

Production and Applications of Enzymes

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With 2 Figures

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The incorporation of enzymes into detergents is the most recent of a series of applications, and has aroused a great deal of interest in other possible uses. A relatively large number of earlier applications of enzymes did not have the benefit of modern day advertising and as a result did not capture the interest of the general population. Yet many of these applications retain their place in industry because of their unique nature.

The concept of using enzymes in industrial applications dates back to 1894 when a patent was obtained (Takamine, 1894) for making diastatic enzymes from fungi. Unfortunately, formation of off flavor hampered use of these early preparations in alcohol production (Takamine, 1914). A few years earlier it was claimed (Röhm, 1908) that pancreatic enzymes could be used in the bating of hides, and these preparations were quickly accepted in the leather industry. Three years later, a process was patented (Wallerstein, 1911) for the chill-proofing of beer using proteolytic enzymes, and again this process was quickly accepted. The use of tryptic enzymes as laundry aids (Röhm, 1913) was not widely accepted although it was used in Europe. The contributions of these three men mark the beginning of industrial enzymes, and of companies that have remained leaders in this field.

Several other applications of enzymes into the commercial area followed. The use of pectinases for the clarification of fruit juices was introduced in the early 30's both in Germany (Mehlitz, 1930) and in the United States (Kertesz, 1930). The use of fungal enzymes for the preparation of sweet sirups (Dale and Langlois, 1940; Langlois, 1940) developed under the stimulus of the sugar shortages of World War II. This development has resulted in a large variety of sirups, and modifications are still being made. The introduction of enzymes, other than from cereal grains, to the baking industry in the early 50's followed much experimental work at Kansas State University (Johnson and Miller, 1949) using enzymes supplied by the commercial producers. Interest in the use of fungal enzymes in the distilling industry, as suggested earlier by Takamine, was revived in the World War II era because of the pressing need for alcohol, but it required the commercial development of fungal amyloglucosidase in the last decade to make the use of microbial enzymes more attractive than malt in this industry.

The present chapter is limited to presenting developments in the enzyme field related to commercial practices. We have attempted to indicate the variety of commercially available enzymes and procedures used to produce them. The extent to which microbial enzymes have enhanced or replaced the enzymes derived from plants and animals is indicated.

A. Research

1. Selection of Organism

The selection of an organism is the first important step in the development of any product. For microbial enzyme production several factors must be considered:

a) Location of Enzyme

Cell-bound enzyme has an advantage in that it is concentrated in tissue which can be readily separated from the fermentation broth. But being cell-bound, it involves the difficulties associated with disintegration of cells (Edebo, 1969), and subsequent separation from other cellular components (Lilly and Dunnill, 1969). Though many enzymes are never released by living cells, (i.e. the "cell-bound" category), relatively few of the commercial enzymes (e.g. yeast invertase) are produced in this manner.

Secreted enzymes, on the other hand, must be concentrated from very dilute solutions of culture filtrate, a process which may be costly. In general, their "purity" is appreciably greater than that of enzymes present in cell-extracts. Most commercial enzymes are of this type.

b) Nature of Organism

The preferred organisms are those which "handle" easily or, in the case of food applications, have the FDA stamp of approval. They are stable in their characteristics of enzyme yields, spore production, and cultural requirements. They present minimal difficulty in filtration, centrifugation, or disintegration (if required). They produce no toxic or other undesirable side products, and they grow on inexpensive substrates.

c) Yield and Purity of Enzyme

Purity is generally measured as specific activity (enzyme per unit weight of protein), which may be quite misleading when polysaccharides, polyesters, etc. are present in large amounts. As a consequence industrial enzymes are usually measured in terms of activity per unit weight. Currently it appears that a reasonable goal is the obtention of a culture filtrate, or cell extract, in which the desired enzyme makes up 10% of the total protein. One organism has been reported to produce a particular enzyme in an amount equal to one third of its total protein

*4x recryst. B. n. x amylase contains high conc
 20 Nov 68 1978*

(Table 1). When that enzyme is β -galactosidase, one wonders whether the organism or the investigator is in error. There are many examples of enzymes which require 1000 (+) fold purification, but relatively few in which "purity" is obtained by a mere 10-fold increase in specific activity (Table 1). Rather unexpectedly, some of these high purity enzymes are found in cell extracts and so represent a large percentage of all protein synthesized (Pardee, 1969). Secreted enzymes represent much less of the total cell protein.

Table 1. "Purity" of enzymes in culture filtrates or cell extracts

| Enzyme | Source | R ^a | Ref. |
|----------------------------|------------------------------------|----------------|-------------------------------|
| Aspartate transcarbamylase | <i>E. coli</i> | 12 | Gerhart and Holoubek (1967) |
| Catalase | <i>Rhodopseudomonas spheroides</i> | 4 | Clayton and Smith (1960) |
| β -galactosidase | <i>E. coli</i> | 16 | Wang and Humphrey (1969) |
| β -galactosidase | <i>E. coli</i> | 3 | Novick and Horinchi (1961) |
| Exo- β 1,3 glucanase | <i>Basidiomycete</i> sp. QM 806 | 7 | Huotari <i>et al.</i> (1968) |
| Exo- β 1,3 glucanase | <i>Sclerotinia libertiana</i> | 8 | Ebata and Santomura (1963) |
| Tyrosinase | <i>Neurospora crassa</i> | 20 | Horowitz <i>et al.</i> (1961) |

$$^a R = \frac{\text{Spec. Act. "Pure" Enzyme}}{\text{Spec. Act. "Crude" Enzyme}}$$

Toxin, Diptheria = 50% of bact. protein Pappenheimer 1965

Selection of an organism cannot, of course, be independent of the conditions involved in the screening procedure. An organism selected for amylase production by screening in surface culture or bran medium may be quite different from that screened in shake culture on starch. Although the trend in enzyme production is away from surface culture, certain enzymes are best produced by it, and there is difficulty producing these enzymes in any other way. Enzymes designed to be used under severe conditions, at high pH, high temperature, etc., must be screened under similar adverse conditions.

A very necessary factor in any screening program is the availability of large numbers of diverse organisms. In addition to the government supported collections (USDA, Peoria, Ill.; U.S. Army Lab., Natick, Mass., and American Type Culture Collection, Rockville, Md.) large companies maintain their own cultures. Frequently investigators find it advantageous to isolate organisms by enrichment procedure from

natural sources such as soil or water. Based on the organisms available and the screening procedures used, it is not at all unusual that different investigators develop enzyme processes based on different organisms (e.g. for amyloglucosidase: *Aspergillus niger*, *A. awamori*, *A. phoenicis*, *Rhizopus delemar*, *R. oryzae*, *Endomycopsis* sp.).

2. Mutation

The selected organism may further be improved through mutation. Table 2 illustrates the type of increases reported in published work. Techniques have been described for obtaining (a) mutants resistant to catabolite repression, (b) constitutive mutants which produce enzyme in the presence of repressor, and (c) constitutive mutants which form enzymes without the addition of inducer (Demain, 1968). The aim of most work along these lines has been the increased production of relatively simple compounds, e.g. aminoacids and antibiotics. The procedures involve resistance to antimetabolites (analogs of the desired product) as a means of selecting mutants. For the production of hydrolytic enzymes, there seems to be no similar simple means for a preliminary selection of the desired mutant, unless enzyme production happens

Table 2. Increases in enzyme production by mutation

| Enzyme | Organism | Enzyme Yield $\frac{(\text{Mutant})}{(\text{Control})}$ | Ref. |
|----------------------------|---------------------------------|---|-------------------------------|
| Aspartate transcarbamylase | <i>E. coli</i> | 500 | Gerhart and Holoubek (1967) |
| Cellulase | <i>Trichoderma viride</i> QM 6a | 2 | Mandels <i>et al.</i> (1970) |
| Dihydrofolate reductase | <i>Diplococcus pneumoniae</i> | 200 | Sirotnak <i>et al.</i> (1964) |
| Dihydrofolate reductase | <i>Streptococcus faecalis</i> | 10—100 | Hillcoat and Blakley (1966) |
| β -galactosidase | <i>E. coli</i> | 4 | Novick and Horinchi (1961) |
| Glucoamylase | <i>Aspergillus foetidus</i> | 1.6 | Underkofler (1970) |
| Homo-serine dehydrogenase | <i>E. coli</i> | 3 | Egorov <i>et al.</i> (1965) |
| Protease | <i>Bacillus cereus</i> | 10 | Levisohn and Aronson (1967) |
| α -amylase | <i>Aspergillus oryzae</i> | 10 | Neubeck (1970, unreported) |

to be correlated with the yield of some simple product. In some instances, a mutant lacking the desired enzyme can be reverted, yielding a culture giving much more enzyme than its grandparent (Egorov, 1965). This again is a procedure which has been more amply demonstrated on products other than enzymes, but which may prove increasingly valuable. The frequency of reversion (using UV) increases with percent kill. At 99+ % kill as many as 6% of the survivors are revertants (Dulaney and Dulaney, 1967).

