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52. Enzyme Applications in Encyclopedia of Chemical Technology, 4ᵗʰ ed., Ed. Kroschwitz, J.I., Volume 9, pp. 567-620, 1994.

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Novo Nordisk / Termamyl LC α-amylase information

53. Aunstrup, K., Andersen, O., Falch, E.A., and Nielsen, T.K. *Production of Microbial Enzymes* in Microbial Technology, 2ⁿᵈ ed., Vol. I, Eds. Peppler, H.J. and Perlman, D., Chapter 9, pp.282-309, 1979.

54. Aunstrup, K., *Production, Isolation, and Economics of Extracellular Enzymes* in Applied Biochemistry and Bioengineering, Volume 2, Enzyme Technology, Eds. Wingard, L.B., Katchalski-Katzir, E. And Goldstein, L, pp. 28-68, 1979.

The impact of environmental issues is apparent within the majority of *Encyclopedia* articles having counterparts in previous editions. For example, the effect of environmental concerns on manufacturing and other processes is evident in articles such as ALKALI AND CHLORINE PRODUCTS, ELECTROPLATING, MINERAL RECOVERY AND PROCESSING, PETROLEUM, and SODIUM CARBONATE. Concern about the environment has also played a role in the types and quantities of materials produced (see CHLOROCARBONS AND CHLOROHYDROCARBONS; COATINGS, MARINE; CORROSION AND CORROSION CONTROL; PIGMENTS, INORGANIC; POISONS, COMMERCIAL) as well as with regard to the technology employed (see COAL CONVERSION PROCESSES; FOOD PACKAGING).

ENZYME APPLICATIONS

Industrial, **567**
Therapeutic, **621**

INDUSTRIAL

Enzymes, like other proteins (qv), are composed of up to 20 different amino acids (qv). They accelerate hundreds of reactions taking place simultaneously in the cell and its immediate surroundings, and are essential for the development and maintenance of life.

Industrial applications of enzymology form an important branch of biotechnology. Enzymatic processes enable natural raw materials to be upgraded and turned into finished products. They offer alternative ways of making products previously made only by conventional chemical processes.

The detergent industry is the largest user of industrial enzymes. The starch industry, the first significant user of enzymes, developed special syrups that could not be made by means of conventional chemical hydrolysis. These were the first products made entirely by enzymatic processes. Materials such as textiles and leather can be produced in a more rational way when using enzyme technology. Foodstuffs and components of animal feed can be produced by enzymatic processes that require less energy, less equipment, or fewer chemicals compared with traditional techniques.

The development of the submerged fermentation technique resulted in tremendous progress in the field of industrial enzymology. This technique was used in the early 1950s for the production of bacterial amylases for the textile industry.

History

In 1833 an amylase from germinating barley was recovered and called diastase (1). Like malt itself, this product converted gelatinized starch into sugars,

primarily maltose. Shortly thereafter, Berzelius proclaimed the existence of non-living catalysts, and Schwann (2) reported on his observation and purification of pepsin.

Throughout the second half of the nineteenth century, several schools of thought debated the connection between biological catalysis and life or vital forces, ie, *vis vitalis*. The Pasteur school firmly believed that alcoholic fermentation was a vital act which could not take place without the presence of viable organisms (3). Another school (4) was convinced fermentation was the result of a common chemical process, and that yeast was a nonviable substance continuously engaged in the process of breaking down other substances. A final group of scientists strongly supported the original concept of Berzelius that enzymes and living microorganisms, at that time known collectively as unorganized ferments or simply ferments, were two very different phenomena (5–7).

In 1878 the term enzyme, Greek for "in yeast," was proposed (8). It was reasoned that chemical compounds capable of catalysis, ie, ptyalin (amylase from saliva), pepsin, and others, should not be called ferments, as this term was already in use for yeast cells and other organisms. However, proof was not given for the actual existence of enzymes. Finally, in 1897, it was demonstrated that cell-free yeast extract ("zymase") could convert glucose into ethanol and carbon dioxide in exactly the same way as viable yeast cells. It took some time before these experiments and deductions were completely understood and accepted by the scientific community.

Early Industrial Enzymes.   Enzymes were used in ancient Greece for the production of cheese (9). Early references to this are found in Greek epic poems dating from about 800 BC. Fermentation processes for brewing, baking, and the production of alcohol have been known since prehistoric times.

The era of modern enzyme technology began in 1874 when the Danish chemist Christian Hansen produced the first industrial batches of chymosin by extracting dried calves' stomachs with saline solutions.

One of the first large-scale industrial productions and applications of enzyme technology to emerge in the twentieth century was the production of fungal amylases by the surface or semisolid culture fermentation of *Aspergillus oryzae* on moist rice or wheat bran. This process, developed by the Japanese scientist Takamene, was inspired by traditional koji fermentation in trays used for the production of foodstuffs and flavoring based on soy protein; this method has been largely replaced by the more efficient submerged fermentation. Takamene's product was called Takadiastase, and is still in use as a digestive aid.

Textile and Leather Industries.   At about the same time Takamene was developing his fermentation technique, enzymes were introduced in the desizing of textiles, ie, the process by which all the starch paste, which has served as strengthening agent and lubricant to prevent breaking of the warp during the weaving process, is removed from the fabric. Historically, textiles were treated with acid, alkali, or oxidizing agent, or soaked in water for several days so that naturally occurring microorganisms could break down the starch. These methods were difficult to control, and sometimes damaged or discolored the material. The application of crude enzyme extracts, from malt or pancreatic glands, in desizing was a significant step forward. The next development was the introduction, on a small scale, of a bacterial amylase from *Bacillus amyloliquefaciens* by Boidin and

Effront in 1917. Mass production did not begin until after World War II, when submerged fermentation was developed as a substitute for the original surface fermentation.

The German chemist and industrial magnate Dr. Otto Röhm, founder and partner of Röhm and Haas in Darmstadt, Germany, was responsible for the further development of industrial enzymes. He studied the leather bating process, ie, the operation in the processing of hides and skins that precedes the tanning step. Bating removes some of the protein that is not essential for the strength of the leather (qv) and that might otherwise prevent the leather from achieving the suppleness and softness of touch required in numerous products. It serves to control the quality of leather; eg, stiff leather used for soles is only lightly bated, but the soft qualities required for gloves result from intense bating. The traditional bating process used dog and pigeon excrements. Röhm's theory was that digestive enzymes were responsible for the bating process, and he correctly concluded that extracts of the pancreas might be used directly for bating.

Detergent Industry. In 1913, Dr. Röhm assumed that dirt in fabrics used by humans was to a large degree composed of fats and protein residues and that the well-known ability of tryptic enzymes to break down fat and protein might be exploited in laundry cleaning (10); trypsin was subsequently added to the wash (11). The concept was immediately commercialized by Röhm and Haas in the presoak detergent Burnus, consisting of soda and small amounts of pancreatin. It sold for the following 50 years in Europe, although it was never a commercial success.

