, and the treatment may involve reaction with electrolytes of opposite charge, such as aluminum salts. In some cases it may also be necessary to add filter aid, e.g., in the form of diatomaceous earth before filtration.

The separation process is done on drum filters or leaf filters; if centrifugation is preferred, high-speed disk centrifuges are used. In many cases it is advantageous to mature the broth before separation, i.e., to let the broth set for some time so that the colloids aggregate and thus ease the separation process. However, economy and contamination risk limit this kind of operation.

2. *Purification*

There are various ways to prepare a commercial product from the pretreated broth. The most common methods are shown in Fig. 2.

The first step usually is to concentrate the enzyme by evaporation or ultrafiltration. Evaporation is performed in traditional or custom-built multistage vacuum evaporators. The liquid must be kept at a low temperature (30°–50°C). Nevertheless it is difficult to prevent activity losses of ~10% even for very stable enzymes. An additional disadvantage of this method is that the low temperature makes microbial growth possible, so that frequent cleaning of equipment and careful control of the product are necessary. Another problem is that the dry-substance content of the broth increases with evaporation, in some cases causing high viscosity and difficult handling.

In these instances ultrafiltration is an advantage. This technique has been developed to a high degree in recent years. Plant-scale equipment is readily available, the process is inexpensive, and it offers the additional advantage that substances of molecular weight below 10,000 are removed from the concentrate. Furthermore, the process can be performed at a low temperature (5°C), thus keeping the activity loss and contamination risk at a minimum. The disadvantages of this method are that the precipitate formed during the concentration process tends to clog the membranes. These membranes are often made of cellulose derivatives which are attacked by the cellulase enzymes present in many fermentation broths of fungal origin. In such instances other membrane types should be used, e.g., composite membranes based on polysulfones on a carrier fabric.

The concentrated enzyme solution is usually turbid due to precipitates formed in the concentration process. It also contains varying amounts of bacteria or other microorganisms. These are removed by a germ filtration process. Because of the small capacity of the germ filters, the process must be preceded by a polishing filtration. However, it should be noted that the previously very popular asbestos-based germ filters now have a limited application because of the health hazards of asbestos fibers. Instead, cellulose filters or membrane filters may be used.

Before the product is ready for sale, the clear filtrate must be mixed with stabilizers and preservatives, and the activity must be standardized to the specified value. Stabilizers are used to increase the storage stability of the enzyme preparation. Their composition varies from one enzyme to the next. Useful agents are salts, proteins, starch hydrolysates, and sugar alcohols.

The number of permitted preservatives is very limited because they should be both enzyme-tolerant and approved food-additive sub-

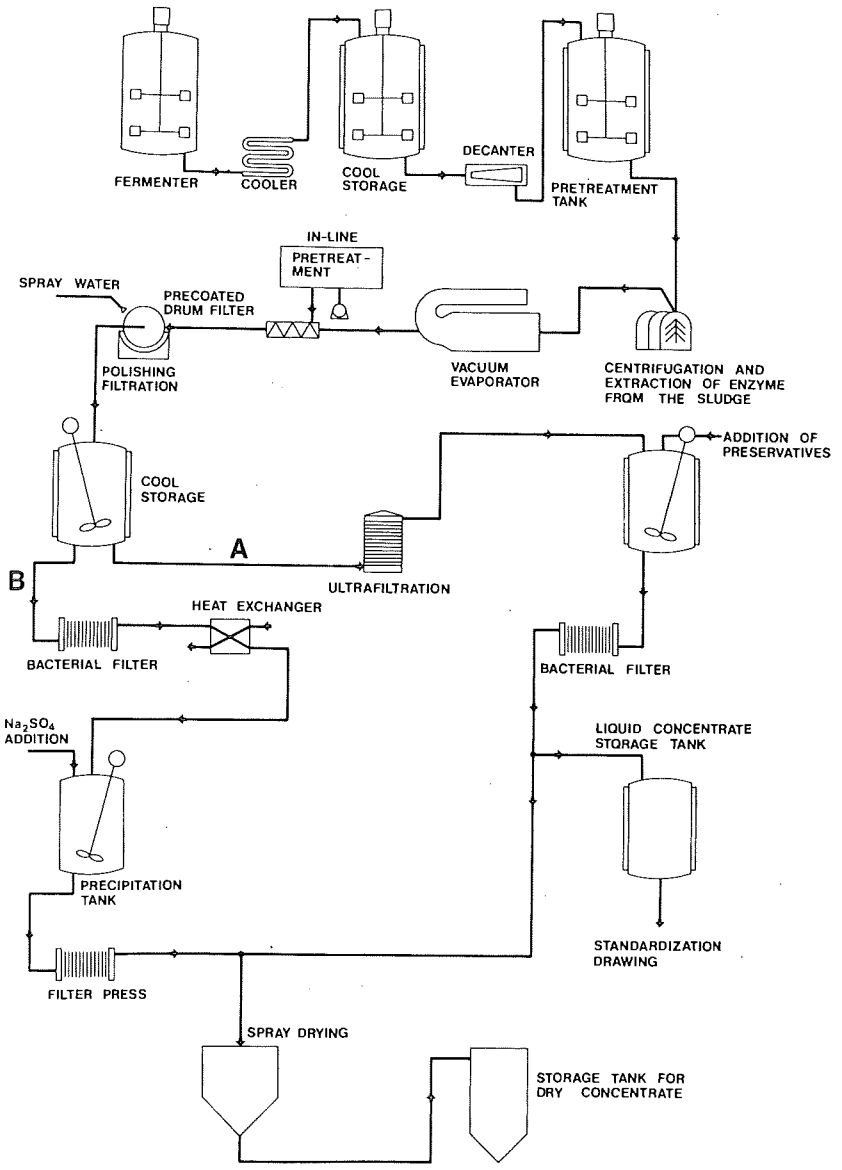

Fig. 2. Examples of enzyme recovery. (Courtesy of T. K. Nielsen, Novo Industri.)

stances. One of the best preservatives is NaCl in a concentration of 18–20%. Other, less efficient, agents are benzoate, parabene, or sorbate. Standardization and testing of the final preparation constitute an important part of the manufacturing process. Details follow later.

Liquid enzyme preparations like the one described are preferred because they are relatively inexpensive to make and safe and easy to use in most applications. In some cases, however, solid enzyme preparations must be used; e.g., for flour treatment or in granulated detergents.

To make a solid enzyme preparation, the simplest method is to spray-dry the clear concentrate. For good results, it is important that the concentration of low-molecular-weight substances, which would tend to make the product sticky or hygroscopic, be low. Consequently, preparations concentrated by ultrafiltration are better suited for spray-drying than those concentrated by evaporation. Spray-drying has some other disadvantages: the process is expensive (about 5–10¢/liter of water removed), the enzymes are subjected to fairly high temperatures and oxidizing conditions, impurities are not removed in the process, and finally the product has a low weight/volume ratio. An advantage is that spray-dried preparations usually are easily soluble.

Precipitation is preferred when some purification is desired in the process. Two methods are used by the industry: solvent precipitation and salt precipitation. Both methods have advantages and drawbacks. Salt precipitation is traditionally done with ammonium sulfate. Since this, however, is not acceptable in detergents, enzymes for this purpose are made with sodium sulfate. The method used is simple. Salt in dry form is added to the clear enzyme solution until the required concentration is obtained, and the precipitate is removed by filtration and dried.

For economic and environmental reasons the salts present in the supernatant are regenerated. Regeneration takes place in plants specially designed for recovery and crystallization of the salt. In this way it is possible to keep the costs of the salt precipitation process at a fairly low level. The advantage of the salt precipitation process is the rather small investment required and the good solubility of the product. The disadvantages are the high salt concentration present in the product, less efficient removal of impurities than in the solvent precipitation process, and the difficulty of maintaining aseptic conditions during the process.