Significant increases in the enzyme productivity of organisms used commercially have been achieved using ultraviolet light and nitrogen mustards as mutagens. Frequently alternate treatments with a variety of mutagens have been more beneficial than a series involving the same mutagen. The original strains of *A. oryzae* used in the production of commercial amylase in the early 40's show only one-tenth to one-fifteenth the productivity exhibited by the presently available mutant organisms¹. The new organisms show significant morphological changes (colour; extent of sporulation) from the original strains. Attempts to demonstrate the increased enzyme activity on starch agar in simple zone tests have not been successful. Selection of the improved organisms was made almost entirely on the basis of screening tests carried out on complex media, using the survivors remaining after mutagenic treatment. Similar results have been obtained with other enzymes and the organisms which produced them (Mandels *et al.*, 1970).

3. Modification of Conditions of Growth

a) General Methods

Enzymes are produced in three ways which may be classified as bran culture, submerged culture, and two step submerged culture.

The surface bran culture method involves growth of the organism on moist acidified bran (either from wheat or rice) in beds through which air is circulated. Bran itself is a good nutrient for amylase production, but the bran may be fortified with other nutrients and salts to improve yields. In another modification air may be passed over the bed. The surface procedure has also been called the koji method when rice is used as the substrate. The terms tray or semisolid culture method are also used. Usually the beds are thin layers one to two inches deep to expedite heat removal. An excellent review of the tray process was published in 1947 (Underkofler *et al.*, 1947). Except for slight modifications, the process is still carried out commercially. More recently, the Japanese (Terui and Takano, 1960) have investigated on a commercial scale a

¹ Unpublished experiments by C. Neubeck.

deep bed bran (and also koji) process using layers of medium several feet thick and several feet in diameter. This process is called the "high heap" method. Another modification of the moist bran process involves tumbling of the growing culture in rotating drums to obtain the required aeration and process control. This method was tested extensively in the laboratory in the 1930-1940 period (Underkofler *et al.*, 1939), and one commercial producer of enzymes is using it. The bran procedures are most widely used for growth of fungi, and the term "moldy bran" is frequently applied to the crude dry product arising from any of the procedures.

The deep tank or submerged process involves growth of the micro-organism in liquid culture within large vessels equipped for aeration and agitation. The tanks are the same as those described for typical submerged fermentation processes. It is the more widely used method now for producing enzymes, because it is easier to control than the moist bran methods and the growing medium can be varied more easily. The process is usually a batch system, but it may be adapted to continuous

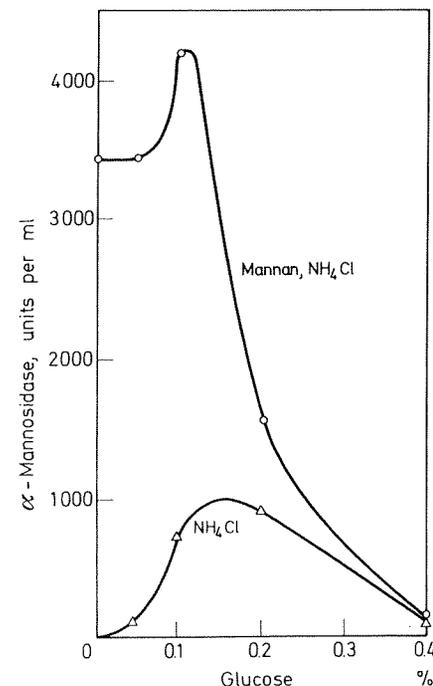


Fig. 1. Effect of glucose concentration on yields of α -mannosidase by washed mycelium (Demain)

fermentation or modified to the extent that supplemental nutrients are added to maintain growth and activity production.

The third procedure is a two step operation in which microbial cells produced in a fermenter are transferred to a reaction vessel in which the conditions differ from those used in growth. α -Mannosidase has been produced in this way (Inamine *et al.*, 1969). *Streptomyces griseus* cells were grown in a rich medium for 17 hours at 28°C, washed, and transferred to a dilute mineral salts solution containing an inducing compound (usually yeast α -mannan). Maximum α -mannosidase concentration, obtained in 18–24 hours, was accompanied by little, if any, growth. This enzyme is useful in converting mannosido-streptomycin to the more active antibiotic form of streptomycin. Advantages of the method include reduced requirement for inducer, high yield of enzyme, and relatively small amounts of contaminating nutrients. The method takes advantage of the fact that conditions for growth (trophophase) differ from those for product elaboration (idiophase). Separation of the phases permits closer control of the optimal conditions for each. The authors are not aware of commercial implementation of this technique for the production of enzymes.

As in the production of many metabolic substances, conditions optimal for growth are not necessarily those which give maximum enzyme yields. Growth is essential, but in general, high concentrations of readily utilizable nutrients should be avoided. Close attention must be given to control of pH, mineral content, temperature and aeration. The growing conditions and nutritional requirements must be worked out empirically for each organism for the selected method of growth. The interrelationships between the several factors influencing enzyme production are very complex. A large part of the research carried out by the industrial producer is concerned with working out the most efficient method of growing the microorganism for enzyme production and translating these results to the plant production units.

Selection of the method of growth will depend on how well the surface or submerged methods can furnish the aeration, temperature and pH control, and supply of nutrients and inducers required for production of the desired enzyme.

b) Inducers

Many organisms are stimulated to increased enzyme synthesis by adding an inducer to the culture. The inducer was formerly considered to be the substrate of the enzyme or a modification of the substrate. More recently it has been shown that products (and modified products)

of the enzyme may also be inducers, e.g. of polysaccharases. This would appear to be a necessary consequence of the inability of polymers to enter the cell. As a result it seems likely that this principle will also apply to induction of other depolymerases, such as proteases and nucleases.

Failure to recognize induction by products of enzyme action resulted from the well-known catabolite repression. Repression occurs at relatively low concentration of the sugar dimers (which are the inducers of the corresponding polysaccharase). Thus at a level of 0.5% cellobiose very little cellulase is produced by organisms growing in shake flasks. However, continuous feeding of very low cellobiose concentrations (< 0.05 mg/ml) greatly increases cellulase yields (Suzuki *et al.*, 1969). The inducing ability of a polymer (the enzyme substrate) results from its hydrolysis to dimer in these systems. The dimer never accumulates to the concentration at which repression occurs, because it is consumed by the organism as rapidly as it is formed.

Any method which can supply the inducer slowly to the growing organism will reduce catabolite repression and result in enhanced enzyme yields. Esters of the inducer have the necessary properties, provided that the organism possesses an appropriate esterase. The esters of inducing disaccharides (Table 3) are considerably more potent than the disaccharides themselves, presumably because the esters do not repress. Glycosides of the dimers may possibly induce, but ethers, because of their resistance to breakdown, would not be expected to do so.

Just how much increase in enzyme yields might one anticipate from the use of modified inducer? After it was found that sucrose monopalmitate increased invertase yields as much as 100-fold (Reese *et al.*, 1969), a screening of organisms was undertaken using this ester as substrate. Under these conditions, *Pullularia pullulans* was found to be an excellent source of invertase, and, unlike yeast, it secreted the enzyme into the culture filtrate. The yields are surprisingly good. The invertase contents of the *unconcentrated* filtrates have the same magnitude as that of a widely-distributed commercial *concentrate*. The specific activities are also quite similar (Reese, unpubl. data).

Similarly, adenosine is a true inducer of purine nucleosidase. But at a level of 0.5% its repressing effect is great enough to prevent the appearance of enzyme. Use of ribonucleic acid (RNA) or of the phosphate ester of adenosine (AMP) by the fungus leads to a low but continuous supply of adenosine and a resultant high enzyme concentration (Reese, 1968).