During World War II, a severe shortage of fats and soap inspired the development of another enzymatic presoaking agent, Bio 38 (Gebrüder Schnyder). Some years later, Bio 40, a product containing a bacterial protease, was launched onto the market. This protease was a considerable improvement over the previous pancreatic products, but still suffered from the disadvantage of a neutral pH optimum, giving it low activity and stability in a washing solution.

In 1958, the microbial alkaline protease Alcalase (Novo Industries) was produced by fermentation of a strain of *Bacillus licheniformis*. It had high stability and activity at pH 8–10, was marketed in 1961, and was incorporated into Bio 40. However, it was not until the successful marketing of the presoaking agent Biotex in 1963 that detergent manufacturers saw the true possibilities of enzymes.

Several new detergent enzymes have emerged on the market (Table 1). Truly alkaline proteases, introduced in 1974 and 1982, were fermented on strains of *Bacillus lentus/firmus*. These enzymes have a pH optimum between 9 and 11, and have taken important market shares from Alcalase.

In 1989, two enzymes based on genetic engineering techniques were introduced, ie, a cloned alkaline protease (IBIS) and a protein engineered Subtilisin Novo (Genencor, California). Lipase and cellulase types of detergent enzymes have also begun to appear.

Starch Industry. Enzymes have been of great value to the starch industry since the 1960s. In the 1950s, fungal amylases were used in the manufacture of specific types of syrups. Early in the 1960s, the enzyme amyloglucosidase made it possible to completely break down starch into glucose. A few years later, most glucose production switched from the old acid hydrolysis method to the enzymatic

Table 1. Available Industrial Enzymes, 1993

Year launched	Enzyme type	Brand names[a]	Company
1950	bacterial α-amylase	BAN	Novo Nordisk
		Canalpha	Solvay Enzymes
		Rapidase	IBIS
1963	protease with a low alkaline pH optimum	Alcalase	Novo Nordisk
		Maxatase	IBIS
		Optimase	Solvay Enzymes
		Superase	Pfizer
		Milezyme	Solvay Enzymes
1965	amyloglucosidase	AMG	Novo Nordisk
		Amigase	IBIS
		Optidex	Solvay Enzymes
		Optilase	Solvay Enzymes
		Diazyme	Solvay Enzymes
		Spezyme	Genencor International
1973	thermostable α-amylase	Termamyl	Novo Nordisk
		Maxamyl	IBIS
		Takatherm	Solvay Enzymes
		Optitherm	Solvay Enzymes
		Kleistase	Daiwa Kasei
1973	microbial rennet (chymosin)	Rennilase	Novo Nordisk
1974	protease with high alkaline pH optimum	Esperase	Novo Nordisk
1976	glucose isomerase	Sweetzyme	Novo Nordisk
		Maxazym	IBIS
		Optisweet	Solvay Enzymes
		Spezyme	Pfizer
		Spezyme	Genencor International
		Takasweet	Solvay Enzymes
1982	low temperature protease with high alkaline pH optimum	Savinase	Novo Nordisk
		Maxacal	IBIS
		Opticlean	Solvay Enzymes
1982	pullulanase	Thermozyme	Novo Nordisk
1986	cellulase for alkaline conditions	Celluzyme	Novo Nordisk
1988	lipase for alkaline conditions made by DNA technology	Lipolase	Novo Nordisk
1991	bleach-stable protease with high pH optimum made by protein engineering	Maxapem	IBIS
		Durazym	Novo Nordisk

[a]First product on market is listed first for each enzyme type.

process, which gave better yields, higher purity, and easier crystallization. This process was further improved by the introduction of a new technique used for the enzymatic pretreatment (liquefaction) of starch. A new and extremely thermostable bacterial α-amylase was introduced in 1973.

In the 1980s the focus was on glucose isomerase, which converts glucose into fructose and thereby doubles the sweetness of the sugar. The first enzyme of this

type for the industrial market was launched in 1976 under the name Sweetzyme (Novo). The combination of this product with amyloglucosidase and α-amylase made it possible to use starch as a raw material for production of a sweetening agent with almost the same composition, amount of calories, and sweetening effect as ordinary cane or beet sugar. The new product, high fructose corn syrup (HFCS) or isosyrup, dominates the market for sweeteners (qv) (ca 1993).

Other Industrial Enzymes. Many new industrial applications of enzymes have been developed since the 1960s. Significant areas of applications include fungal proteases as substitutes for calf chymosin in cheesemaking, enzymes for brewing and for the wine (qv) and juice industries, and enzymes for the baking industry (see BAKERY PROCESSES AND LEAVENING AGENTS; BEER). Other areas, like paper (qv), pulp (qv), and bioremediation, are working intensively at implementing enzyme technology (see HAZARDOUS WASTE TREATMENT).

Genetic Engineering of Enzymes

With the usefulness of the first industrial enzymes, scientists and companies began to look for sources of new and better enzymes. Originally, attention was focused on animal and plant material, ie, the pancreas and malt. Although these could well serve as cheap raw materials, it became clear in the 1950s and 1960s that enzymes of microbial origin offered greater advantages; eg, microbes can be grown quickly and efficiently in fermentation tanks, and natural productivity of an enzyme can be increased by mutating microbes and screening for higher yielding mutant strains. Intense efforts were made to screen nature, in particular soil samples, for microbes producing enzymes that were suitable for industrial development. As a consequence, two dozen new enzymes were marketed within a few decades. Most of these production organisms belong either to the genus *Bacillus* (gram-positive bacteria) or *Aspergillus* (filamentous fungi) and most enzymes are either amylases or proteases. Amylases and proteases are enzymes that are usually secreted, ie, released from the cells into the growth medium. Therefore, the likelihood of high yields and simple purification procedures are increased. Since many *Aspergilli* and *Bacilli* have a characteristic ability to secrete amylases, proteases, and other enzymes, they were a natural target group for screening. Many of these species are easily isolated, are robust to handle under laboratory conditions, and grow well in fermentors on cheap industrial media to high cell densities.

Interesting enzymes were also detected in other types of microorganisms. Further development of most of these enzymes had to be given up for one or more reasons, ie, it was impossible to grow the microorganism successfully under industrial conditions; the microorganism was pathogenic, toxinogenic, or not completely safe to handle; enzyme purification was prohibitively expensive because, for instance, the enzyme was cell associated or contaminated with undesirable enzymes or other compounds; and yields of the desired enzyme could not be increased by classical mutagenesis and screening for higher yielding strains. Genetic engineering now offers new solutions to these problems.

Genetic engineering makes it possible to take DNA out of a donor cell, modify the DNA in a test tube, and introduce the modified recombinant DNA into a new

host cell in such a way that the recombinant DNA becomes a stable part of the genetic material of the host. Thus it is possible to transfer the gene encoding a particular enzyme from any exotic organism into a well-known production organism like a *Bacillus* or an *Aspergillus;* ie, a production organism may be obtained that both produces the desired enzyme of the exotic donor and has all the desirable properties of a safe industrial microorganism.