Solvent precipitation may be performed with ethanol, acetone, or other water-soluble solvents. The solvent is added to the enzyme solution in the proper concentration and the precipitate is filtered off and dried. The supernatant solvent is purified and recovered by distillation.

This process is advantageous in that it results in a product of high purity and high activity. The product may be more difficult to dissolve than the salt-precipitated product. Investments in explosion-proof

equipment and recovery equipment are high, and the solvent loss must be kept at a very low level to operate economically.

The precipitation processes may be improved by various modifications such as fractional precipitation or multiple precipitation with intermediate purification steps. Precipitation with tannin, lignin, lignosulfonic acid, and other materials has been described in the literature, but these processes have had limited use.

In several instances, the purification process includes a step that removes an undesirable side effect. In principle, this can be done by any selective method of separation of inactivation. The problem is that removal of the undesirable component must be completed without substantial loss of the main component. An example of such a process is the removal of lipase in the production of microbial rennet by *M. miehei*. The lipase activity present in the broth after fermentation is reduced to less than 10% by leaving the broth at a pH below 3.5 for a few hours. The loss of rennet activity is insignificant (Schleich, 1971).

Another example is the removal of transglucosidase activity from glucoamylase preparations. This has been a serious problem in industry, and over the years many patent applications have been filed on methods to solve the problem. A number of methods are listed in Table VIII.

TABLE VIII
PATENTED METHODS FOR THE REMOVAL OF TRANSGLUCOSIDASE FROM GLUCOAMYLASE PRODUCTS

Year	U.S. Patent No.	Principle
1962	3,042,584	Adsorption on clay mineral
1962	3,047,471	Precipitation with lignin and tannic acid
1962	3,067,108	Precipitation with sulfonated compounds
1963	3,101,302	Adsorption to clay mineral with solvent
1963	3,108,928	Treatment at pH 9–10 and 22–55°C
1964	3,117,063	Precipitation with lignin or tannic acid and sulfonated compounds
1964	3,134,723	Precipitation with Ba, Ca, Sr, Cd, Pb, Mn, or Zn at pH 4.5–8.5
1965	3,268,417	Treatment with protease at pH 6–9
1967	3,303,107	Treatment at pH 1–3 and temperatures up to 70°C
1968	3,380,891	Treatment with sulfonated fatty acids at pH 1.5–4
1968	3,380,892	Treatment with cation-forming precipitate with oxalic acid at pH 1.5–3
1969	3,483,084	Treatment with chloroform
1969	3,483,085	Treatment with hetero poly(acid)s such as phosphomolybdic acid

3. *Finishing of Solid Enzyme Preparations*

The dried preparations from salt or solvent precipitation form large lumps which are unattractive to sell. The simplest way to prepare a marketable product is to grind the preparation to a fine powder in a mill and standardize the activity by the addition of inert substances such as salt, lactose, or other suitable substances. In the preparation of enzymes for flour treatment, standardization is usually done with flour.

Such a preparation will be satisfactory in many ways but it has one disadvantage: the particles are very small and the dust formation by handling will be relatively high. Since exposure to enzyme dust may cause allergic reactions, it is preferable to make the enzyme preparations in a way that minimizes the dust-forming ability. A number of such methods have been developed. The simplest one is admixture of dedusting compounds such as polyethylene glycol; another method is granulation with inorganic salt, e.g., by fluid bed agglomeration. The methods preferred at present involve granulation and coating with inert wax. In the so-called prilling process the enzyme is mixed with melted wax and spray-cooled. This forms granules of about 0.5–0.8 mm diameter in which the enzyme particles are uniformly distributed.

An improved process is shown in Fig. 3. The enzyme in this process is mixed with an inert filler (e.g., salt), a binder (e.g., carboxymethylcellulose), and water to make a paste. The paste is then extruded and shaped into spheres in a so-called marumerizer. After drying, the spheres are coated with a layer of wax material. A uniform particle size, e.g., 0.5–2 mm diameter, is obtained in this way, and dust formation is insignificant.

4. *Immobilization of Extracellular Enzymes*

This chapter will not describe the many methods developed to immobilize enzymes. Reference is made to Volume 1 of this series.

V. STANDARDIZATION AND CONTROL

The final step of the manufacturing process is standardization of the enzyme activity and control of other properties of the product. Manufacturers and customers have a common interest in the reliable performance of these tests, both for economic reasons and to ensure safe use of the product.

The number of tests has grown considerably in the last few years and will probably increase much more in the future. The manufac-

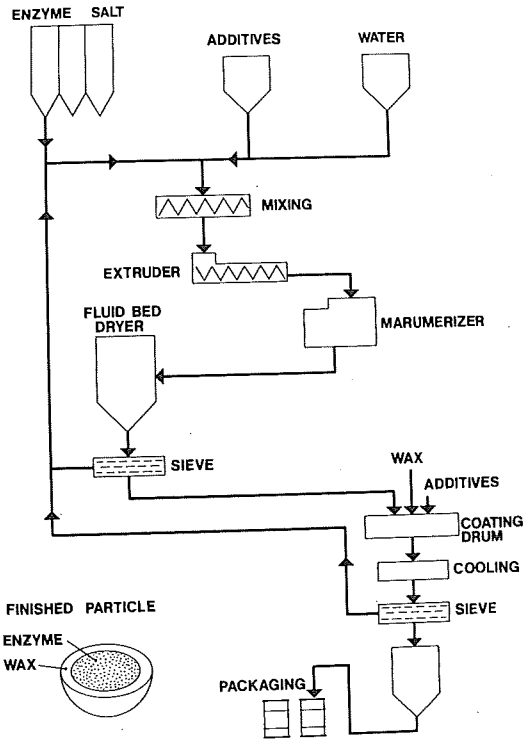

Fig. 3. Preparation of dust-free enzyme. (Courtesy of T. K. Nielsen, Novo Industri.)

turer, therefore, has to reckon with a substantial expense for this part of the manufacturing process, and he has to invest in extensive facilities for control laboratories.

A. Standardization

In principle, enzyme preparations are always sold on an activity basis. The manufacturer will promise or guarantee a certain effect in application, and the customer expects this effect to take place when he uses the product. He will also expect properties to be the same from batch to batch. To ensure that the enzyme concentration is maintained at the desired level, the manufacturer must determine the enzyme activity and standardize the product with inert material. Accurate and reliable analytical methods are necessary, and means are required to ensure that the activity level does not change in time.

The Commission on Biochemical Nomenclature has recommended defining enzyme activity as reaction rate, 1 catal being the amount of

enzyme which converts 1 mole of substrate per second. This definition can only be used if the substrate is well defined and the enzyme reaction known. Industrial enzymes, as a rule, will react with variable, natural substrates, and the enzyme reaction is often not known in detail. It has been necessary, therefore, to use other ways to define the enzyme activity. The following example illustrates this.

A glucoamylase unit may be defined as the amount of enzyme that hydrolyzes starch at such a rate that 1 mole of glucose per second is released under specified reaction conditions. The method will give reliable and reproducible results as long as the same batch of starch and enzyme is used. However, experience proves that the reaction rate depends on the starch quality, and the test is sensitive to the presence of α-amylase, a side activity of all glucoamylase preparations. Consequently, this method cannot be employed for standardization. A simple, well-defined substrate like maltose, which is not hydrolyzed by α-amylase, can solve the problem. This method also has drawbacks: pure maltose is difficult to obtain and maltose preparations are often contaminated by maltotriose which is hydrolyzed at a higher rate. Furthermore, glucoamylase is not used for maltose hydrolysis; hence the method cannot be applied in comparing glucoamylases of *Aspergillus* and *Rhizopus*, which have different ratios between hydrolysis of starch and maltose.