In apparent contradiction of the above, it is possible to obtain good enzyme yields at concentrations of an inducer that normally repress. This is achieved by decreasing the rate of consumption of the inducer, (a) by some means of inhibition, such as lower-than-optimal temperature;

(b) by use of compounds at concentrations which are slightly toxic; or (c) by restricting one or more of the factors required for growth. It thus appears that catabolite repression is not a matter of catabolite concentration, but of the rate at which the inducer is metabolized. An alternate procedure, when there is a family of inducers, is to select the

Table 3. Effect of modified inducers on enzyme yield

| Enzyme | Organism | Inducer | Yield | Ref. | |
|-----------------------------------|------------------------------------|-----------------------------|-------------------|----------------------------|-----------------------------|
| Cellulase EC 3.2.1.4 | <i>Trichoderma viride</i> | Cellulose | 22.5 ^a | Reese <i>et al.</i> (1969) | |
| | | Cellobiose | 0.2 ^a | | |
| | | Cellobiose dipalmitate | 4.8 ^a | | |
| | <i>Pestalotiopsis westerdijkii</i> | Cellulose | 35.9 ^a | | |
| | | Cellobiose | 0.2 ^a | | |
| | | Cellobiose octaacetate | 20.1 ^a | | |
| | <i>Pseudomonas fluorescens</i> | Cellulose | 514 | | Suzaki <i>et al.</i> (1969) |
| | | Cellobiose (slow feeding) | 200 | | |
| | | Sophorose | 397 | | |
| Dextranase EC 3.2.1.11 | <i>Penicillium funiculosum</i> | dextran | 1080 ^a | Reese <i>et al.</i> (1969) | |
| | | isomaltose | 2 ^a | | |
| | | isomaltose dipalmitate | 1098 ^a | | |
| | | | | | |
| Invertase EC 3.2.1.26 | <i>Pullularia pullulans</i> | sucrose | 1.3 ^a | Reese <i>et al.</i> (1969) | |
| | | sucrose | 108 ^a | | |
| | | monopalmitate | | | |
| Purine nucleosidase EC 3.2.2.1 | <i>Aspergillus ambiguus</i> | Adenosine | 0 | Reese (1968) | |
| | | Yeast RNA | 57 | | |
| | | Adenosine 5'PO ₄ | 90 | | |

^a Unit values in International units. Others as defined by authors.

more slowly consumed member. Xylidine (Fahraeus, G., pers. comm.) induced much more laccase in *Polyporus versicolor* than did other, more rapidly consumed substrates, and sophorose ($\beta 1 \rightarrow 2$ diglucose) is a better inducer of cellulase than cellobiose ($\beta 1 \rightarrow 4$ diglucose) for some organisms (Table 3). In a similar way the D-isomers (the *un natural* forms) of tyrosine and phenylalanine are considerably more active inducers of tyrosinase in *Neurospora* than are the natural forms (L-isomers) of the same aminoacids (Horowitz *et al.*, 1961). In some cases, the toxicity of the agent may modify its inducing effect.

c) Surfactants as Promoters of Enzyme Production

Surfactants (esp. Tween 80)² at low concentrations have long been known to enhance the growth of some bacteria. Their effects in increasing enzyme yields have been reported recently (Reese and Maguire, 1969), although it is quite possible that the effect was recognized earlier and kept a trade secret. It may be that the sometimes observed increase in yields in tanks (vs. shake flasks) is due to the addition of antifoam agents (i.e. surfactants). More frequently, anti-foams depress the dissolved oxygen in a growing culture and produce poor activities.

Table 4. Effect of addition of surfactant (Tween 80) to culture medium on enzyme yields (Reese, unreported)

| Enzyme | Source | R ^a (Yield + Surfactant) (Yield - Surfactant) |
|-----------------------|---------------------------------------|---|
| Cellulase | Many fungi | 20x |
| Invertase | Many fungi | 16 |
| $\beta 1,3$ glucanase | Many fungi | 10 |
| β -glucosidase | Many fungi | 8 |
| Xylanase | Many fungi | 4 |
| Amylase | Many fungi | 4 |
| Nucleosidase | Many fungi | 5 |
| Esterase | Many fungi | 6 |
| Dextranase | <i>Penicillium funiculosum</i> QM 474 | 2 |
| Pullulanase | <i>Aerobacter aerogenes</i> QM B 1591 | 1.5 |

^a R = Ratio of yield in shaken flasks (29°) containing appropriate culture media plus Tween 80 (0.1%), to yield under identical conditions lacking Tween.

The addition of surfactants to culture media increases the yields of many enzymes. The results presented in Table 4 were obtained with secreted enzymes. No data are available for cell-bound enzymes. In some tests, no enzyme is detectable in the controls, but several units of activity per ml can be found in the presence of surfactants (i.e. infinite enhancement). The values shown in Table 4 are based on the surfactant Tween 80 at 0.1%. For some systems, higher Tween concentrations give better yields, but there usually is a critical upper level. For some systems, other (nonionic) surfactants such as Triton³ are superior to Tween. In general, the enhancement factor is greatest for organisms which do not normally secrete much enzyme and least for those organisms already selected for their high yields. But even in the latter, an appreciable

² Tween is a trademark of Atlas Chemical Co.

³ Triton is a trademark of the Rohm and Haas Company, Phila.

increase may be anticipated. A doubling in cellulase was observed with the active cellulolytic fungus, *Trichoderma viride*, and in dextranase with the highly productive *Penicillium funiculosum*.

The mechanism by which surfactants increase enzyme secretion is not known. It seems likely that the "leakiness" of cell membranes may vary from one organism to another and thus account for the variability between strains in the amount of enzyme normally secreted. Surfactants may be expected to accumulate at the cell membrane and thereby further increase (or modify) leakiness. As the amount of cell-bound enzyme appears to remain constant (feed-back control), increased secretion leads to increased enzyme production as the cell attempts to maintain the cell-bound level. In the case of surfactant-stimulated production of α -amylase by *A. oryzae*, the higher enzyme production may be accompanied by a lower mycelial weight. At some critical level of surfactant, the effect on the cell wall becomes so great that the cells lyse, and growth as well as enzyme production cease.

An alternate explanation offered for the increased yields is that the surfactant protects the enzyme from inactivation. Thus, Tween protects laccase from inactivation by phenol (Fahraeus, G., pers. comm.), but this effect has not been demonstrated for many of the observed increases.

The enhancement of enzyme yields by carbohydrate esters (Table 3) may be due, in part, to their surfactant properties. This is certainly true of sucrose monopalmitate, which has been found to increase yields, not only of invertase, but of many other enzymes listed in Table 4. Both Tween 80 and sucrose monopalmitate are nonionic surfactants relatively nontoxic to the organisms tested. At first, it was believed that only this type of surfactant possessed the stimulating effect. Later sodium oleate proved superior to Tween 80 for producing cellulase by some fungi. The anionic surfactants tend to be more toxic than the nonionics. The cationic agents, which are even more toxic, have not been found useful in this work.

4. Immobilization

Most enzyme reactions take place in a solution of enzyme and substrate in a batchwise process, at the completion of which the enzyme is discarded. As the activity of the enzyme may be scarcely diminished in reactions carried out under optimum conditions, this practice is very wasteful. Immobilization of enzyme by various means prevents its diffusion and permits its later separation from the reaction mixture by simple filtration. Enzyme can be recovered in this way and reused many times, greatly improving the economy of operation. The easy removal

of enzyme from the treated product may be a distinct advantage in food industry applications. In batch operations the processor usually depends on subsequent processing steps for removal or inactivates the enzyme in some other manner.

Enzyme localised on columns can be used in a continuous single reaction, or by successive layering of different enzymes, a series of reactions can be carried out, with the final product emerging in the effluent. In like manner, enzymes may be localized in animal tissues to carry out a required action.

Four means of localization have been described.

a) Microencapsulation (Chang *et al.*, 1967; Chang and Poznansky, 1968)

Semipermeable microcapsules of cellular dimensions (10–20 μ dia 200 \AA thick) can be formed, each containing enzyme, or some other large molecule. Useful membranes have been made of collodion or other polymeric materials. These membranes are permeable to the substrates and products of enzymic action, but the enzyme itself is too large to leak out. A great many enzymes have been successfully encapsulated, and the method appears to have general applicability. A limitation appears, however, in that large substrate molecules (proteins, polysaccharides) cannot permeate into the capsule, and the system is not applicable to such hydrolyses.

b) Covalent Bonding of Enzyme to Insoluble Carrier (Silman and Katchalski, 1966)

Enzymes have been bound to insoluble cellulose derivatives by various methods such as (a) to insoluble carboxymethyl cellulose using the azide derivative, (b) to insoluble cellulose using the diimide reaction, etc. More recently⁴ H. Weetall described the covalent coupling of 1-amino-acid oxidase to porous silica glass particles.