The process of isolating a particular gene by genetic engineering techniques is commonly referred to as molecular cloning or simply cloning (see GENETIC ENGINEERING). The efficiency of expression of a gene, ie, the amount of protein produced, depends on a number of properties of both the gene itself and the host organism. For industrial purposes, enzyme expression has to be very high, usually several grams per liter of fermentation liquid. To achieve this, it may be sufficient to choose a suitable host that resembles the donor organism. However, in most cases modifications by genetic engineering are necessary to increase the activity of the gene. Even then, combination of a suitable host with a gene designed to be very active may not be sufficient. One reason for this may be that the enzyme cannot be secreted, ie, transported across the cell membrane and cell wall of the host into the medium.

Almost all important industrial enzymes are naturally secreted. The substrates of these enzymes are large polymeric molecules such as proteins (qv), starch (qv), and cellulose (qv) that cannot be taken up by the cell and digested by internal enzymes. Consequently, the cell must secrete appropriate enzymes to utilize the chemical energy of the polymers. The cell does this by attaching to the enzyme a string of amino acids called a signal peptide. The signal peptide is recognized by and bound to the cell membrane, whereby the entire enzyme is attached to the inner surface of the membrane. While the signal peptide serves as an anchor, the enzyme is pulled through the membrane and subsequently released on its outer surface following removal of the signal peptide. A particular signal peptide usually works well with its natural enzyme in its natural host. When cloned in another host, the yield of the enzyme can be increased by replacing the native signal peptide with one known to function well in the new host. If the enzyme is naturally intercellular, it may not be possible to obtain secretion with any signal peptide because the enzyme rapidly folds in a way that prevents secretion. Thus the ability to be secreted also depends on the properties of the enzyme itself.

Host Microorganisms. As of this writing, only microorganisms are used as recombinant production organisms for industrial enzymes; therefore, tissue cultures and transgenic plants and animals are not dealt with herein. The choice of host microorganism for production of industrial enzymes is often critical for the commercial success of the product. Potential hosts should give sufficient yields, be able to secrete large amounts of protein, be suitable for industrial fermentations, produce a large cell mass per volume quickly and on cheap media, be considered safe based on historical experience or evaluation by regulatory authorities, and should not produce harmful substances or any other undesirable products. Few microorganisms fulfill all the above criteria and are used for production of industrial enzymes. Important hosts are presented as follows.

Escherichia coli. The genetics of this gram-negative bacterium are very well known. For this reason, many of the first efforts to produce recombinant products

from this microorganism were successful. However, because of the importance of the other criteria listed above, many efforts failed. *E. coli* is only used to produce the milk-clotting mammalian protease chymosin [*9001-98-3*] (rennin).

Bacilli. This very diverse group of gram-positive bacteria includes several historically well-known enzyme producers that fulfill all the above criteria including the ability to secrete large amounts of enzyme. It is generally known that recombinant *B. subtilis, B. licheniformis,* and *B. alkalophilus* strains are used for enzyme production.

Aspergilli. The filamentous fungi also include a number of well-known enzyme producers. Among these, *A. oryzae* was used as a host for lipase, the first industrial enzyme produced from a genetically engineered organism and sold on a large scale. *A. niger* is used for the production of chymosin. Recombinant production organisms derived from these two species may be used for production of several enzymes.

Yeast. Several yeast species, including *Saccharomyces cerevisiae* (baker's yeast) and *Kluyveromyces lactis,* are good candidates for the production of certain industrial enzymes, although their ability to secrete is much inferior to *Bacilli* and *Aspergilli.* The best-known example of *K. lactis* is used for commercial production of chymosin [*9001-98-3*].

It is increasingly evident that even when an efficiently expressed gene is inserted into a suitable host, the optimal production strain is rarely obtained. Among the properties that may need to be improved are genetic and product stability.

One method used to achieve genetic stability is to insert the plasmid or the recombinant DNA directly into the chromosome. Since no cell can afford to lose a chromosome, this assures that the recombinant DNA is not lost as long as it remains an integral part of the chromosome.

Microorganisms that secrete proteins often secrete proteases. Many secreted enzymes are actually resistant to proteolytic degradation, at least in their natural host, but some are not. To maintain product stability, it may be necessary to remove or reduce the proteolytic activity of the host. This can be achieved by classical mutagenesis, particularly in those cases where the microorganism only produces one protease, or by replacing normal genes on the chromosome with genes that have been inactivated in the test tube. This precise modification normally does not affect other properties of the host, and proteases may be inactivated one after another. This approach, however, requires that suitable genetic engineering techniques be applied to the particular host strain, and that each of the proteases is cloned.

Engineering of New Enzymes. There are approximately 10 million plant and animal species on Earth, and most of these contain hundreds of enzymes different from the enzymes of any other species. Furthermore, new enzymes are continuously created in nature by evolutionary processes. Sophisticated screening systems are now being developed to find new enzymes in microorganisms growing in hot springs, the deep sea, or other extreme environments. The prospect that a microorganism may not be cultivable no longer represents an insurmountable obstacle to the use of its genes to genetic engineering, and screening for microorganisms may be supplemented by screens for genes.

It is possible to modify and improve natural enzymes with protein engineering. Because the properties of any enzyme are determined by its three-dimensional structure, which in turn is determined by the linear combination of amino acids, it is possible, by a precise and site-specific modification of a gene, to change any amino acid into any other, or design a synthetic gene of any composition, and hence design a protein of any three-dimensional structure as long as basic physical and chemical rules are obeyed. Protein engineers can only predict the structural consequences of minor changes in enzymes (ca 1993). Protein-engineered enzymes on the market include a detergent protease that has been stabilized against chemical oxidation by replacement of two amino acids of the native enzyme.

It is likely that any new enzymes isolated by screeners will be quickly and routinely cloned by genetic engineers, and be sequenced and expressed as almost pure proteins. Protein chemists can then evaluate the properties of the new enzyme and determine its three-dimensional structure. This vast amount of information allows the protein engineers and their computers to design the enzymes of the future.

Abzymes.   To design an improved enzyme, protein engineers must know the structure of a protein resembling the desired enzyme. Since the number of different known types of enzymes is less than 1000, only a limited number of chemical reactions can be catalyzed by known naturally occurring enzymes. Catalytic antibodies, or abzymes, may overcome this limitation. Abzymes are antibodies having the ability to complex with the transition state of a chemical reaction, thereby lowering the free energy of the intermediate. Since the reaction rate is determined by the difference in free energy between the reactants and the transition state, the lowering of the energy of the transition state speeds up the process until chemical equilibrium, which is independent of catalysis, is reached. Since the immune system of mammals is able to produce approximately 100 million antibodies, it is likely that an antibody that recognizes any transition-state molecule, or a stable analogue of this, can be raised. The concept has been shown to work with at least seven different types of reactions, including a specific ester hydrolysis which was stimulated one millionfold by an abzyme. A cost-effective industrial abzyme must still be developed.

Catalytic Activity

Enzymatic Catalysis.   Enzymes are biological catalysts. They increase the rate of a chemical reaction without undergoing permanent change and without affecting the reaction equilibrium. The thermodynamic approach to the study of a chemical reaction calculates the equilibrium concentrations using the thermodynamic properties of the substrates and products. This approach gives no information about the rate at which the equilibrium is reached. The kinetic approach is concerned with the reaction rates and the factors that determine these, eg, pH, temperature, and presence of a catalyst. Therefore, the kinetic approach is essentially an experimental investigation.