The problems described are simple compared with the analytical difficulties of other important enzymes, such as milk-coagulating enzymes, proteases, and pectinases. In all cases, the substrate is variable, side activities are important, and enzyme products of different origin do not react in the same way to changes in reaction conditions.

It is unlikely that standard methods applicable to all enzyme preparations and with an absolute unit definition will be developed. Instead, enzyme manufacturers must develop their own analytical methods based on reactions which they believe to be reliable and relevant to the application of their enzyme. To overcome the variation in substrate, it is customary to introduce enzyme standards in the analytical methods and to adjust the result accordingly. In this way it is possible in most cases to standardize the enzyme preparations with a variance of less than 10%.

B. Control

New enzyme products must be thoroughly tested to ensure their safety in the application intended. For use in food production new enzymes must be approved by relevant government bodies; in the United States it is the Food and Drug Administration (FDA). The

production process must comply with "good manufacturing practices," and a representative sample of the product is put through thorough toxicological tests. This is an expensive and time-consuming process (2–5 years). Requirements of other applications vary, but enzymes such as detergent enzymes that may come into contact with consumers must undergo a test program as rigid as that for food enzymes.

Routine control of enzyme products involves the following:

- tests appropriate to the application, such as the absence of transglucosidase in glucoamylase or the determination of the protease level in amylase;
- storage stability tests and analysis of the concentration of preservatives and stabilizers such as Ca^{2+};
- appearance tests of color, odor, amount of precipitate, and particle size;
- tests for undesirable impurities such as heavy metals, mycotoxins, and antibiotic activity;
- determination of enzyme dust level in granulated enzyme preparations;
- microbiological tests which usually involve a total viable count and tests for absence of pathogens or indicator organisms such as coliforms. A test for absence of production organism is generally included.

VI. PROTEOLYTIC ENZYMES

Extracellular proteases are probably the most widespread microbial enzymes. They are easy to detect and isolate, and they often occur in large concentrations. Consequently, they have been popular research objects, and there is an extensive literature on production and properties of proteases from various microorganisms.

Proteases with a wide variety of specificities are known, but very few are produced industrially. The most important are the alkaline serine proteases and neutral metalloproteases, which are formed by *Bacillus* species and the acid proteases from *Aspergillus* and *Mucor*.

A. Serine Proteases

Serine proteases owe their name to the fact that serine is an essential amino acid of the active center. They are endoproteases, and they have an alkaline pH optimum. The microbial serine proteases are usually very stable and have a strong proteolytic activity with a low specificity. Serine proteases are inhibited by diisopropyl fluorophosphate or phenylmethylsulfonyl fluoride.

1. Subtilisin Carlsberg

This enzyme was discovered in 1947 by Linderstrom Lang and Ottesen at the Carlsberg Laboratory. Subsequent work at this laboratory and by others has resulted in an extensive knowledge about the composition and properties of the enzyme. The amino acid sequence and tertiary structure of the enzyme are known.

The commercial utilization of the enzyme started in 1960 when it was discovered that the enzyme had excellent properties for use in detergents. Subtilisin Carlsberg is now the most widely used detergent protease.

Subtilisin Carlsberg is produced by *B. licheniformis*. Due to the uncertainty of *Bacillus* taxonomy at the time the enzyme was discovered, the producing organism was named *B. subtilis*. Unfortunately, this name is still erroneously used by many authors.

a. Production Method: A simple and good laboratory method for production of the enzyme was developed at an early stage (Güntelberg, 1954). The organism was grown on glucose, casein hydrolysate, and salts; and it was demonstrated that yields increased if glucose was fed during the fermentation. The organism has no growth requirements, but it is necessary to include organic nitrogen sources in the medium to obtain a high yield. Many strains of *B. licheniformis* will produce protease in high yields, for instance, the strain NRRL B 3723 (Feldman, 1971).

In commercial production the organism is grown at temperatures between 30° and 40°C and at neutral pH. A concentrated medium with a high content of protein or protein hydrolysate is used (Table VII), and it may be an advantage to feed carbohydrate during the fermentation.

The enzyme production starts when the maximum cell count is reached after 10–20 hr of growth and continues at an almost constant rate throughout the fermentation period if the fermentation is run properly. Laboratory experiments with production in continuous fermentation have been reported (Jensen, 1972). The continuous production process does not present special problems; whether it is feasible or not is entirely a question of economy.

When starch hydrolysates are used as a carbon source the α-amylase and α-glucosidase of *B. licheniformis* hydrolyze both α-1,4- and α-1,6-glucosidic bonds so that the starch is completely metabolized. However, the level of these enzymes is usually insignificant in the broth. The protease hydrolyzes all proteins of the medium, and at the end of the fermentation period the protease is practically the only protein dissolved in the broth. The yield is usually high. It is possible

to obtain a yield of enzyme protein of over 10% of the initial protein content of the medium.

The enzyme is primarily sold in the form of dust-free granulates for detergent use. Commercial granulates contain 1–5% active enzyme protein. The enzyme is also stable in liquid form, and the enzyme content of liquid preparations is about 2%.

b. Properties and Application: Subtilisin Carlsberg is an alkaline protease (pH optimum in hydrolysis of casein about 10). Over 80% of the activity is maintained in the pH range 8–11. The enzyme is stable at pH 5–10 at 25°C, and up to ~50°C for 1 hr at pH 8.5. The enzyme does not depend on Ca^{2+} for stability and will maintain stability in the presence of sequestering agents such as tripolyphosphates and ethylenediaminetetraacetic acid. Inactivation of the enzyme is rapid at pH values below 4 or above 11.5 and at temperatures above 70°C. Oxidizing agents such as hypochlorite and hydrogen peroxide destroy the enzyme rapidly, but stabilized hydrogen peroxide, such as in sodium perborate, does not harm the enzyme activity. The specificity is broad; only 30–35% of the peptide bonds in casein will not be hydrolyzed by the enzyme.

Subtilisin Carlsberg has performed excellently in detergents for many years. The optimal enzyme concentration in most detergents is ~0.015–0.025% active enzyme protein. The pH of the suds is usually ~9, which is close to the optimum of the enzyme. The proteolytic action prevails during the washing process up to 55°–60°C. In this temperature range heat inactivation sets in, and it coincides with the temperature at which H_2O_2 is released in perborate-containing detergents.

Proteases are obviously useful for washing of clothes stained with blood or other proteinaceous matter. It has also been proven that proteinases improve the general washing efficacy because proteinaceous impurities make dirt adhere to the fabric.

The use of detergent enzymes depends on washing habits. They are more widely used in Europe where perborate and high-temperature wash bring out the advantages of detergent enzymes. Because Subtilisin Carlsberg is a durable and inexpensive protease, it may be used in many fields where proteolysis under alkaline conditions is needed, for instance, in preparation of protein hydrolysates for food or feed purposes.

2. *Subtilisin Novo (Subtilisin BPN)*

Subtilisin Novo or Subtilisin BPN is produced by *Bacillus amyloliquefaciens*. This new species is separated from *B. subtilis* (Welker

and Campbell, 1967), because the strains used for industrial enzyme production have many properties in common, primarily the properties of their extracellular enzymes which were different from those of the neotype of *B. subtilis,* the Marburg strain.

B. amyloliquefaciens has been used for industrial enzyme production for over 50 years. Practically all protease preparations of bacterial origin produced before 1960 were made from this organism. At present, Subtilisin Novo is only used to a minor extent. It is the proteolytic side activity of the amylase preparations that are used for some detergents.

a. Production Methods: Most strains of *B. amyloliquefaciens* will produce protease in good yields. Welker and Campbell (1967) have listed a number of strains that are suitable for production of the enzyme. Medium and growth conditions are similar to those used for *B. licheniformis,* but the protease yield is usually somewhat lower.