The enzymes must, of course, be linked at some distance from the active site of the enzyme. Such insolubilized enzymes retain their activities, but their immobility may reduce the reaction rate. The properties of such covalently linked enzymes are not always the same as those of the free enzyme. The anionic or cationic nature of the carrier may alter the pH optimum for the reaction (Goldstein, 1969). The binding of the enzyme to carrier may result in steric hindrance and impose restrictions on the specificity of the bound enzyme. This is most

⁴ ACS 1969 Meeting.

apparent where the substrate is a large molecule, such as protein, rather than a small molecule like a peptide. The products of protein hydrolysis may differ, those products from bound enzyme being of larger size than those from soluble enzyme. K_m and V_{max} values may also be affected.

c) Localization of Enzyme in the Aqueous Phase of a Two-Phase System (Reese and Mandels, 1958)

An enzyme can be dissolved in an aqueous phase and retained on a column of a hydrophilic solid such as cellulose. Substrate in the solvent phase diffuses into the aqueous phase where reaction with the enzyme occurs. Products diffuse back into the mobile phase and pass out of the column. Using invertase, such a system retained nearly complete activity for a number of weeks. This system resembles the micro-encapsulated enzyme procedure, except that a solvent phase substitutes for a semipermeable membrane.

d) Retention of Enzyme by Ultrafiltration Membranes

In the preceding methods, the enzyme is confined to small droplets, capsules, or inert carrier. Enlargement of a capsule to the size of a fermenter is theoretically possible, and this principle has been applied with practical modifications. The enzyme is free in solution as in the batch procedures, but an ultrafilter serves to separate the reaction products from the enzyme and substrate. Substrate is continuously pumped into the system, and product is removed by ultrafiltration to provide a continuous system. This method is applicable only to systems where the substrate is a large or an insoluble substance, so that it – with the enzyme – are retained inside the membrane. Successful demonstration of experimental runs on cellulose saccharification (Ghose and Kostick, 1969) and on starch hydrolysis (Butterworth *et al.*, 1969) have been made. Success depends upon the availability of suitable membranes and practical application on their cost.

All four means of immobilization have been used successfully in laboratory operation. Machinery has been developed for large-scale production of microcapsules with various contents. Chemical procedures have been described for covalent bonding of enzyme to carrier. It has been reported (Anon, 1970) that Tanabe Seiyakii Co., Osaka, is now using immobilized enzymes commercially to catalyze the hydrolysis step in the production of D or L amino acids. It has also been reported (Takasaki *et al.*, 1969) that glucose isomerase can be retained

by *Streptomyces* cellular structures by heating and that this preparation can be used for a series of isomerizations. The preparation apparently diminishes in strength with the number of passes.

It seems likely that the use of penicillin-amidase supported on cellulose by chemical coupling with triazines will soon be employed commercially to convert penicillin to other forms. The use of amyloglucosidase supported on a cellulosic support also appears to be a good possibility for the near future.

B. Commercial Enzymes

1. Preparation of Enzymes for Industrial Use

The preparation of marketable enzyme products is essentially the same for enzymes prepared from different sources (plant, animal, microbial) since all are protein. The precise details vary and the use for which the product is prepared usually determines the number of processing steps and degree of purification.

The basic steps and their variations involve:

1. Solution of the above enzyme in water by extraction from the crude enzyme-containing material. Some very crude products simply represent a dry form of the enzyme-producing cellular structure and even this first step may be bypassed. In some cases an autolysis or activating step may be required prior to the water extraction.

2. Removal of cellular debris and other insoluble material from the aqueous enzyme extract using centrifugation, filtration, or both.

3. Precipitation of the enzyme from the aqueous solution by protein precipitants. The precipitate is then dried in air or *in vacuo*. The enzyme solution may be concentrated prior to the more selective precipitation step.

Fig. 2 is a diagram indicating some treatments which have been used in the preparation of enzymes on a commercial scale. Most of the equipment can be found in food-processing plants. Nearly all processing operations (except drying) are carried out at low temperature (0–10° C) to minimize denaturation of the enzyme. Enzyme products may be removed at several points to obtain a series of products differing in absolute activity and purity. The same enzyme source may be the starting point for a large variety of different products. The simplest products representing dried cellular material are used in such operations

as the bating of hides. Liquid products are employed in textile starch desizing and fruit juice clarification. High activity products are used in fruit juice clarification and several other food-processing applications discussed below.

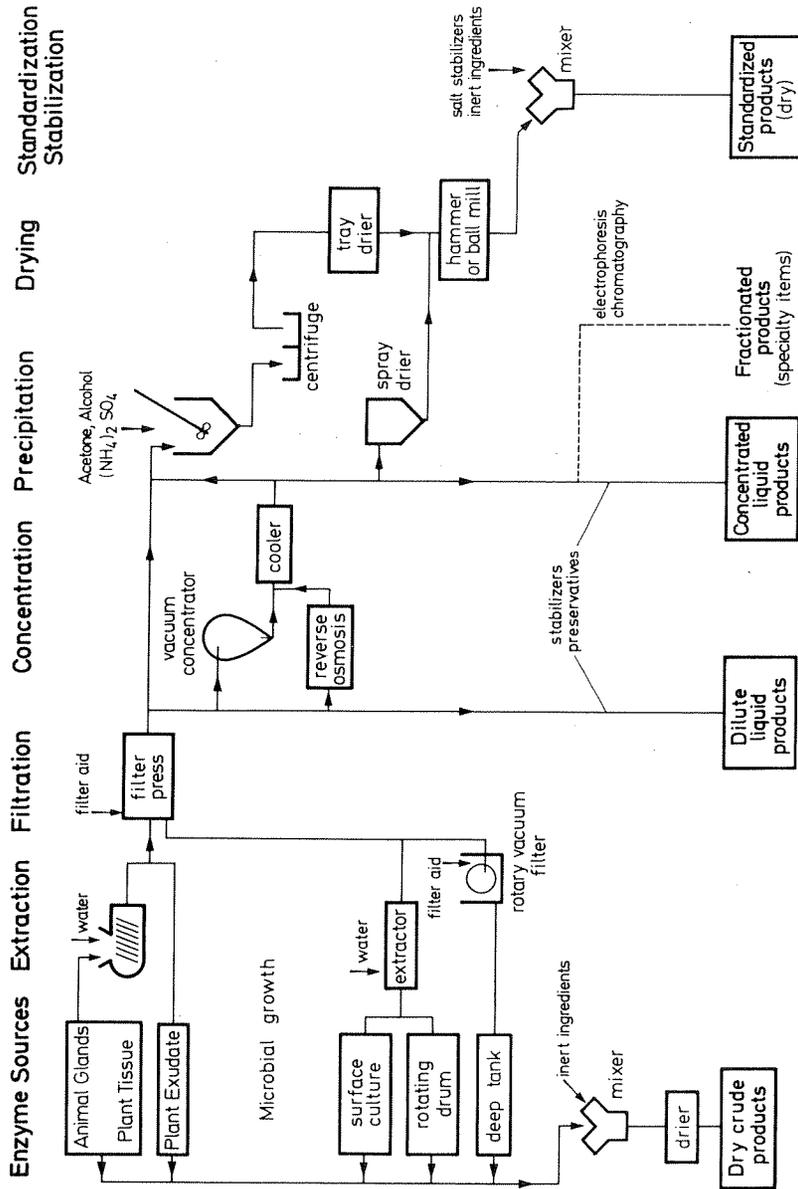


Fig. 2. Preparation of commercial enzymes

A listing of the media ingredients, filtration aids, precipitating agents, and formulating materials used by major enzyme producers in the preparation of microbial enzymes for food processing was presented by representatives of the industry (Beckhorn, 1965). A large variety of ingredients and process variables were required to encompass the various products prepared by the three manufacturers represented (Wallerstein Co., Rohm and Haas Company, Miles Chemical Co.).

The broad variety of different enzyme products has evolved because of the many applications in which they are being used. Enzymes are used in industrial applications which differ greatly in production volumes and degree of sophistication. Very active materials require very precise measurements and very effective mixing for efficient use. The small manufacturer has difficulty in using such products, and diluted standardized products fit his needs better. Automated industrial processes frequently utilize liquid products, because they lend themselves to pumping operations more readily than solids. It is often less expensive to prepare a liquid product and stabilize it against microbial spoilage and enzyme denaturation than to concentrate, precipitate, and dry the product for the user to redissolve. Unfortunately some enzymes are too labile to be handled readily in this way and they must be provided in dry form.