The characteristics of enzymes are their catalytic efficiency and their specificity. Enzymes increase the reaction velocities by factors of at least one million

compared to the uncatalyzed reaction. Enzymes are highly specific, and consequently a vast number exist. An enzyme usually catalyzes only one reaction involving only certain substrates. For instance, most enzymes acting on carbohydrates are so specific that even the slightest change in the stereochemical configuration is sufficient to make the enzyme incompatible and unable to effect hydrolysis.

Usually the degree of specificity of an enzyme is related to its biological role. Subtilisins, proteolytic enzymes secreted by certain bacteria, are relatively undiscriminating about the nature of the side chains adjacent to the peptide bond to be cleaved, although these enzymes preferably hydrolyze peptide bonds on the carboxylic side of aromatic or aliphatic nonpolar side chains. This reflects the fact that the function of these enzymes is to hydrolyze larger proteins into smaller peptides that can be absorbed more easily by the microorganism. The digestive enzyme trypsin splits bonds on the carboxylic side of lysine and arginine residues only. The enzymes involved in blood clotting are even more specific than trypsin.

Another characteristic of enzymes is their frequent need for cofactors. A cofactor is a nonprotein compound that combines with the otherwise inactive enzyme to give the active enzyme. Examples of cofactors are metal ions such as Ca^{2+}, Cu^{2+}, Co^{2+}, Fe^{2+}, and Mg^{2+}, and organic molecules such as nicotinamide adenine dinucleotide (NAD) and flavin adenine dinucleotide (FAD).

Enzymes accelerate reactions by stabilizing the transition states, the highest energy species on the reaction pathway, and thereby decreasing the activation barrier. In other words, the combination of enzyme and substrate creates a new reaction pathway whose transition-state energy is lower than it would be if the reaction were taking place without the participation of the enzyme. Enzymes have evolved to bind the transition states of substrates more strongly than the substrates themselves. Therefore, compounds that mimic the structure of the transition state are often potent inhibitors of the enzyme-catalyzed reaction.

**Enzyme Kinetics.** A simple enzyme catalyzed reaction can be described:

$$E + S \rightleftharpoons ES \xrightarrow{k_{cat}} E + P \tag{1}$$

The enzyme, E, and the substrate, S, initially combine to form an enzyme–substrate complex, ES, which is held together by physical forces. In the second step the chemical process occurs, whereby the enzyme and the product, P, are liberated. This step is controlled by a first-order rate constant k_{cat}, called the catalytic constant or the turnover number. When deriving kinetic expressions, it is generally assumed that the concentration of enzyme is negligible compared to the concentration of substrate. Furthermore, it is assumed that what is being measured is the initial velocity v for the formation of products, ie, the rate of formation for the first few percent of the product. Under these conditions the products have not accumulated, the substrates have not been depleted, and the reaction velocity is generally linear with time. It is found experimentally that v is directly proportional to the concentration of enzyme $[E_0]$, but varies with the substrate concentration $[S]$ (Fig. 1). At low $[S]$, v is directly proportional to $[S]$. At higher $[S]$, however, this relation begins to break down, and at sufficiently high $[S]$ v tends toward a limiting value v_{max}. The Michaelis-Menten equation (eq. 2) expresses this relation quantitatively.

$$v = \frac{k_{cat}\,[E_0]}{1 + K_m/[S]} \quad \text{where } v_{max} = k_{cat}\,[E_0] \tag{2}$$

k_{cat} represents the maximum amount of substrate that the enzyme can convert to products per unit time; for most enzymes this lies in the range 1 to 10^4 s^{-1}. K_m is the Michaelis constant, and is the substrate concentration at which $v = \frac{1}{2}$ v_{max}; K_m for most enzymes lies in the range 10^{-6} to 10^{-1} M. The parameter k_{cat}/K_m is called the specificity constant because it determines the specificity between competing substrates. At low substrate concentrations k_{cat}/K_m is an apparent second-order rate constant. In order to obtain an accurate determination of k_{cat} and K_m, it is necessary to measure v at [S] where [S] $< K_m$ and at [S] where [S] $> K_m$. In cases where lack of solubility of the substrate makes measurements of v at [S] $> K_m$ impossible, only k_{cat}/K_m can be determined accurately; the individual kinetic parameters k_{cat} and K_m cannot be determined in these cases.
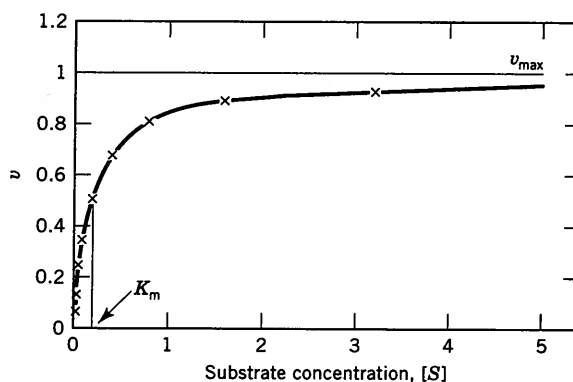
Fig. 1. Reaction velocity v as a function of substrate concentration for a reaction obeying Michaelis-Menten kinetics.

Kinetic parameters are often derived from a set of experimental data by rearranging the data into a linear form, eg, plotting $1/v$ as a function of $1/[S]$ (a Lineweaver-Burk, or double-reciprocal, plot) allows the determination of k_{cat} and K_m from the intercepts of the axes. However, the most accurately determined rates measured at high [S] tend to cluster around the origin, whereas the rate values at low [S], which are least accurately measured, are far from the origin; the latter data tends most strongly to determine the slope. In other words, rearranging the data also rearranges the error distribution, thereby invalidating the assumptions behind linear regression. This method is subject to large errors. However, software using nonlinear regression makes it possible to determine kinetic parameters directly from a plot of v as a function of [S], thus making linear rearrangements unnecessary.

In Figure 2, a double-reciprocal plot is shown; Figure 1 is a nonlinear plot of v as a function of [S]. It can be seen how the least accurately measured data at low [S] make the determination of the slope in the double-reciprocal plot difficult. The kinetic parameters obtained in this example by making linear regression on the double-reciprocal data are $v_{max} = 1.15$ and $K_m = 0.25$ (arbitrary units). The
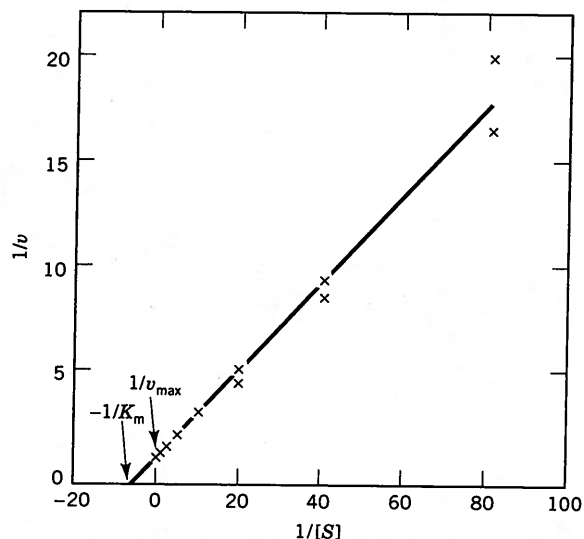
Fig. 2. The double-reciprocal plot of $1/v$ as a function of $1/[S]$. Two determinations of v for each $[S]$ are shown. $1/v_{max} = 0.87$ and $-1/K_m = -4.05$ (arbitrary units). Figure 1 is a nonlinear regression plot of the same data where $v_{max} = 1.00$ and $K_m = 0.20$ (arbitrary units).

same kinetic parameters obtained by software using nonlinear regression are $v_{max} = 1.00$ and $K_m = 0.20$ (arbitrary units).