During fermentation several other extracellular enzymes will be produced; primarily α-amylase, β-glucanase, neutral proteinase, and hemicellulase. The latter two enzymes are unstable and are usually present in low concentrations in the final preparation if no special precautions are taken.

Methods for removal of the α-amylase activity by fractional precipitation with calcium acetate and solvents (Keay and Anberson, 1971) or by oxidation with hypochlorite (Hoerle, 1976) have been described but have found only limited commercial application. Commercial preparations are usually made in liquid form, as dedusted powders or dust-free granulates for detergents. As a rule, they contain large amounts of α-amylase. The content of Subtilisin Novo is generally less than 1%.

b. Properties and Application: Subtilisin Novo is closely related to Subtilisin Carlsberg, and this is reflected in the stability and activity. In short, the temperature and pH range is a little narrower for Subtilisin Novo than for Subtilisin Carlsberg. The enzyme has a limited application—mainly in combination with α-amylase in detergents.

3. *Proteases from Alkalophilic Bacillus Species*

In 1967, it was discovered that *Bacillus* strains that grow at pH values over 10 produce proteolytic enzymes that are active and stable at pH values up to 12 (Aunstrup *et al.,* 1972). A large number of strains were isolated and several different proteases were found. All were serine proteases, and, apart from their better stability at high pH values, the properties were similar to the Subtilisin-type proteases. The molecular basis of the good alkali stability has not been subjected to

intensive studies, but it is characteristic that the isoelectric points of these enzymes are around pH 11 (as compared to pH 9.7 for Subtilisin Carlsberg).

a. Production Methods: Preparation methods for the proteases have been described in several patents (Aunstrup *et al.*, 1973; Horikoshi and Ikeda, 1977), in which suitable strains are also mentioned. The production methods are similar to those used for making Subtilisins, but the fermentation process is characteristic in that pH must be kept above 7.5 at all times. Otherwise the culture will die and lyse.

The high pH may be obtained by addition of alkali such as sodium carbonate, or the medium may be prepared with salts of metabolizable acids such as lactates. Some of the strains are amylolytic so that starch hydrolysates may be used as the carbon source. The strains may secrete small amounts of other alkalophilic enzymes, but the proteases will usually constitute over 90% of the enzyme content of the broth.

The enzymes are prepared in granulated form for detergents and as dedusted powder for dehairing. The protease content of the preparations is generally from 1 to 2% active enzyme protein.

b. Properties and Application: The new proteases are stable and active in the pH range 6–12 and may be used at temperatures up to 60°C. In detergents they are generally superior to Subtilisin Carlsberg. This is particularly pronounced if the alkalinity is high or the traditional sequestering agent, tripolyphosphate, is replaced by other sequestering agents such as citrate or gluconate.

The proteases are very useful in the dehairing process. It is customary to swell the hides with lime and loosen the hair with sodium sulfide. This process is effective, but highly undesirable for ecological reasons and because of the risk involved in handling sulfides. A satisfactory result may be achieved when these alkaline proteases are used in combination with lime. However, the application has so far been limited mainly for economic reasons.

B. Metalloproteases

The metalloproteases or neutral proteases have received much less attention than the Subtilisins, scientifically as well as commercially, although they too are widely distributed in nature. The metalloproteases are endoproteases: Zn^{2+} is essential to activity, and Ca^{2+} is important in maintaining stability. Sequestering agents such as EDTA inhibit the enzymes. The pH optimum is close to neutral, and stability in general is not as good as in the serine proteases. Metalloproteases are produced by several *Bacillus* species, such as *B. amyloliquefaciens, B. cereus,* and *B. polymyxa. B. thermoproteolyticus* produces

a remarkable protease (Thermolysin) with a high thermostability (up to 80°C). This enzyme has been the subject of detailed investigations, and the amino acid sequence has been established.

a. Production Methods: The metalloprotease of *B. amyloliquefaciens* is formed together with α-amylase and alkaline protease in the normal fermentation process used for production of these enzymes (Keay *et al.*, 1972). NRRL B-3411 is a suitable strain. To prepare the enzyme without interfering enzymes, mutants free from alkaline protease may be used (Murray and Prince, 1970), but methods for fractionation in good yields have also been developed (Keay *et al.*, 1972). Since the metalloprotease is unstable and may be destroyed during fermentation, a short fermentation time is necessary to obtain maximum yields of this enzyme. Thermolysin is prepared in a high-temperature (55°C) fermentation with a fermentation time of about 24 hr (Endo, 1962).

b. Application: Metalloproteases from several *Bacillus* species have been developed industrially for use as rennet substitutes (Murray and Prince, 1970), but the application has been abandoned as it was unsuccessful due to excessive casein hydrolysis. At present metalloproteases are used for bating in tanneries, to hydrolyze barley proteins in breweries, and for proteolysis in the food industry.

C. Acid Proteases

All microbial acid proteases are of fungal origin. Aspartic acid is present in the active center, and there is a considerable homology with the acid proteases of animal origin.

1. Mucor Proteases

As early as 1921 a milk-coagulating protease was made from *Mucor rouxii* (Kohman *et al.*, 1927). It was not successful because the milk-coagulating activity relative to the proteolytic activity was too low. Since then numerous attempts have been made to find a good milk-coagulating enzyme. In a large screening program performed in the early 1960s, Arima and his co-workers (Arima *et al.*, 1968) succeeded in finding a strain of thermophilic *Mucor* which produced a satisfactory enzyme. The strain belonged to the species *M. pusillus*, and the enzyme formed had a higher milk-coagulation-to-protease ratio than any other microbial protease known. The enzyme was subsequently found to give excellent results in actual cheese-making trials. In 1965, it was discovered (Aunstrup, 1968) that the related species *M. miehei* forms a similar enzyme of equally good milk-coagulating properties. Both enzymes are now used as milk coagulants.

M. pusillus and *M. miehei* are thermophiles. The growth temperature range is 20–55°C for *M. pusillus* and 30–60°C for *M. miehei.* A number of morphological and biochemical features distinguish the two species (Cooney and Emerson, 1964). The fact that the two proteases are distinctly different in composition and action, although they belong to the same group of proteases, is of particular interest. It is also interesting that only the *M. miehei* enzyme is a glucoprotein and it is more heat-stable than the *M. pusillus* enzyme.

a. Production Methods: Despite their similarities, the two species require widely different production methods.

M. pusillus is cultivated on a semisolid medium consisting of 60% wheat bran and water for 3 days at 30°C. The enzyme is then extracted with water. The yield is approximately 3200 Soxhlet units per gram of wheat bran (Arima, 1964). Ammonium salts added to the bran will improve the yield.

M. miehei is grown in submerged culture, e.g., in a medium containing 4% starch, 3% soybean meal, 10% ground barley, and 0.5% $CaCO_3$ for 7 days at 30°C. The yield reported corresponds to approximately 3500 Soxhlet units per milliliter of broth (Aunstrup, 1968). A suitable strain is CBS 370.65.

During cultivation the organisms will secrete other enzymes such as lipase, esterase, amylase, and cellulase. If autolysis of the mycelium occurs during fermentation, intracellular protease will leak out. This enzyme has a low milk-coagulating activity, and its presence in the preparation should be avoided. The amylase is only present in small amounts and it is of no significance to the application. Lipase and esterase are usually undesirable in the final preparation.

In the recovery process, the mycelium is first removed by filtration and a concentrated liquid product is prepared by vacuum evaporation or reverse osmosis. Finally, sodium chloride in a concentration of about 20% is added as a preservative. To refine the process, a step for removal of the lipase is usually introduced. This may be done by treatment at pH values between 2 and 3.5 for a short time. If nonspecific protease occurs in the broth, it may be removed by adsorption to silicium dioxide at pH 5. (Moelker and Mattijsen, 1967).