The various enzyme products are marketed on the basis of activity. Sometimes the activity is determined by a standard enzyme assay, but more frequently the activity is determined by methods based upon the industrial application of the enzyme. Large consumers with well-equipped laboratories find it expedient to purchase concentrates, but smaller plants prefer to purchase enzymes at standardized activities. Materials compatible with the intended application are added to the enzyme products by the enzyme manufacturer to adjust the activity to the required value. Stabilizers, activators and other materials to improve the response of the enzyme are also added. In the case of enzymes for food applications, the usual bacteriological tests (e.g. absence of Salmonella, gas formers, etc.) are applied to the products.

Most commercial enzymes in wide demand are not extensively fractionated. However, in some applications (baking, dextrose production), the presence of contaminating enzymes must be very low or rigidly controlled. One advantage of microbial enzymes results from the fact that an organism can be selected and grown under conditions where the level of contaminating enzymes can be kept low. In applications where high purity enzymes are required (e.g. pharmaceutical syntheses) the procedures required fall outside of the usual commercial practice. At the present time, chromatographic and electrophoretic techniques for the preparation of these high purity enzymes are restricted to pilot plant

or laboratory scale where better process control can be obtained. The cost of such operations puts high purity products in the speciality rather than in the industrial class. The next five to ten years will probably see more extensive exploitation of these techniques in the commercial field as the demand for new and more highly purified enzymes arises. Some use of preferential adsorption has been made in the removal of transglucosidase from fungal amyloglucosidase. Many adsorbents and techniques have been disclosed in patents for this purpose. Among these are cellulose (Kerr, 1927), clays (Inglett, 1963), lignin (Cayle, 1923; Hurst and Turner, 1962), anionic ion-exchange resins (Corman, 1967) and Sephadex (Garbutt, 1967).

2. Comparison of Industrial and Laboratory Preparations of Enzymes

The simple flow diagram given for the preparation of industrial enzymes could serve as well to illustrate operation on a laboratory scale. The magnitude of the commercial operation, however, brings about critical differences between plant and laboratory procedures. Commercial producers of enzymes find it necessary to carry out laboratory and pilot plant work to develop the most efficient series of steps to be followed in the manufacture. Usually a process is developed from a microbial source, because animal and plant tissue represent a limited and essentially uncontrolled supply of raw material.

It was noted above that the initial step in preparing a microbial enzyme is the selection of the proper organism, the method of growth, and the cultural conditions required for maximum activity. These screening techniques must also conform to procedures which have utility in scaling up to equipment that the producer has available or can be engineered at practical cost. Ordinary flasks or jars, either shaken or stationary, are generally used for the growth at this stage using 50–1000 g of medium. The exact procedures, i.e. media, agitation conditions, temperature profile, etc., followed by the commercial producer are usually considered to be proprietary. The effects of the various screening techniques must be empirically determined for each new product, and usually some adjustments are necessary as mutants of greater productivity are developed. The next step in the transfer of the process to plant equipment must be carried out in large laboratory or pilot plant equipment (e.g. 5–100 gallons capacity) which can then be scaled up to the available plant equipment. The pilot equipment is operated at practical levels of such variables as power consumption, aeration, temperature control, medium sterilization etc. Agitation and aeration

requirement during scale-up are discussed in other chapters of this volume, and these will not be discussed here except for the observation that submerged fungal cultures used for enzyme production are highly aerobic and tend to become very viscous. Scale-up, therefore, can be a difficult problem. The maintenance of a pure culture and proper growth temperature in commercial enzyme production deserves some additional comment here, because the control of these factors on a commercial scale may be very critical.

a) Maintenance of Pure Culture in Commercial Scale

Laboratory media in small quantities (e.g. 100 g of either submerged culture or surface culture) can be readily sterilized and maintained without contamination by simple closures of cotton or use of a sterile air supply, provided that the inoculum is handled properly. Under plant conditions, the sterilization of 1000 pounds or more of material requires adequate distribution of heat throughout the mass of medium and the prevention of leakage around rotating shafts, inoculation and sampling ports. Frequently the extent of contamination in raw materials is much higher in bulk, and this may be a problem because of longer make-up time. The sterilization of liquid media is more readily accomplished than the high solids bran media, but some degradation of medium is usually obtained in media of either the submerged or surface type. Maintenance of pure culture in the surface method is further complicated by the exposure of a large surface area (1000 square feet or more) to contamination. Control of such a large area over the usual two to four day growing period with sterile air is a formidable task. Protection from contamination in the initial stage depends on acidification of the bran. Rapidly growing organisms can effectively prevent contamination by other organisms by establishing a surface barrier, but this is not always the case. The rotating drum method using bran has many of the advantages of the submerged culture method as far as maintenance of pure culture, however, mycelium fragility and compaction during rotation can pose a problem in large masses of culture.

b) Maintenance of Proper Growth Temperature

The temperature of small quantities of growing culture can be readily controlled in the laboratory by immersing the growth vessel in constant temperature water baths or in rooms with good temperature control and high ratio of air change. Laboratory equipment can be operated over a wide range of temperatures. Changes in temperature can be made

in a matter of minutes. Large equipment must rely on cooling jackets or coils for temperature control in submerged culture. Surface culture requires water-cooling or circulation of air to control the temperature. The amount of heat generated in the growing process for either method is very large. Essentially all carbohydrate in the medium is consumed with the evolution of CO_2 , H_2O and heat. A typical 2500-pound surface culture charge containing about 1000 pounds of solids (≈ 400 –600 pounds of starch) can produce enough heat during the most active stage of growth (from 20–40 hours) to raise the temperature of the entire mass of growing culture 5°C or more per hour if the heat is not removed. Heat removal is slow because of the low heat transfer rate through the bran medium. The large bulk of material in a rotating drum may also result in a major problem of heat removal. In submerged culture, the same quantity of heat is liberated for the same carbohydrate consumption, but the heat is more readily removed because of better mixing and heat transfer in the fermenter, and greater dilution of medium (e.g. 1000 pounds solids in 10,000 pounds of medium).

There are several commercial enzymes which can be produced with the same efficiency by submerged or surface culture with respect to the units of activity produced per weight of nutrient consumed. The decision about which method to use in production is made on the basis of which one gives the fewest control problems. The selection of organism can also influence this decision. Frequently the choice is that process which gives the lowest level of contamination with undesirable enzymes. Sometimes the superiority of the submerged process – e.g. growth of *Bacilli* to produce liquefying diastase and alkaline protease – is overwhelming, and the decision is clear. There are, however, some fungal cultures which produce activity poorly in submerged culture – e.g. *Rhizopus* to produce amyloglucosidase – and the surface method is the one of choice.

c) Differences in Laboratory and Commercial Enzyme Processing

There are also major differences between large-scale enzyme processing and the usual laboratory methods of preparing enzymes. A complete laboratory procedure involving the cooling of the grown culture, extraction, filtration and precipitation can often be carried out in less than four hours with good temperature control at all steps. Drying generally adds another two hours. Normal commercial transfer (i.e. material handling) and refrigeration facilities usually require an 8 to 16 hour period for each required operation. The time scale, therefore, increases by a factor of about ten. Most enzymes are processed at

5 – 10°C at all stages up to the drying stage to minimize the effect of longer processing times, but microbial control requires close attention even at these temperatures. Preservatives may be added to the extracts during processing to minimize the growth of contaminants.

Drying varies greatly depending on the enzyme. Vacuum driers at low temperature may be required, but most commercial enzymes can be dried in a current of air at 40 – 50°C with only slight loss in activity. Some enzymes (e.g. laundry enzyme) are now being dried in spray driers.

Development of a new enzyme requires laboratory and plant work to determine the correct processing conditions. Modifications in processing may be required to increase the yield to a profitable level. Several industrial enzymes show a recovery of 70–80% of the culture activity in the final dry product. Liquid products show a higher recovery because the final precipitation and drying steps are eliminated.

3. Applications

Enzymes derived from animal and plant sources have been employed in relatively large amounts for many years. Many of these applications will probably continue to use the enzymes derived from these sources, because certain of these enzymes have specific qualifications for the purpose or involve an inexpensive byproduct from some other process. As the cost of the plant or animal source increases, the search for a microbial replacement will be accelerated. Some commercial applications of enzymes, especially those of microbial origin, represent new, rapid, controlled processes for carrying out conversions which were formerly brought about slowly or erratically by microorganisms contaminating a natural raw material. There are a number of industrial applications in which the living cell continues to be the source of microbial enzyme; the best known of these is the use of yeast in alcohol production.