Enzyme Inhibition. Enzyme inhibitors (qv) are reagents that bind to the enzyme and cause a decrease in the reaction rate. Irreversible inhibitors bind to the enzyme by an irreversible reaction, and consequently cannot dissociate from the enzyme or be removed by dilution or dialysis. Examples of irreversible inhibitors are nerve gases such as diisopropylphosphofluoridate [55-91-4] (DFP).

Reversible inhibition is characterized by an equilibrium between enzyme and inhibitor. Many reversible inhibitors are substrate analogues, and bear a close relationship to the normal substrate. When the inhibitor and the substrate compete for the same site on the enzyme, the inhibition is called competitive inhibition. In addition to the reaction described in equation 1, the competing reaction described in equation 3 proceeds when a competitive inhibitor I is added to the reaction solution.

$$E + I \rightleftharpoons EI \qquad \cdot \tag{3}$$

At high $[S]$, where the number of substrate molecules greatly outnumber the number of inhibitor molecules, the effect of a competitive inhibitor is negligible. Therefore, v_{max} is unchanged. However, the apparent K_m increases because of a higher degree of inhibition at low $[S]$. The effect of adding a competitive inhibitor is illustrated in Figure 3.

Effect of Temperature and pH. The temperature dependence of enzymes often follows the rule that a 10°C increase in temperature doubles the activity. However, this is only true as long as the enzyme is not deactivated by the thermal denaturation characteristic for enzymes and other proteins. The three-dimen-
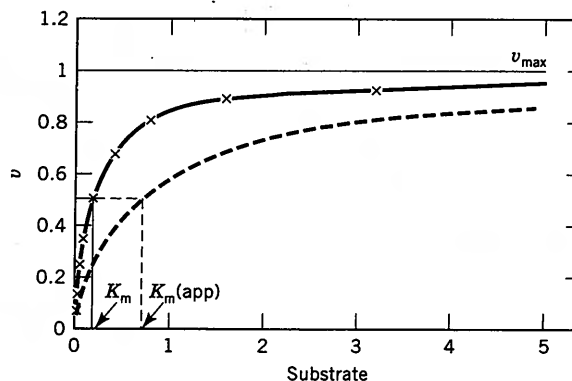
Fig. 3. The effect on kinetic parameters of adding a competitive inhibitor. Reaction velocity v as a function of [S] is shown. (–×–) Uninhibited reaction; (----) inhibited reaction. As indicated on the figure, the parameter K_m is increased by adding the competitive inhibitor; both curves eventually reach the same v_{max}.

sional structure of an enzyme molecule, which is vital for the activity of the molecule, is governed by many forces and interactions such as hydrogen bonding, hydrophobic interactions, and van der Waals forces. At low temperatures the molecule is constrained by these forces; as the temperature increases, the thermal motion of the various regions of the enzyme increases until finally the molecule is no longer able to maintain its structure or its activity. Most enzymes have temperature optima between 40 and 60°C. However, thermostable enzymes exist with optima near 100°C.

The pH dependency of enzyme-catalyzed reactions also exhibits an optimum. The pH optima for enzyme-catalyzed reactions cover a wide range of pH values. For instance, the subtilisins have a broad pH optima in the alkaline range. Other enzymes have a narrow pH optimum. The nature of the pH profile often gives clues to the elucidation of the reaction mechanism of the enzyme-catalyzed reaction. The temperature at which an experiment is performed may affect the pH profile and vice versa.

Enzyme Assays. An enzyme assay determines the amount of enzyme present in sample. However, enzymes are usually not measured on a stoichiometric basis. Enzyme activity is usually determined from a rate assay and expressed in activity units. As mentioned above, a change in temperature, pH, and/or substrate concentration affects the reaction velocity. These parameters must therefore be carefully controlled in order to achieve reproducible results.

Spectrophotometry, a simple and reliable technique, is often used in rate assays. This method can be used when the substrate or the product of the reaction absorbs in the uv or the visible region. In other cases, a nonabsorbing system can be coupled to a system in which the substrate or product absorbs in the uv or visible region.

An example of a direct spectrophotometrical assay is the use of synthetic peptide p-nitroanilide substrates to determine protease activity. The p-nitroaniline group liberated from the substrates by the protease can be determined spectrophotometrically at 410 nm. An example of an indirect (coupled) spectrophotometric assay is the determination of α-amylase using p-nitrophenylmalto-

heptaoside. Initially, the substrate is cleaved by the α-amylase and subsequently one of the reaction products, p-nitrophenylmaltotrioside, is cleaved by α-glucosidase, liberating p-nitrophenyl, a chromophore that can be measured at 405 nm.

Potentiometry is another useful method for determining enzyme activity in cases where the reaction liberates or consumes protons. This is the so-called pH-stat method. pH is kept constant by countertitration, and the amount of acid or base required is measured. An example of the use of this method is the determination of lipase activity. The enzyme hydrolyzes triglycerides and the fatty acids formed are neutralized with NaOH. The rate of consumption of NaOH is a measure of the catalytic activity.

Enzyme Nomenclature. The number of enzymes known exceeds two thousand. A system of classification and nomenclature is required to identify them unambiguously. During the nineteenth century, it was the practice to identify enzymes by adding the suffix -in to the name of their source. Names such as papain, ficin, trypsin, pepsin, etc, are still in use. However, this system does not give any indication of the nature of the reaction catalyzed by the enzyme or the type of substrate involved.

By the end of the nineteenth century a more descriptive system was in use. The suffix -ase was appended to the name of the substrate involved in the reaction, eg, amylase, cellulase, protease, lipase, urease, etc. Names that reflected the function of the enzyme with the suffix -ase were also used, eg, invertase, transferase, isomerase, oxidase.

A system based partly on historical names, partly on the substrate, and partly on the type of reaction catalyzed is far from satisfactory. In 1956, the International Union of Biochemistry set up a Commission on Enzymes to consider the classification and nomenclature of enzymes. The Commission presented a report in 1961 whose recommendations for naming and classifying enzymes were subsequently adopted (12). Enzymes are classified on the basis of the reactions they catalyze. Despite its apparent complexities, the system is precise and very descriptive, accommodating existing enzymes and serving as a systematic basis for the naming of new enzymes. All enzymes are placed in one of the six principal classes.