Usually these enzymes are marketed in liquid form; however, solid preparations are used in some areas. They are made by solvent precipitation or direct spray-drying of the purified broth.

b. Application: The commercial preparations are sold in concentrations ranging from 10,000 to 150,000 Soxhlet units. Since the specific activity of the enzyme is approximately 5 million Soxhlet units, the concentration of active enzyme protein in the preparation is about 0.2–3%.

When *Mucor* protease is applied to coagulate milk, a number of parameters influence the activity in a way different from that of calf rennet. There is also a difference between enzymes from *M. pusillus* and *M. miehei.* The variation in temperature, Ca^{2+} concentration and pH are of special importance. By adjusting the reaction conditions during milk coagulation, it is possible to produce cheese of a quality similar to cheese made with calf rennet with both *Mucor* proteases.

2. *Protease from Endothia parasitica*

This enzyme was discovered in 1963 by Sardinas. The species, which is pathogenic to chestnut trees, is apparently the only one of the genus that forms this type of protease (Sardinas, 1966).

a. Production Methods: A suitable strain, e.g., ATCC 14.729, is grown in submerged culture in a medium composed of 3% soybean meal, 1% glucose, 0.3% $NaNO_3$, 1% skim milk, 0.05% KH_2PO_4, and 0.025% $MgSO_4 \cdot 7H_2O$. Fermentation takes place at 28°C and pH 6–7. After 48 hr an enzyme activity of about 2000 Soxhlet units is obtained.

Due to the instability of the enzyme, recovery must take place quickly, at low temperature, and preferably without oxygen. After the mycelium is removed by filtration, the broth is concentrated and the enzyme precipitated by solvent. This enzyme is always marketed as a solid preparation.

b. Application: The milk-coagulating activity is less dependent on pH variation in milk than calf rennet, and the dependence on variation in Ca^{2+} concentration follows that of calf rennet. The thermostability is low; the enzyme is inactivated in less than 5 min at 60°C. It has found limited use as milk coagulant mainly because of its high proteolytic activity. Only in the production of Emmenthal cheese has it proved superior to the *Mucor* proteases. The reason is that this cheese undergoes a high-temperature treatment where the protease is rapidly inactivated. There have been no reports on the presence of lipase or other undesirable enzyme activities in this preparation.

3. *Aspergillus Proteases*

The most important application of *Aspergillus* proteases is for production of soy sauce, of which consumption in Japan alone is over 10^9 liters yearly. In this process bran cultures of *A. oryzae* and the closely related *Aspergillus sojae* are used to hydrolyze the soybean proteins almost completely to amino acids. The extensive hydrolysis is possible because these fungi produce several proteinases and exopeptidases.

Commercial protease preparations made from *A. oryzae* contain acid, neutral, and alkaline proteases. Several components of each type

have been isolated. As a result, the preparations show proteolytic activity in the pH range 4–11. Commercial proteases are also made from black *Aspergillus* species, primarily *A. phoenicis* (syn. *A. saitoi*) and *A. niger* var. *Macrosporus*. These organisms produce acid proteases only, and they are active in the pH range 2–6.

a. Method of Production: The *Aspergillus* proteases can be produced in high yield only by semisolid cultivation. Wheat bran or rice bran is used, and it has been reported that a high ratio of inorganic nitrogen to carbon in the medium is important to obtain good yields (Yoshida and Ichishima, 1964).

A. oryzae NRRL 2160, *A. saitoi* ATCC 14.332, and *A. niger* ATCC 16.513 are useful strains for production of protease. During cultivation several other enzymes are formed in high concentrations: α-amylase, glucoamylase, cellulase, and pectinase. Usually, all these enzymes will be present in the final product unless it is highly purified.

The usual recovery method involves extraction of the bran with water and precipitation of the extract with solvent. More refined, laboratory purification methods are used to make the highly purified preparations used for certain medical purposes. The *Aspergillus* proteases are marketed in solid form.

b. Application: Aspergillus proteases are primarily used in digestive aids, where the acid pH-optimum and the large number of concomitant enzyme activities are beneficial. The market for such preparations in Japan was 520 million yen in 1972 (Samejima, 1974).

In the United States *A. oryzae* is used extensively for flour treatment. The protease exerts a gentle hydrolysis of the gluten, resulting in a considerably reduced mixing time of the dough and an improved bread quality. It is important that the protease be inactivated at a low temperature in the baking process so that extensive degradation of the gluten may be avoided.

Apart from flour treatment, *Aspergillus* proteases find limited application. They may be used in hydrolysis of meat or fish protein under acid conditions to aid the separation from fats, but for economic reasons this application is limited.

VII. AMYLOLYTIC ENZYMES

Starch is an important raw material for food, alcoholic beverages, and various other products. Several enzymatic methods are used to process starch, ranging from slight hydrolysis to reduce the viscosity of starch gels to complete hydrolysis to dextrose. Some of the most important processes and enzymes are summarized in Table IX.

TABLE IX
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
		B. licheniformis	5.5–9	110	DE 10–20[a]
Debranching	Pullulanase	*K. pneumoniae*	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 Crystalline dextrose
Isomerization	Glucose isomerase	(*Streptomyces* sp.) *B. coagulans* *Actinoplanes* sp.	6.5–8.5	60–65	Fructose syrup

[a] DE = dextrose equivalent.

The α-amylases catalyze a random hydrolysis of the α-1,4-bonds of the starch molecule. The result is a rapid decrease of viscosity and formation of dextrins and small amounts of glucose and maltose. Glucoamylase hydrolyzes from the nonreducing end of starch and dextrin molecules and splits off one glucose molecule at a time. α-1,6-Bonds are attacked but at a slower rate than the α-1,4-bonds. Pullulanase and isoamylase attack α-1,6-bonds of the starch or dextrin molecules.

A. Bacterial α-Amylase

The α-amylase from *B. amyloliquefaciens* was developed for industrial use by Boidin and Effront in 1913 (Boiden and Effront, 1917). The nomenclature of the producing organism has been somewhat uncertain. Initially it was called *B. mesentericus,* then *B. subtilis,* and now *B. amyloliquefaciens.* It is worth mentioning that the commercial amylase is different from the saccharifying amylase produced by the typical *B. subtilis,* the Marburg strain.

The α-amylase is quite heat-stable and may be used in starch hydrolysis up to 90°C. However, the starch industry was interested in a still higher hydrolysis temperature. That was made possible in 1972 by the introduction of an α-amylase made by *B. licheniformis.* This enzyme allows starch hydrolysis up to 105–110°C (Madsen *et al.,* 1973).

The *Bacillus* amylases are metalloenzymes. The *B. amyloliquefaciens* amylase consists of four subunits bound together by one zinc atom. The subunits are separable, and enzyme molecules with multiple subunits may be formed, but the four-subunit structure has the highest activity (Robyt, 1973).

Calcium ion stabilizes the enzymes and is customarily added to the reaction liquids. Amylase from *B. licheniformis* is less dependent upon Ca^{2+} stabilization as is the enzyme from *B. amyloliquefaciens.*

a. Production Methods: The *Bacillus* amylases are always prepared in submerged culture. The medium used is relatively rich and may contain ground grain such as corn and a protein source such as soybean meal or corn steep liquor. Additional carbohydrates may be added during the fermentation but this must be done carefully to avoid overdosage, as glucose represses the amylase formation.