Although a process for the production of diastatic enzyme from fungi was patented (Takamine, 1894) nearly seventy five years ago, the contamination of the preparation with bacteria prevented its use as a replacement for malt diastase. Almost 40 years elapsed before other microbial enzymes were developed and found their place in industrial processes. These microbial enzymes have either supplanted, partially replaced, or surpassed plant and animal enzymes.

Microbial enzymes are generally not exact counterparts of the plant or animal enzyme which they replace, but there has been an unfortunate tendency to carry over the nomenclature from the past and apply it to the microbial enzyme. This has been particularly true in the case of

Table 5. Uses and sources of industrial enzymes

| Industry | Application | Enzyme | Source | US Suppliers ^a of microbial enzyme for use |
|-----------------|--|----------------------------|--|---|
| Baking | Bread dough modification | protease | fungus | 4, 7, 10 |
| | Flour supplementation | amylase | malt at mill fungus at bakery | — 4, 7, 10 |
| | Bleaching of natural flour pigments | lipoxidase | soybean flour | — |
| Beer (Brewing) | Mashing | amylase | malt | — |
| | Chillproofing | protease | fungus, bacterial, papain, pepsin | 10 |
| | Low dextrin (low calorie) | amyloglucosidase | fungus | 4, 10 |
| Candy | Soft center candies, invert sugar fondants | invertase | yeast | 1, 8, 9, 10 |
| (Confectionery) | Recovery of sugar scrap | amylase | bacterial | 7 |
| Cereal | Preparation of precooked cereals | amylase | malt, fungus | 4, 7, 10 |
| Coffee (Cocoa) | Removal of mucilage from berries | pectinase | fungus | 7 |
| | Thinning of concentrates | amylase, pectinase | fungus | 4, 7, 10 |
| Dairy | Milk curd formation for cheese making | rennet | fungus calf stomach | 5 — |
| | Protein hydrolyzates | proteases | papain, pancreas, fungus, bacterial | 4, 7, 10 |
| | Removal of oxygen from egg whites | glucose oxidase + catalase | fungus | 1, 4 |

^a Listing of United States Suppliers of Microbial Enzymes:

1. Fermco Laboratories,
2. Grain Processing Corporation,
3. Lederle Laboratories,
4. Miles Laboratories, Inc.,
5. Chas. Pfizer and Company,
6. Premier Malt,
7. Rohm and Haas Company,
8. Standard Brands Inc. (Clinton Corn Processing Company),
9. Universal Foods,
10. Wallerstein Company.

Table 5. (continued)

| Industry | Application | Enzyme | Source | US Suppliers ^a of microbial enzyme for use |
|-------------------------------------|---|------------------------------------|---|---|
| Distilling | Premalt (liquefaction) | amylase | bacterial malt | 4 — |
| | Mash conversion (saccharification) | amylase amyloglucosidase | malt fungus | — 2, 4 |
| Fishing | Thinning of condensed solubles | protease | bacterial | 7 |
| Fruits Fruit juices | Clarification, filtration and concentration of juices | pectinase | fungus | 4, 7, 10 |
| | Debitting of grape fruit | naringinase | fungus | 7 |
| Laundry aids and dry cleaning | Presoak and washing | alkaline protease | bacterial | 5 |
| | Spot removal | protease, lipase, amylase | mixture of fungus, bacterial, pancreatic | 10 |
| Leather | Bating | protease | pancreatic, bacterial fungus | 7, 10 |
| Meat packing | Tenderizers | protease | papain, bromelain | 7, 10 |
| | Recovery of meat scraps | protease | fungus bacterial | 7 |
| Oil | Fracturing of oil wells | hemicellulase- cellulase | fungus | 7 |
| Paper | Modification of starches for sizes and coatings | amylase | bacterial | 4, 7, 10 |
| Pharmaceutical | Digestive Aids | protease, lipase, amylase | pancreatic fungus | — 4 |
| | Enhanced spreading of injectables | hyaluronidase | animal testis | — |
| | Test papers for glucose | glucose oxidase + peroxidase | fungus, horse- radish bacterial, | 4 3 |
| | Antiinflammatories | Streptokinase, trypsin | bacterial, pancreatic | 3 |

Table 5. (continued)

| Industry | Application | Enzyme | Source | US Suppliers ^a of microbial enzyme for use |
|--------------|---|-------------------|-------------------------------|---|
| Photographic | Recovery of silver from used films | amylase | fungal | 4, 7, 10 |
| Starch | Various corn sirups | amylase | fungal | 4, 7, 10 |
| | Dextrose production | amyloglucosidase | fungal | 4, 2 |
| | Fructose production | glucose isomerase | microbial and used internally | 8 |
| Textile | Starch desizing | amylase | bacterial | 4, 6, 7, 10 |
| Wines | Improved yield of juice Clarification and filtration | pectinase | fungal | 4, 7, 10 |

microbial enzymes which replace those derived from malted cereals. Although the microbial enzyme may not be an exact counterpart of the enzyme derived from other sources, it is usually possible, by selection of organism and growing process, to tailor-make an enzyme which can perform under the required conditions. Some times combinations of microbial enzymes are required.

It may be well to review the progress which has been made in the replacement and supplementation of commercial plant and animal enzymes during the period from 1930–1969 and also those commercial processes which have evolved from applications of microbial enzymes. A very comprehensive monograph covering the use of enzymes in food is available (Reed, 1966). A fairly complete listing of enzymes used commercially, and of their sources is given in Table 5.

a) Commercial Enzymes of Plant Origin and the Extent of their Replacement by Microbial Enzymes

α) Cereal Amylases

Cereal amylases represent the most widely used commercial enzymes. The amylases derived from malted barley, and to a lesser extent malted wheat, have been employed for centuries in the preparation of malted beverages. In this application, both the liquefying endoamylase (α -amylase) and the saccharifying exoamylase (β -amylase) are required to bring about the required liquefaction of the grain in the mashing

process, and the production of fermentable sugar (maltose) utilized by the yeast to produce alcohol. The liquefaction of the starch in the mash can be carried out by the microbial liquefying amylase obtained from *Bacillus subtilis*. The liquefying amylase derived from *Aspergilli* (*flavus-oryzae*, generally) is less suitable because of its lower heat stability. The saccharification of the starch may be accomplished by amyloglucosidase derived from either *Aspergillus niger* or *Rhizopus oryzae*. Fungal amyloglucosidase produces glucose rather than maltose, but the extent of conversion to fermentable sugar is greater than with malt α -amylase, because less reversion products (i.e. isomaltose) are produced. The yields of alcohol are therefore improved by the fungal supplements. In addition, the amyloglucosidase is less affected than malt amylase by the presence of bacteria and low pH in the fermentation step. In the production of beverage alcohol the complete replacement of malt is not indicated, because the malt reportedly imparts certain desirable flavours not obtained with the fungal preparations. Large distillers are interested in preparing their own amyloglucosidase for captive use (van Lanen and Smith, 1969). Malt still occupies a strong position in the preparation of beer because of considerations of flavor. One commercial application of fungal amyloglucosidase in the preparation of low dextrin beer has been disclosed (Gablinger, 1968), and has had wide promotion as "low calorie beer". The resulting beer also has improved clarity.

The cereal amylases also find extensive use in the baking field as flour supplements. It is the α -amylase activity which must be added to flour to give the needed high gassing power (active release of CO₂) in fermenting dough. Flour usually has an excess of β -amylase, but, since the early part of the 20th century, malt α -amylase in the form of malted wheat or barley flour has been added at a level of 0.25–0.5% at the mill to bring about nearly complete conversion of damaged starch to fermentable sugar. In modern bakery practice fungal α -amylase (from *Aspergillus flavus oryzae*) is added to increase the fermentation rate at the dough stage and impart other desirable properties, such as crust color and soft crumb, to the finished bread. The fungal supplements are more suitable than additional malt, because the fungal supplements can be prepared at high activities, either without protease or with a predetermined level of protease. The fungal supplements also have a lower level of bacterial contamination. The capability of controlling the protease level in the supplement is critically important in baking operations. The ease with which this factor can be controlled by standardized fungal preparations has stimulated this application of microbial enzymes. Fungal protease in carefully measured amounts are used in commercial baking operations to lower the time and work input required to mix and mellow dough prepared from modern strong

flours. Tableted forms of diastase and protease provide a wide latitude in the baker's choice of flour supplements.