Number	Class	Type of reaction catalyzed
1	oxidoreductases	transfer of electrons
2	transferases	group-transfer reactions
3	hydrolases	transfer of functional groups to water
4	lyases	addition of groups to double bonds or the reverse
5	isomerases	transfer of groups within molecules to yield isomeric forms
6	ligases	formation of C–C, C–S, C–O, and C–N bonds by condensation reactions coupled to ATP cleavage

Each class is divided into groups or subclasses according to the type of reaction catalyzed. These groups are further subdivided according to the nature of

the substrate involved. Each enzyme is then assigned a four-digit classification number (EC number) based on this division, and a systematic name to identify the reaction catalyzed. A trivial name may be used if the systematic name is too long or cumbersome. For example, lactase (trivial name) catalyzes the conversion of lactose to galactose and glucose. It is given the systematic name of β-D-galactoside galactohydrolase, and the number EC 3.2.1.23, ie, 3-hydrolases; 3.2-glycosidases; and 3.2.1-hydrolyzing O-glycosyl bonds.

Production of Industrial Enzymes

Until about 1950, the predominant method of producing industrial enzymes was by extraction from animal or plant sources; by 1993, this accounts for less than 10%. With the exception of trypsin, chymosin, papain [9001-73-4], and a few others, industrial enzymes are now produced by microorganisms grown in aqueous suspension in large vessels, ie, by fermentation (qv). A small (5%) fraction is obtained by surface culture, ie, solid-state fermentation, of microorganisms (13).

Enzymes are usually sensitive to harsh physical and chemical conditions, and care must be taken during recovery and purification to avoid inactivation of the enzyme. This demands careful selection of production processes and conditions for each individual enzyme. Different methods are subsequently applied to assure the stability and activity of the enzymes during storage and application.

Fermentation.   The volume of industrial fermentors range from 20 m^3 to several hundred m^3, in a few cases exceeding 1000 m^3. A conventional fermentor (14) is shown in Figure 4. In most cases, oxygen is required by the microorganism, and air is supplied through a bottom sparger at a rate of 0.5 to 2 tank volumes per minute. The liquid is often agitated to improve gas transfer and mixing. Heat caused by microbial metabolism and agitation is removed through a cooling jacket or coil. Baffles are placed near the wall to increase mixing efficiency and prevent vortex formation. For microorganisms that are very sensitive to shear stress, air lift fermentors (15) can be used. In the air lift fermentor, the rising air bubbles provide the mixing and circulation of the culture broth.

To prevent contamination with undesirable microorganisms, the fermentor and auxiliary equipment must be sterilized before inoculation. This is achieved by steam, ie, at least 20 min at 121°C. The incoming air is filtered.

Specific procedures exist for storing (16,17) and propagating microorganisms to obtain reproducible fermentations. The stock culture is stored frozen ($\leq$ −80°C) or freeze-dried. To prepare the inoculum (seed) mixture, an aliquot is taken and grown in consecutive solid or liquid cultures of increasing volume. The volume of the last step, the seed fermentor, is typically 4–12% of the main fermentor volume.

The media contains the nutrients for the organism, often agricultural products, eg, starch, soy protein, and palm oil. The initial concentration of raw materials can be as high as 250 kg/m^3. Various salts, eg, potassium phosphate, are added to provide basic elements. Sometimes vitamins or specific growth factors are also added. A batch fermentation can be divided into a growth phase during which little enzyme is produced and a production phase during which growth is slow. Many enzymes are produced as a result of depriving the microorganism of
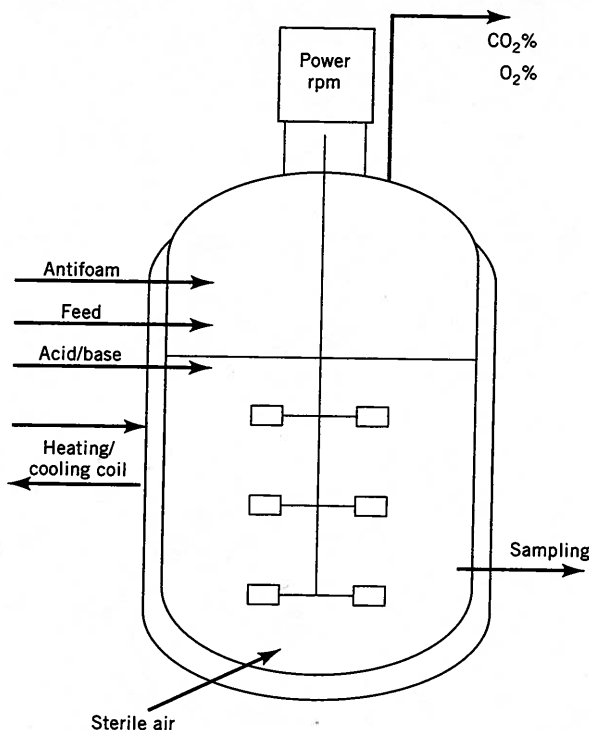
Fig. 4. A conventional fermentor. Foam, total pressure, P_{O_2}, pH, temperature, and weight must be monitored and controlled.

the carbon and/or nitrogen source. Therefore, a slow feed of the limiting nutrient can be introduced to prolong the production period. Such a fed-batch fermentation lasts 3–10 days. In some cases, a continuous fermentation is used where media is constantly added and broth is removed at the same rate. Reported yields of enzymes vary from 1 kg/m^3 broth to 50 kg/m^3 (18).

Temperature, pH, and feed rate are often measured and controlled. Dissolved oxygen (DO) can be controlled using aeration, agitation, pressure, and/or feed rate. Oxygen consumption and carbon dioxide formation can be measured in the outgoing air to provide insight into the metabolic status of the microorganism. No reliable on-line measurement exists for biomass, substrate, or products. Most optimization is based on empirical methods; simulation of quantitative models may provide more efficient optimization of fermentation.

Recovery. The principal purpose of recovery is to remove nonproteinaceous material from the enzyme preparation. Enzyme yields vary, sometimes exceeding 75%. Most industrial enzymes are secreted by a microorganism, and the first recovery step is often the removal of whole cells and other particulate matter (19) by centrifugation (20) or filtration (21). In the case of cell-bound enzymes, the harvested cells can be used as is or disrupted by physical (eg, bead mills, high pressure homogenizer) and/or chemical (eg, solvent, detergent, lysozyme [9001-63-2], or other lytic enzyme) techniques (22). Enzymes can be extracted from dis-

rupted microbial cells, and ground animal (trypsin) or plant (papain) material by dilute salt solutions or aqueous two-phase systems (23).

Ultrafiltration (qv) (uf) is increasingly used to remove water, salts, and other low molecular-weight impurities (21); water may be added to wash out impurities, ie, diafiltration. Ultrafiltration is rarely used to fractionate the proteins because the capacity and yield are too low when significant protein separation is achieved. Various vacuum evaporators are used to remove water to 20–40% dry matter. Spray drying is used if a powdery intermediate product is desired. Lyophilization (freeze-drying) is only used for heat-sensitive and highly priced enzymes.