Fermentation usually takes place at a temperature between 30° and 40°C, and pH is around neutral. If pH falls below 6, the amylase activity will be lost because of denaturation. In order to prevent this and to improve stability of the amylase, $CaCO_3$ may be added to the medium. The α-amylase formation starts as soon as the bacterial count approaches its maximum of 10^9 to 10^{10} cells/ml after about 10–20 hr. It

continues until the utilizable carbon source is exhausted—usually after 100–150 hr. It is interesting that *B. amyloliquefaciens* will utilize maltose and the lower saccharides up to a pentasaccharide at a very slow rate, whereas *B. licheniformis* utilizes these carbohydrates much faster. Apparently, these carbohydrates are not hydrolyzed by the α-amylase but have to be hydrolyzed by an α-glucosidase before they can be utilized. Both microorganisms possess this enzyme, but *B. amyloliquefaciens* has much less than *B. licheniformis*. The presence of this enzyme may also explain the transglucosylation reactions which have been observed in some crude α-amylase preparations. The α-glucosidases are unstable and are formed in small amounts; consequently, they are not present in the commercial products.

Both organisms form serine protease during cultivation. *B. licheniformis* does not form other extracellular enzymes in significant amounts, but *B. amyloliquefaciens* will form a neutral protease, β-glucanase, and hemicellulase. Of these, only the proteases are undesirable in starch hydrolysis because they catalyze the formation of melanin products.

Consequently, most commercial α-amylase products are now protease-free. This condition can be obtained in various ways. The simplest method is to heat the broth to a temperature at which the protease activity is destroyed but only a minimum of the amylase is lost. Another possibility is separation of the protease by adsorption to clay minerals (e.g., bentonite) or fractional precipitation. The advantage of the latter method is that the protease can be recovered. However, it is difficult to remove the protease activity completely in this way. The method is quite expensive because of the chemicals required and because some α-amylase activity is lost in the process. The most sophisticated solution to the problems is to use a mutant incapable of producing the proteases. Such mutants may cause problems in the preparation of the medium as it is necessary to use prehydrolyzed protein. The fermentation yield is decisive in the economy of the production process; high-yielding strains, therefore, are important. Outtrup reported a 25-fold increase in productivity of a *B. licheniformis* strain (Outtrup and Aunstrup, 1975) in six mutational steps. Bacterial α-amylases are usually marketed as liquid preparations which are preserved with 20% sodium chloride.

Activities of commercial preparations vary by a factor of 100 and are adjusted to the application in question. There is a tendency to produce more concentrated products because of freight and packing savings. The most active liquid preparations may contain 2% active amylase protein, and the most active solid preparations 5% active amylase protein.

b. Properties and Application: B. licheniformis amylase has a wider pH range than the *B. amyloliquefaciens* amylase. It is much more heat-stable and less dependent on Ca^{2+}. This enzyme has only two disadvantages: it is very difficult to inactivate by heat treatment, and it is more expensive if activities are compared at low temperatures. At the application temperature the *B. licheniformis* amylase, in fact, costs less than the *B. amyloliquefaciens* amylase.

The enzymes are not stable at pH values below 6, and their optimum activity is ~6.5–7. This means that the pH must be raised to this level in many applications (e.g., treatment of raw starch) where initial pH is low.

Bacterial α-amylases have two large fields of application. The earliest is desizing, for which amylase is used to remove starch sizes from the warp of cotton fabrics. The most important use is starch liquefaction in preparation of glucose syrups, dextrose, or fructose/glucose syrups. In both applications the *B. licheniformis* enzyme has proved superior to the *B. amyloliquefaciens* enzymes due to better heat stability.

Liquefaction of starchy raw materials for production of alcohol is another important application. Bacterial α-amylases have almost completely superseded the traditional malt in this process. The brewing industry also employs α-amylase with advantage in the liquefaction of various starch raw materials that are used as adjuncts.

B. Fungal α-Amylase

α-Amylase is the main component of the old enzyme preparation Taka-diastase prepared from *A. oryzae*. The enzyme is called Taka-amylase A by some authors. The molecule is different from the *Bacillus* amylase in a number of points: no subunits have been detected; it contains eight half-cystine groups and one SH group; it is a glycoprotein with 8 moles mannose, 1 mole xylose, and 2 moles hexosamine. Ten calcium ions are associated with the molecule, nine of which may be removed by dialysis. The molecular weight is ~51,000. *A. oryzae* amylase has pH optimum at 4.8–5.8, and it is less heat-stable than the *B. amyloliquefaciens* amylase.

α-Amylase is also produced by *A. niger*. The properties of this enzyme are similar to those of the *A. oryzae* enzymes, but some *A. niger* strains produce an additional acid amylase which is fairly stable down to pH 2 and somewhat more heat-stable. Despite the obvious practical advantages of this enzyme, it has found only limited application, probably because of low yield and, consequently, high price.

a. Production Method: The traditional production method is cultivation of the fungus on wheat bran. A number of enzymes apart from the

α-amylase are produced this way, but by adjusted medium composition and a selected strain it is possible to optimize the α-amylase production. Products made this way usually contain fairly high concentrations of proteinase. *A. oryzae* amylase may also be produced by cultivating a strain in submerged culture in media similar to those used to make *Bacillus* amylases. It is characteristic of this fermentation that the viscosity created by the mycelium is high, so that aeration and stirring become a problem. Glucose inhibits the amylase formation, and addition of glucose during fermentation must therefore be done with care. Amylase preparations made by submerged fermentation contain fewer other enzymes than preparations made in semisolid cultivation. Primarily, the protease content is much lower. Both preparations have little glucoamylase activity.

b. Properties and Applications: Aspergillus amylase is more saccharifying, i.e., produces more sugars, than do the equivalent *Bacillus* enzymes. It is possible to obtain over 50% of maltose when starch is hydrolyzed by this enzyme. Syrups of high maltose concentration are useful for a number of purposes where special functional properties are required.

Another important application of *A. oryzae* amylase is in the baking industry, where the enzyme is added to flour with a low amylase content. The amylase degrades starch in the dough, and the maltose formed serves as substrate for the baker's yeast during leavening. The low temperature stability of the *A. oryzae* amylase is important as it prevents extensive degradation of the crumb during baking.

C. Glucoamylase

Glucoamylases have been found in several genera of fungi, but only organisms belonging to *Aspergillus, Rhizopus,* and *Endomyces* have been used for commercial production. All three types of glucoamylases are capable of completely hydrolyzing starch to glucose. Enzymes from *Rhizopus* and *Endomyces* are somewhat less thermostable than the *Aspergillus* enzyme. The optimum temperature for starch hydrolysis is 60°C for the *Aspergillus* enzyme and 55°C for the other two enzymes. The difference is important because it is difficult to prevent microbial contamination in the hydrolysis process at temperatures below 60°C. Consequently, the *Aspergillus* enzyme is the preferred glucoamylase. The other two enzymes find limited use, primarily in the Soviet Union and Japan.

Glucoamylase is produced by strains of the *A. niger* group. Patent literature describes processes based on several species belonging to this group, e.g., *A. niger, A. awamori, A. phoenicis, A. diastaticus,* and

A. usamii. No comparative investigations have been published on the enzymes from these organisms, but the differences appear to be as small as the differences in morphology between the species. The following, therefore, describes the enzyme as *A. niger* glucoamylase.