A number of other commercial processes involving starch modification which formerly employed malt amylase, such as the desizing of starched textiles and the preparation of starch sizes and cold swelling starches, have been captured by microbial enzymes. These applications generally use amylases derived from *Bacillus subtilis*, because the high thermal stability of these enzyme preparations allow operation at high temperature and because the less extensive hydrolysis of the starch by the bacterial preparations gives more desirable products. The liquefying activity of bacterial amylases can usually be prepared more cheaply than the fungal amylase.

β) Plant Proteases

Three other plant enzymes, papain, bromelain, and to a lesser extent ficin, have found acceptance in the food industry as proteases. Papain is derived from the latex of the fruit, leaves, and trunk of *Carica papaya*, and bromelain from the fruit and stems of pineapple plants. These enzymes are used to prevent the hazing of beer when chilled (Chill-Proofing) by modifying the protein. Other applications for these plant proteases are in meat tenderizers and digestive aids. Ficin from the latex of *Ficus carica* is used to a much lower extent, perhaps because of its marked action on native protein and difficult handling. Proteases from *Aspergillus flavus-oryzae*, and to a lesser extent from *Bacillus subtilis*, have been used to replace and supplement these plant proteases in all applications, but papain continues to have the widest acceptance.

γ) Lipoxidase

Lipoxidase derived from soybean flour is widely used to decolorize the natural pigments in wheat flour. Although lipoxidase activity is exhibited by many microbial (fungal) preparations, these have not been able to replace the soybean lipoxidase.

b) Commercial Enzymes of Animal Origin and the Extent of their Replacement by Microbial Enzymes

α) Pancreatic Preparations

Crude and purified proteases derived from the pancreas have a long history of commercial use. Many of the applications employ a cruder form, pancreatin, but some drug applications require purified trypsin.

Processes carried out at a pH of 7–9 represent the most efficient use of this type of enzyme. The first industrial process involving this type of protease was in the bating of hides to remove the debris from the skin after liming, and to impart softness and air exchange to the finished leather. Although pancreatic bates are still in general use proteases derived from *Aspergillus flavus-oryzae* and *Bacillus subtilis* now supplement them.

Pancreatic preparations are also used in the preparation of hydrolyzed protein products and bacteriological media. Fungal and bacterial proteases alone and in mixture can be used to prepare similar products. The cost and attainment of needed properties determine the choice of enzyme.

Pancreatic preparations have been widely used as digestive aids, because they contain proteases, amylase and lipase. They have been prescribed for patients who have pancreatic disorders or after removal of the pancreas. The various activities present in the pancreatic preparations can be duplicated by *in vitro* methods from blends of microbial enzymes derived from *Bacillus subtilis*, *Aspergillus flavus-oryzae* and *Aspergillus niger*. Cellulase derived from *Aspergillus niger* is often added to the microbial preparation. The pancreatic preparations still hold the major share of the market, but this could be a useful application for the right combination of microbial enzymes.

Pancreatic preparations also make up a large part of the "spot removal" enzymes employed in dry cleaning establishments to remove stubborn stains. The activity of these preparations is usually enhanced by adding microbial amylase, protease, and lipase. Pancreatic preparations were patented (Röhm, 1913) and used in Europe as presoaks long before the era of modern-day laundry aids. These applications represent the forerunner of enzyme-containing detergents, but the pancreatic preparations could not withstand the high temperatures and pH used in the washing cycle. The development of alkaline protease from selected strains of *B. subtilis* has solved many of the problems associated with this application, and advertising has accomplished the rest of this development.

β) Pepsin

Pepsin derived from the mucosa of hogs has found use in chill-proofing beer and as a digestive aid. Microbial proteases (and papain) can replace pepsin in chillproofing, but there are no commercially available microbial proteases which show the low pH optimum (1.8–2.2) exhibited by pepsin.

γ) Rennet

Rennet is used extensively in the production of cheese. This enzyme is found in the fourth stomach of the calf; it converts casein into para-casein, which, in the presence of calcium, precipitates to form an elastic curd. Much commercial rennet is contaminated with pepsin which tends to act strongly on casein and thereby produces a weak curd with off flavor. Many microbial proteases can clot milk, but only recently have microbial preparations been produced which can replace calf rennet to prepare cheeses of good flavor. Microbial preparations from *Endothia parasitica* and *Mucor pusillus* var. Lindt seem to be the most promising, and these have been used commercially. The new sources of rennet were reviewed recently (Sardinas, 1969).

c) Commercial Processes which Use Microbial Enzymes

α) Invertase

Invertase prepared from *Saccharomyces cerevisiae* and *S. carlsbergensis* is used in the preparation of soft center candies, fondants, and invert sugars.

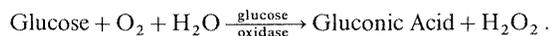
β) Pectinases

Pectinases are prepared from various species of *Aspergilli* and *Penicillia*. The commercial preparations contain a variety of pectic activities which include pectin esterase, polygalacturonase, polymethylgalacturonase and pectin transesterase. Before these enzymes were introduced in the early 30's (Willaman and Kertesz, 1931), processors of fruit juices found difficulty in obtaining high yields, and high pressing or filtration rates. Shelf stability was also a problem. The pectinases are now used to aid in the clarification, filtration, and concentration of many fruit juices, especially apple, grape, and berry juices. The pectinases are valuable in improving the yield of juice in the preparation of wines and hasten the aging process. Concentrated fruit juices can be prepared, stored under refrigeration, and shipped without gelation. The juice concentrates can be diluted to single strength at any convenient location or time. Jellies may be prepared from the concentrates by adding pectin.

Pectinases are also used in the processing of green coffee beans to hasten the removal of the jelly which surrounds the coffee cherry. Natural fermentation may give a coffee bean of inferior quality.

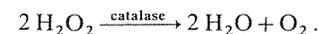
γ) Glucose Oxidase

Glucose oxidase is produced by a number of *Aspergilli* and *Penicillia* (*A. niger* and *P. notatum*). It catalyzes the following reaction:



It is used widely in specific test procedures for the presence of glucose, for example in the diagnosis of diabetes or in analyzing food products where the formation of hydrogen peroxide denotes the presence of glucose. It also finds use in quantitative analytical procedures for glucose in which the glucose oxidase reaction is coupled with horseradish peroxidase and an organic compound acting as a hydrogen donor.

The largest commercial uses of glucose oxidase require the presence of catalase to remove the peroxide formed in the glucose oxidase reaction:



This system is used to remove sugar present in low concentration in egg whites. The system may also be used to remove small amounts of oxygen from the head space of sealed containers by adding a packet containing glucose, oxidase, and catalase.

δ) Sirup Enzymes

Certain amylase preparations derived from *Aspergillus oryzae* can increase the extent of conversion of corn sirup to fermentable sugars without producing the unwanted crystallization characteristic of sirups made by the simple acid hydrolysis of starch (Dale and Langlois, 1940). A considerable amount of work by manufacturers of corn sirup and enzyme suppliers has been carried out after this original observation. As a result a whole series of corn sirups representing various degrees of conversion and combinations of dextrose, maltose, and oligosaccharides were developed using various microbial enzymes alone and in mixture. More recently starch has been converted almost quantitatively to crystalline glucose by the enzyme process. This new process awaited the development of a commercial source of amyloglucosidase. Amyloglucosidases derived from *A. niger* and *R. oryzae* are used in the production of glucose, and *A. oryzae* Amylase alone or mixed with amyloglucosidase is used to prepare the various sirup compositions. Some sirup manufacturers have found it advantageous to prepare their own amyloglucosidase.

Although acid hydrolysis is most commonly used to prepare the starting sirup, the liquefying amylase from *B. subtilis* can be used to thin the initial starch paste without the need for acid resistant tanks. The process then becomes an all-enzyme process. The all-enzyme process produces a smaller amount of reversion products than the acid-enzyme process.

ε) Glucose Isomerase

After manufacturers developed sirups with properties useful in various applications, it became clear that increasing the sweetness of sirups by

converting a large part of the glucose to fructose would be a good thing. A corn sirup as sweet as sucrose could be produced in this way. The major drawbacks to the use of alkali to accomplish the desired isomerization are the low degree of isomerization (33%), the large amount of decomposition products, and the salt formed in neutralizing the alkali. There has been a great deal of interest in microbial glucose isomerase since 1960, particularly in Japan where sucrose is in short supply. It was reported (Takasaki *et al.*, 1969) that the commercial production of glucose-fructose sirups with the glucose isomerase of *Streptomyces* is practical. Some U.S. producers of corn sirup are also marketing sirups prepared with glucose isomerase.