Sufficient color reduction is often achieved by recovery and purification methods. However, sometimes specific color removal is achieved by adsorption to, eg, activated carbon.

Purification.   Enzyme purity, expressed in terms of the percent active enzyme protein of total protein, is primarily achieved by the strain selection and fermentation method. In some cases, however, removal of nonactive protein by purification is necessary. The key purification method is selective precipitation of the product or impurities by addition of salt, eg, sodium sulfate, or solvent, eg, ethanol or acetone; by heat denaturation; or by isoelectric precipitation, ie, pH adjustments. Methods have been introduced to produce crystalline enzyme preparations (24).

Many laboratory techniques have been described to purify proteins (25), but they are often too costly for industrial enzymes, especially column separations. However, aqueous two-phase extraction (26) and ion exchange are used.

Product Requirements.   When an enzyme is recovered from fermentation broth, it is usually present in an aqueous solution or processed to a dried state. Both types of preparation have to be formulated to comply with requirements appropriate to their final application.

Requirements related to the storage of enzymes from the time of manufacture to the time of application include enzyme stability, ie, the catalytic activity of the enzyme must remain after prolonged storage at relevant temperatures; microbial stability, ie, industrially recovered enzymes in aqueous solution are potentially excellent growth media for microorganisms, so it is usually necessary to prevent microbial growth; and physical stability, ie, the enzyme must remain in solution to avoid inhomogeneous dosage. Some applications demand special requirements. These can be of a technical nature such as having no precipitate, off-odor, and off-color when mixed into a liquid detergent; and/or an absence of side activities, eg, transferase activity must be absent in saccharifying enzymes like amyloglucosidase, and protease must be absent in cell wall-degrading enzymes for the upgrading of vegetable proteins. Other requirements in certain applications derive from approval considerations, eg, only food-grade ingredients, absence of certain microorganisms, and kosher restrictions on enzymes for food applications.

Enzyme Stability.   Loss of enzyme-catalytic activity may be caused by physical denaturation, eg, high temperature, drying/freezing, etc; or by chemical denaturation, eg, acidic or alkaline hydrolysis, proteolysis, oxidation, denaturants such as surfactants or solvents, etc. pH has a strong influence on enzyme stability, and must be adjusted to a range suitable for the particular enzyme. If the enzyme

is not sufficiently stable in aqueous solution, it can be stabilized by certain additives; a comprehensive treatment with additional examples is available (27).

Many enzymes need a certain ionic strength to maintain an optimum stability and solubility, eg, bacterial α-amylases show optimal stability in the presence of 1–2% NaCl. Some enzymes may need certain cations in low amounts for stabilization, eg, Ca^{2+} is known to stabilize subtilisins and many bacterial α-amylases. Antioxidants (qv) such as sodium sulfite can stabilize cysteine-containing enzymes which, like papain, are often easily oxidized.

Polyalcohols, such as glycerol, sugar, sorbitol, and propylene glycol may prevent denaturation (28). Also substrates or substrate analogues often stabilize by conferring an increased rigidity to the enzyme structure.

If a protease is present in solution it is necessary to minimize its activity in order to avoid interference with processing steps, including autolysis during the formulation of a protease. This can be obtained by reducing water activity by means of propylene glycol (25–50%); by adjusting pH to a range where the protease is inactive, eg, low pH in formulations of alkaline proteases; or by adding compounds that reversibly inhibit the protease, eg, formate or borate (29).

Microbial Stability. Microbial growth is hindered by reducing water activity and adding preservatives. An overview is available (30). Reduction in water activity is typically obtained by including approximately 50% of a polyalcohol such as sorbitol or glycerol. Furthermore, 20% of a salt like NaCl has a pronounced growth inhibiting effect.

Some alcohols, eg, propylene glycol, not only lower water activity but also have an additional preservative effect caused by the way they interfere with the cell membrane transport system of the contaminating microorganisms. Surfactants (qv) may show a similar effect.

Organic acids may inhibit growth when present in the undissociated form because of their ability to change the pH inside the cell. The most efficient are benzoic acid and sorbic acid, but formic, acetic, and propionic acid also have this effect. The parabens, ie, *p*-hydroxy benzoic acid esters, are also used because of their antimicrobial effect over a broad pH range.

These systems are allowed in many food applications, but there also exists a range of nonfood preservatives active over a broad pH range. However, these may not be compatible with all enzymes because of their inhibitory or denaturing effects. A useful reference on this subject is available (31).

Physical Stability. Physical stability depends primarily on the purity of the enzyme. Impurities remaining from the fermentation broth may precipitate or form a hazy solution. Unwanted sedimentation is often related to Ca^{2+} or acidic polysaccharides. The solubility of some enzymes can be increased by optimizing the ionic strength or changing the dielectric constant of the solution by adding low molecular-weight polyols.

Formulations. Any formulation is a compromise between the previously mentioned requirements. For example, the fermentation broth may contain enzyme-stabilizing substances, but the application of the enzyme or precipitation problems in the formulation may demand a high degree of purification that eliminates the stabilizers. Alternatively, the pH necessary for good microbial or physical stability may differ from the pH that gives optimum enzyme stability, or a

preservative that is effective at the optimum pH for enzyme stability may have a denaturing effect on the enzyme.

The formulation of an enzyme is normally considered a way to store and transport the enzyme until its application. One common exception is immobilized enzymes where formulation is an active part of their application.

It is sometimes possible to add properties in liquid formulations that provide additional functions. Examples in development or in commercial use as of 1993 include microencapsulation (qv) of enzymes for protection against bleach when dispersed in a liquid detergent; addition of certain polymers to protect the enzyme after it has been added to liquid detergents (32), or to boost activity in the final application; addition of surfactants or wetting agents, eg, α-amylases for textile desizing in order to improve their effect; and addition of buffers to keep the application pH under control.

Immobilization.   Enzymes, as individual water-soluble molecules, are generally efficient catalysts. In biological systems they are predominantly intracellular or associated with cell membranes, ie, in a type of immobilized state. This enables them to perform their activity in a specific environment, be stored and protected in stable form, take part in multi-enzyme reactions, acquire cofactors, etc. Unfortunately, this optimization of enzyme use and performance in nature may not be directly transferable to the laboratory.

Cost reduction is the primary argument for using immobilized enzymes, especially when comparing this method with soluble enzyme or nonenzymatic methods; nevertheless, satisfactory technical solutions can be found among the latter two alternatives. When immobilized, expensive enzymes can be re-used, and this may compensate for the cost of immobilization. However, enzyme production methods are constantly being improved and costs reduced accordingly. Costs in this context also include process costs, eg, equipment, energy, product recovery, enzyme inactivation, etc. Enzyme stability factors, eg, temperature, pH, proteases, oxidation, and solvents/organics, also are important, but are not often regarded as a cost issue because the desired stability is not always found with soluble enzymes. Continuous processes involving immobilized enzymes enable large substrate volumes to be handled by comparatively small reactors and allow the re-use of enzymes.

A significant advantage of immobilized enzymes is the total absence of catalytic activity in the product. Moreover, the degree of substrate-to-product conversion can be controlled during processing, eg, by adjusting the flow rate through a packed-bed column reactor of immobilized enzyme.