The kinetic and molecular properties of *A. niger* glucoamylase have been studied in detail (Freedberg *et al.*, 1975), but a general agreement on the results has not yet been reached. At least two enzyme components are found in the cultured broth. They have similar activities except for the ability to attack raw starch and glycogen. The molecular weight is 60,000–100,000, and the molecules contain 13–18% carbohydrate. The optimum pH is 4.2–4.5, and the enzyme catalyzes the hydrolysis of α-1,4- and α-1,6-glucosidic bonds at a decreasing rate as the chain length decreases. The temperature stability is good up to 60°C at optimum pH.

a. Production Methods: The *Aspergillus* strain is grown in submerged culture in a rich medium containing a high concentration of starch. A good composition is 20% corn and 2.5% corn steep liquor (Smith and Frankiewies, 1975). The starch must be liquefied by a heat-stable bacterial α-amylase before sterilization. During the hydrolysis and in the initial stage of fermentation a large concentration of glucose is present in the medium. Unlike most other hydrolytic enzymes, formation of glucoamylase is not repressed by glucose. During fermentation pH drops to 3–4; it may be controlled by NaOH or by introduction of NH_3 into the aeration system (Dworschack and Nelson, 1972). Fermentation takes place at 30–35°C for about 4–5 days. During this time the starch is completely hydrolyzed, and the glucose formed is metabolized by the fungus. The metabolic products are mainly CO_2, but a significant amount remains in the broth in the form of organic acids and sugar alcohols. Minor side activities such as protease, cellulase, lactase, α-amylase, and transglucosidase are formed during fermentation.

The α-amylase activity will be denatured quickly in the broth if pH is below 4.5, and small amounts are of no importance for the application. The transglucosidase is undesirable in the application and must removed if present. Good strains which produce very little transglucosidase are known (e.g., *A. awamori* NRRL 3112) (Smiley, 1967). With most strains the removal of transglucosidase is a considerable problem in the recovery process, and many methods for its removal have been patented (Table VIII). It is not known to what extent these methods have been used commercially. Several of the methods are unattractive because of loss of glucoamylase activity in the process.

Glucoamylase is almost exclusively marketed in liquid form. The

microbial stability is good owing to the low pH (~4.5) and the high dry-substance concentration caused by the presence of sugar alcohols and organic acids. The sugar alcohols (mannitol) also have a stabilizing effect on the activity when the enzyme is stored. Solid preparations may be made in the usual way, but it is often necessary to reduce the amounts of sugar alcohols and organic acids first, for instance, by ultrafiltration.

b. Properties and Application: The content of active enzyme protein in commercial products is usually high, up to ~5%. The products contain small amounts of other enzyme activities such as protease, α-amylase, and cellulase.

The primary application of glucoamylase is in starch saccharification, which is described in detail by Antrim *et al.* (this volume). Glucoamylase has another useful application, viz., production of alcohol from starchy materials. Glucoamylase from *Rhizopus* was used for this purpose more than 70 years ago in the so-called amyloprocess. It was not very successful, presumably because the technology required for the process was too advanced for its time. In 1947, a new technique was developed at the Northern Regional Research Laboratory, Peoria, Illinois. *A. niger* was the enzyme source, and the process is still used in some large distilleries. The method is similar to that normally used in the production of glucoamylase, but all of the broth is transferred to the mash to be saccharified.

In many distilleries commercial glucoamylase is used extensively, combined with bacterial α-amylase, for liquefaction of the starch. Claims of improved alcohol yield relative to malt have been made and may be due to the presence of enzymes capable of hydrolyzing glucosides other than starch, for instance gums or cellulose. Glucoamylase finds minor application in the manufacture of low-calorie beer. It is added to the fermentation vat and hydrolyzes the dextrins to glucose, which is subsequently fermented by the yeast.

VIII. OTHER ENZYMES

A. Pectinase

This term covers at least six different enzymes which take part in the hydrolysis of the pectin molecule. Commercial products contain a mixture of these enzymes adapted to the application in question. The enzymes are produced by cultivation of *A. niger* or *A. wentii* in submerged or semisolid culture. Pectin may be added to the medium as an inducer.

The enzyme composition depends on the method of cultivation. In submerged culture, polygalacturonase is the predominant enzyme, whereas pectinesterase is scarce. Semisolid culture produces a mixture of enzymes that is more satisfactory for most applications.

Pectinases are used in preparation of fruit juice and wine. By means of pectinase the yield may be improved, viscosity adjusted, or special effects such as clarification obtained. It is possible, for instance, to improve the stability of cloudy orange drinks or to make clear lemon drinks by application of special pectinase preparations. Application of pectinases is a question of experience; it is made difficult by seasonal and geographic variations of the composition of the fruit that has to be treated. Furthermore, the complexity of the enzyme preparation makes exact standardization of the various pectinases extremely difficult. Extensive laboratory tests are required for each new crop of fruit in order to determine the proper pectinase treatment.

B. β-Glucanase

Commercial β-glucanase preparations are used to hydrolyze barley gums in the brewing industry. Some 20 years ago a considerable market was expected in the chicken feed industry, but it has not yet materialized. The purpose was to improve the feed value of certain types of hard barley by hydrolyzing the gums.

β-Glucanase is produced by *B. amyloliquefaciens* and synthesized concomitant with α-amylase and protease. Commercial preparations are optimized for the glucanase content by adjusting fermentation conditions accordingly. The enzyme is an endoglucanase, and the hydrolysis products are saccharides with 3–5 glucose molecules. The enzyme is stable at pH values above 5 and up to 50°C.

Fungal β-glucanase is made by fungi of the *A. niger* group. It is often formed as a side activity in pectinase preparation. Commercial preparations are optimized for production of β-glucanase. Since the enzyme preparations usually contain several β-glucosidases apart from the β-glucanase, an extensive hydrolysis (to glucose) is possible. The enzyme has a pH-optimum ~5 and may be used in combination with bacterial glucanase in the brewing industry for mashing or during fermentation if desired.

C. Pullulanase

This is used as a debranching enzyme in starch hydrolysis. Several microorganisms produce pullulanase, but the only commercial product available is prepared from *Klebsiella aerogenes* (previously known

as *Aerobacter aerogenes*). The enzyme has a pH-optimum at 5 and is rapidly inactivated at temperatures above 50°C.

Pullulanase is produced in submerged culture in a medium containing hydrolysates of starch high in amylopectin (Bulich, 1976) and in nitrogen sources, such as corn steep liquor. The fermentation takes place between 25 and 35°C, pH is maintained between 7 and 8. A suitable strain is *K. aerogenes* NRRL B 7580. The enzyme is partly cell-bound and the ratio of cell-bound enzyme to free enzyme varies according to the composition of the medium. The ratio is influenced by the carbohydrate component of the medium, and by using high-molecular-weight amylopectin it is possible to obtain over 75% free enzyme. Before recovery the cell-bound enzyme is released from the cells by the use of a nonionic surfactant.

D. Dextranase

Dextranases are used to a limited extent in the sugar industry when dextrans occur in the sugar-containing juice. Commercial preparations are made from *Penicillium funiculosum* or *Penicillium lilacinum*, pH-optimum is ~5, and they may be used up to 50° or 60°C. The enzyme from *P. lilacinum* is preferred because it has the best heat stability.

Dextranases have been advocated as a means of removing dental plaque. Enzymes such as the aforementioned two are of little value, as the dextrans of dental plaque are usually of the α-1,3-linkage type, whereas the penicillium enzymes hydrolyze α-1,6-bonds only. Enzymes suitable for removal of dental plaque have been described in the literature, but no preparations have yet been marketed.

E. Cellulase

Cellulase has enjoyed much publicity because of its potential application in waste treatment. The current market is small; the most important use is for digestive aids.

The best organism for production of cellulase is *Trichoderma reesei*. Enzyme from this organism is preferred because it shows relatively good activity against native cellulose. Most *Aspergillus* enzyme preparations contain cellulolytic activity against cellulose derivatives, such as carboxymethylcellulose, but no activity against native cellulose.

Cellulase may be prepared in semisolid or submerged culture. The enzyme may be adsorbed to cellulose present in the medium. Sophorose is said to be an inducer of the enzyme. Most cellulase preparations are solid; this only reflects the state of the art. If a bigger field of application is discovered, liquid preparations will probably

soon be developed. The products are relatively expensive, and the high cost is one of the elements that have prevented general use.