η) Laundry Enzymes

It was noted above that the use of pancreatic enzymes in the washing of clothes was first patented in 1913, but the unstable nature of pancreatic preparations limited their use to presoak operations. The use of pancreatic enzymes in Europe to presoak clothes never died out completely. This vast market was activated by the development of alkaline protease in 1963–1964 by Novo Industries of Denmark and Royal Netherlands Fermentation Industries from the submerged culture of selected strains of *Bacillus subtilis*. The microbial enzyme therefore resulted in this major development. The value of the enzyme lies in its ability to solubilize stains caused by proteinaceous material. The alkaline protease is stable at high pH in the presence of phosphates and other ingredients of detergents. Proteolytic activity is also well retained up to 60° C. It seems likely that presence of other enzymes (e.g. lipase, amylase) with high tolerance to heat and detergent would be good supplements to the protease and would extend the range of stains which can be easily removed. Some products now on the market do contain added amylase, but most do not. The development of the commercial enzymes used in detergents has been reviewed in a number of articles (Hoogerheide, 1968; Koch, 1969; Wieg, 1969). The development of laundry enzymes has stimulated the entry of several new manufacturers into the industrial enzyme field.

θ) Lactase

Fluid milk contains about 5% of lactose. Lactose has low solubility and low sweetness, and a significant part of the world's population does not tolerate lactose in the diet. For these reasons the conversion of lactose to the component sugars, glucose and galactose would be worthwhile. Commercial lactases have been developed from lactose-fermenting yeasts and shown to have utility in the hydrolysis of lactose in a variety

of milk products (Sampey and Neubeck, 1955). The market for these lactase preparations has not developed to any extent. There has also been some interest in using lactase to hydrolyze the lactose in the milk solids added to bread dough to get a more lively fermentation. This process, also is not used commercially.

d) Outlook for Microbial Enzymes

Although the transition from animal and plant enzymes to microbial enzymes was relatively slow in "catching on", it seems likely that the present trend will continue. Commercial facilities for preparing enzymes by growing microorganisms are now available in a number of companies. The fullest possible utilization of these plants is economically sound. The excess or idle capacity available in plants for making antibiotics offers a source of production for the submerged growth of microorganisms suitable for enzyme production. The state of the art for making enzymes has advanced rapidly over the last two decades, and the problems of scale-up from the laboratory to the plant are now less formidable. The relative ease (i.e. compared to plants or animals) by which microorganisms can be screened, mutated, and otherwise developed for the production of a specific enzyme reduces the necessity for finding plants or animals as enzyme producers. A much greater variety of organisms is available, and the microorganisms are much more efficient.

There are obvious hazards in looking for enzymes in the realm of pathogenic organisms, and there are, of course, limitations by the U.S. government which restrict the utilization of enzymes derived from microorganisms in foods until they have been shown to be safe. As a result of these regulations, only a limited number of microorganisms were generally recognized as safe (GRAS) at the time (1958) when the regulations went into effect. The GRAS list included certain enzymes prepared in accordance with sound manufacturing practice and derived from *Saccharomyces cerevisiae* (invertase), *S. fragilis* (lactase), *Bacillus subtilis* (carbohydrase and protease), *Aspergillus flavus-oryzae* group (carbohydrase and protease) and *A. niger* (carbohydrase, cellulase, glucose, oxidase, catalase, pectinase, and lipase). Certain other microorganisms have been specifically cleared after presenting proof of the safe nature of the organisms and enzymes to the proper authorities. The same restrictions do not apply at present in many other applications, but good manufacturing practice demands that neither the producer nor the user of an enzyme product be exposed to health hazards.

There are several areas where microbial enzymes may have a good chance of commercial success.

α) Medical Uses

Reports (Chang *et al.*, 1967; Chang and Poznansky, 1968) about investigations on the application of encapsulated microbial enzymes to medical uses are available. By careful modification of the material, capsules can be obtained which are acceptable to body tissues, i.e., they produce neither clotting nor antibody formation. In this, they have an advantage over enzymes covalently linked to insoluble substrates. The application of encapsulated enzymes to a system in which, for one reason or another, a necessary enzyme is deficient has been investigated. The capsules may be inserted into the animal, or blood may be passed over a shunt system containing capsules, which is outside the body. Catalase deficiency in mice was overcome by encapsulated catalase and urea in the blood was removed with encapsulated urease. Should the products of enzymatic action be toxic, other capsules containing adsorbents may be simultaneously incorporated to lessen the toxicity. The method appears to have potential in the treatment of various enzyme-deficiency diseases.

One of the more hopeful prospects for a significant volume of pure enzyme is in the field of tumor therapy. *L-asparaginase* can induce remission in certain tumors in the mouse, rat, and dog, and suppress human leukemia by depleting an amino acid essential for neoplastic cells. This discovery has stimulated the search for *L-asparaginase*. Asparaginases are available from a number of microbial sources including fungi, yeast, and bacteria, but thus far, tumorinhibitory activity has been demonstrated only with the asparaginase from *Escherichia coli*, *Erwinia aroidae*, and *Serratia marcescens*. Asparaginase from *E. coli* has been prepared on a large scale.

From preliminary experimental evidence, collagenase derived from *Clostridium histolyticum* may prove to be useful as a collagenolytic agent for the treatment of ruptured vertebral discs, the removal of necrotic tissue following cryoprostectomy, and the promotion of allograft survival in teeth. This enzyme may have some therapeutic utility in medicine and surgery where debridement is a part of the treatment. The major areas of possible use are in the treatment of severe burns and various dermal lesions such as decubiti, Ischemic ulcers, and other infective soft tissue lesions.

β) Dental Applications

There have been a considerable number of investigations into the use of dextranase derived from a variety of sources to eliminate plaque formation on human teeth. Many of these studies have proved inconclusive, and a significant amount of further work will be necessary to

decide if dextranase can remove all plaque formed on the human teeth, and thereby significantly reduce the amount of dental caries occurring in human populations. It is conceivable that, once an enzyme or a combination of enzymes is found which will effectively perform this function, it will be included in formulations by commercial concerns now producing dental cleaning materials.

γ) Food Processing

As mentioned above, enzymes have found significant use in various applications in the processing of food materials. It is conceivable that a considerable amount of enzyme will be produced for use as processing aids to provide more efficient recoveries of the desired materials than are obtained through the less controllable chemical or mechanical processes. It is also conceivable that a significant number of enzymes will be discovered in the near future which will in effect do nothing more than offer over-all cost reduction in performing various steps in existing manufacturing processes.

The mushrooming demand for snack foods has stimulated an ever-increasing demand for new and different flavors to satisfy this market. It is conceivable that a significant number of enzymes will be isolated that will, either directly or indirectly, assist in flavor modifications for the production of new snack foods. It is likely that attempts will be made to provide enzymes for the selective modification of protein and carbohydrates by shortening their chain length. They might then impart new and different flavors or functionality to snack foods. The oils in which many deep fat-fried snack foods are prepared could be changed by a limited hydrolysis with selective lipases to impart an entirely new and different flavor. Work along these lines is progressing at a number of flavor and food companies.

It is extremely likely that enzymes will be used to aid in solving socio-economic-religious problems which arise during attempts to feed the undernourished populations with proteins obtained from exotic sources. These unusual food sources might include alfalfa or other plants, raw cellulose, soya beans, fish, and oil-grown microorganisms. Enzymes will probably find extensive use in the removal of certain materials now contained in existing food sources that render them unacceptable for human use. One example of this is the high level of undesirable RNA contained in single-cell protein derived from microbial growth on petroleum hydrocarbons.

δ) Waste Treatment

With the present interest in maintaining a clean environment increased emphasis will be placed on waste treatment, be it solid, liquid, or air. It

is likely that enzymes from microorganisms can assist in the degradation and effective destruction of solid and liquid wastes. In this area producers will not be restricted to organisms on the GRAS list to obtain the necessary activity. The enzymes now used in some of these applications fall considerably short of the desired goal.

Enzymes from microbial sources offer unlimited potential for efficient use in a wide diversity of applications. The successful exploitation of their capabilities requires only a combined effort between three essential disciplines, engineering, chemistry and biochemistry.

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