F. Lactase (β-Galactosidase)

This enzyme is intracellular in bacteria and yeasts, but it is extracellular in many fungi, for instance, *Aspergilli*, *Mucor* sp., *Rhizopus* sp., and *Penicillium* sp. Commercial preparations are made from *A. oryzae* and *A. niger*. They have acid pH-optima and are consequently well suited for use in digestive aids. Lactase from *A. oryzae* has pH-optimum of 4.8, and will have about 10% of its maximum activity at pH 2. Heating to 60°C for 10 min causes an 85% loss of activity. The *A. niger* lactase is more resistant; the pH-optimum is 3.5, 50% of maximum activity is at pH 2, and it will resist heating for 1 hr at 55°C without significant loss of activity.

The *Aspergillus* lactases are produced by semisolid cultivation in acidified wheat bran at 30°C. *A. oryzae* ATCC 20423 (Kiuchi, 1975) and *A. niger* ATTCC 13496 (Cayle, 1971) are suitable strains. The enzyme is extracted from the bran with water and may be precipitated directly with solvent or purified by adsorption to bentonite at pH 4, washing and releasing the enzyme at pH 7; it is then recovered by solvent precipitation. These enzymes are usually of high purity and therefore rather expensive. They are used mainly for digestive aids.

G. Lipase

Lipases are formed by many microorganisms. The traditional sources for commercial products are *Rhizopus* sp., *Mucor* sp., *Aspergillus* sp., and *Candida* sp. Some of the microorganisms are known to produce several lipases, and the specificity of the lipases varies both with regard to fatty acids and position in the triglyceride molecule.

The method preferred for preparation is semisolid fermentation, and the enzymes are recovered in the usual way. Lipids are not necessary for induction of the enzyme production and may in some instances inhibit enzyme synthesis. Lipase may be recovered as a by-product in the production of microbial rennet by *M. miehei* by absorption on clay minerals at pH 5 and elution at pH 10 (Moskowitz *et al.*, 1975). *Candida* lipases are usually prepared in submerged culture. Hydrocarbons have been reported as carbon sources, but not in commercial production.

Numerous applications have been proposed (Seitz, 1974), but their use has been limited. The most important market is digestive aids, for which microbial lipases replace pancreatic lipase, which is expensive and scarce. It is also used to improve the flavor of special types of

cheese. Lipases have not been introduced in detergents, although extensive investigations have been made and several patents issued on the use of lipases for this purpose.

IX. CONCLUSION

The extracellular enzymes are the "work horses" of the enzymologist, not very sophisticated but tough and hard-working. In many cases their use is hampered by high prices or properties which do not fit the process so well. Future developments will probably be directed to alleviation of these problems: yield improvements to decrease costs and screening for new organisms, modification of structure genes, or derivation of enzyme molecules by chemical means to improve enzyme properties.

No matter to what extent these attempts are successful, there is little doubt that the hydrolytic activities of the extracellular enzymes will maintain their position as important industrial tools. Their specificity, mild reaction conditions, and low toxicity are properties that will all become increasingly significant.

In regard to the economic question, which is conclusive in most business decisions, enzymes have the advantage of high specific activity. The actual raw-material costs are therefore insignificant, and the costs of preparation are open to attack by genetic and biochemical engineering—a real challenge.

REFERENCES

Arima, K. (1964). U.S. Patent No. 3,151,039.
Arima, K., Yu, J., Iwasaka, S., and Tamura, G. (1968). *Appl. Microbiol.* **16**, 1727.
Aunstrup, K. (1968). British Patent No. 1,108,287.
Aunstrup, K. (1977). *In* "Biotechnology and Fungal Differentiation" (J. Meyrath and J. D. Bu'Lock, eds.), p. 157. Academic Press, New York.
Aunstrup, K., and Outtrup, H. (1973). British Patent No. 1,303,633.
Aunstrup, K., Outtrup, H., Andresen, O., and Dambman, C. (1972). *Proc. Int. Ferment. Symp., 4th, Tokyo,* p. 229.
Aunstrup, K., Andresen, O., and Outtrup, H. (1973). U.S. Patent No. 3,723,250.
Boidin, A., and Effront, J. (1917). U.S. Patent No. 1,227,374.
Bulich, A. A. (1976). U.S. Patent No. 3,963,575.
Cayle, T. (1971). U.S. Patent No. 3,629,073.
Churchill, B. W., Steel, D. R., and Buss, D. R. (1973). U.S. Patent No. 3,740,318.
Cooney, D. G., and Emerson, R. (1964). "Thermophilic Fungi." Freeman, London.
Dwonschack, R. G., and Nelson, C. A. (1972). U.S. Patent No. 3,660,236.
Endo, S. (1962). *J. Ferment. Technol.* **40**, 346.
Feldman, L. I. (1971). U.S. Patent No. 3,626,957.

Freedberg, I. M., Levin, Y., Kay, C. M., McCubbin, W. D. and Katchalsky-Katzir, E. (1975). *Biochim. Biophys. Acta* 391, 361.
Güntelberg, A. V. (1954). *C.R. Lab. Carlsberg, Ser. Chim.* 29, 27.
Hoerle, R. D. (1976). Danish Patent Application No. 1618/77.
Horikoshi, K., and Ikeda, Y. (1977). U.S. Patent No. 4,052,262.
Jensen, D. E. (1972). *Biotechnol. Bioeng.* 14, 647.
Keay, L., and Anderson, R. G. (1971). U.S. Patent No. 3,592,737.
Keay, L., Moseley, M. H., Anderson, R. G., O'Connor, R. J., and Wildi, B. S. (1972). *Biotechnol. Bioeng. Symp.* No 3, 63.
Kiuchi, A., and Tanaka, Y. (1975). U.S. Patent No. 3,919,049.
Kohman, H. A., Irwin, R., and Stateler, E. S. (1927). U.S. Patent No. 1,654,176.
Lehmann, R., Pfeiffer, H. F., Schindler, J., and Schreiber, W. (1977). U.S. Patent No. 4,062,732.
Madsen, G. B., Norman, B. E., and Slott, S. (1973). *Staerke* **25**, 304.
Moelker, H. C. T., and Mattijsen, R. (1967). French Patent No. 1,592,965.
Moskowitz, G. J., Como, J. J., and Feldman, L. I. (1975). U.S. Patent No. 3,899, 395.
Murray, E. D., and Prince M. P. (1970). U.S. Patent No. 3,507,750.
Outtrup, H., and Aunstrup, K. (1975). *Proc. Int. Congr. IAMS, Ist,* **5**, 204.
Outtrup, H., Andresen, O., and Aunstrup, K. (1972). British Patent No. 1,296,839.
Priest, F. G. (1977). *Bacteriol. Rev.* 4I, 711.
Robyt, J. F., and Ackerman, R. J. (1973). *Arch. Biochem. Biophys.* **155**, 145.
Samejima, H. (1974). *Enzyme Eng.* **2**, 363.
Sardinas, J. L. (1966). U.S. Patent No. 3,275,453.
Schleich, H. (1971). U.S. Patent No. 3,616,233.
Seitz, E. W. (1974). *J. Am. Oil Chem. Soc.* **51**, 12.
Smiley, K. (1967). U.S. Patent No. 3,301,768.
Smith, J. A., and Frankiewies, J. R. (1975). German Patent Aus. No. 25-54-850.
Smythe, C. V., and Drake, B. B. (1949). U.S. Patent No. 2,480,090.
Smythe, C. V., Drake, B. B., and Neubeek, C. E. (1950). U.S. Patent No. 2,530,210.
Welkcer, N. E., and Campbell, L. L. (1967). *J. Bacteriol.* 94, 1124.
Yamada, N., and Tomoda, K. (1966). U.S. Patent No. 3,293,114.
Yoshida, F., and Ichishima, E. (1964). U.S. Patent No. 3,149,051.