Choice of Method.   Numerous enzyme immobilization techniques have been described in the literature; comprehensive books on this and related subjects, including industrial applications, are available (33–36). The more general techniques and some selection criteria are included herein.

Because enzymes can be intracellularly associated with cell membranes, whole microbial cells, viable or nonviable, can be used to exploit the activity of one or more types of enzyme and cofactor regeneration, eg, alcohol production from sugar with yeast cells. Viable cells may be further stabilized by entrapment in aqueous gel beads or attached to the surface of spherical particles. Otherwise cells are usually homogenized and cross-linked with glutaraldehyde [111-30-8] to

form an insoluble yet penetrable matrix. This is the method upon which the principal industrial applications of immobilized enzymes is based.

Extracellular microbial enzymes can be immobilized in the form of proteins purified to varying degrees. Cross-linking methods are based on intra- and intermolecular binding, usually involving the coupling of lysine amino groups by using the bifunctional reagent glutaraldehyde. In many cases this leads to insolubilized, active, and stabilized enzyme. Additional chemicals and proteins may be used in the cross-linking process.

Other immobilization methods are based on chemical and physical binding to solid supports, eg, polysaccharides, polymers, glass, and other chemically and physically stable materials, which are usually modified with functional groups such as amine, carboxy, epoxy, phenyl, or alkane to enable covalent coupling to amino acid side chains on the enzyme surface. These supports may be macroporous, with pore diameters in the range 30–300 nm, to facilitate accommodation of enzyme within a support particle. Ionic and nonionic adsorption to macroporous supports is a gentle, simple, and often efficient method. Use of powdered enzyme, or enzyme precipitated on inert supports, may be adequate for use in nonaqueous media. Entrapment in polysaccharide/polymer gels is used for both cells and isolated enzymes.

Membrane reactors, where the enzyme is adsorbed or kept in solution on one side of an ultrafiltration membrane, provides a form of immobilized enzyme and the possibility of product separation.

Microemulsions or reverse micelles are composed of enzyme-containing, surfactant-stabilized aqueous microdroplets in a continuous organic phase. Such systems may be considered as a kind of immobilization in enzymatic synthesis reactions.

The choice of a suitable immobilization method for a given enzyme and application is based on a number of considerations including previous experience, new experiments, enzyme cost and productivity, process demands, chemical and physical stability of the support, approval and safety issues regarding support, and chemicals used. Enzyme characteristics that greatly influence the approach include intra- or extracellular location; size; surface properties, eg, charge/pI, lysine content, polarity, and carbohydrate; and active site, eg, amino acids or cofactors. The size, charge, and polarity of the substrate should also be considered.

Industrial-Scale Applications. The breakthrough for immobilized enzyme technology came in the mid-1960s. The first significant development was the production of L-amino acids by continuous optical resolution of synthetic racemates using amino acylase adsorbed onto DEAE-Sephadex (33). The introduction of immobilized penicillin G acylase, and then immobilized glucose isomerase [*9055-00-9*] (IGI) for the production of high fructose corn syrup (HFCS) demonstrated the enormous industrial potential of immobilized enzymes. IGI is the last in a series of three enzymes used to convert starch to HFCS; ie, liquefaction by α-amylase [*9000-90-2*], saccharification by glucoamylase [*9032-08-0*], and isomerization of glucose by IGI to approximately 45% fructose, which is fractionated chromatographically to 55% fructose and 45% glucose. Starch sources other than corn can be used.

The success of IGI was a result of high quality immobilized preparations developed at a time when raw sugar was expensive (1975). When the technology

was established and further improved, it remained competitive when raw sugar prices dropped.

A number of factors, concerning both enzyme and process, favored the immobilization of glucose isomerase. The enzyme was expensive because of its low specific activity and its low yield resulting from its intracellular formation, by-product formation could be minimized by using short residence times at enzyme-optimal pH, and particles with physical properties suited to large enzyme columns were needed to handle the huge volumes generated by the starch industry. A commercial product (Sweetzyme) based on glutaraldehyde cross-linked *Bacillus coagulans* cells also was introduced.

Commercial IGIs of the 1990s are based on various enzyme sources, largely *Streptomyces* spp., and include immobilization techniques incorporating the adsorption of purified glucose isomerase (37). An example of an IGI is made by cross-linking *S. murinus* cells with glutaraldehyde and then extruding the resulting matrix into particles. Typical reaction conditions and performance are listed in Table 2.

The second most important group of immobilized enzymes is still the penicillin G and V acylases. These are used in the pharmaceutical industry to make the intermediate 6-aminopenicillanic acid [551-16-6] (6-APA), which in turn is used to manufacture semisynthetic penicillins, in particular ampicillin [69-53-4] and amoxicillin [26787-78-0]. This is a remarkable example of how a complex chemical synthesis can be replaced with a simple enzymatic one; four steps, involving toxic and corrosive chemicals, solvents, and a $-40°C$ process step, can be avoided. It is estimated that all 6-APA is now produced by the enzymatic route. Similar reactions on cephalosporins can be made.

Enzyme techniques are primarily developed for commercial reasons, and so information about immobilization and process conditions is usually limited. A commercially available immobilized penicillin V acylase is made by glutaraldehyde cross-linking of a cell homogenate. It can be used in batch stirred tank or

Table 2. Typical Operating Parameters for Immobilized Glucose Isomerase[a] and Penicillin V Acylase[b]

Immobilized enzyme	Glucose isomerase	Penicillin V acylase
enzyme reactor	2–5 m height, 0.6–1.5 m diameter	batch +/− recycled packed bed
substrate, %	ca 45 w/w glucose	10 w/v penicillin V
initial flow or activity	1.2–6.8 bed vol/h	100,000–200,000 units/kg
temperature, °C	55–60	35
pH	7–8	7.5[c]
conversion	42–45% fructose	98% 6-APA
productivity, t/kg immobilized enzyme	15 (dry subst)	0.25
at residual act., %	10	50

[a]Ref. 37.
[b]Ref. 38.
[c]Adjusted with NH_3.

recycled packed-bed reactors with typical operating parameters as indicated in Table 2 (38). Further development may lead to the creation of acylases and processes that can also be used for attaching side chains by enzymatic synthesis.

During the 1980s, molecular biology techniques were used successfully to reduce enzyme production costs; this is part of the reason the number of immobilized enzyme processes used in industry are relatively few when compared with soluble enzyme processes, and certainly less than expected in the 1970s. Acylases other than IGI and immobilized penicillin are in use, although they are not of similar significance. Examples from the starch and dairy industry are glucoamylase and lactase. It is expected that the pharmaceutical, organic chemical, and oils and fats industries will increase their use of this technology. Proteases are used in peptide synthesis and nitrilase in the synthesis of acrylamide. Of particular interest is the use of immobilized lipases for optical resolution, ester synthesis, and the production of specific lipids by interesterification (35). These have already demonstrated the feasibility of using immobilized enzyme technology in nonaqueous or low water systems.

Granulation of Enzymes. Although the trend is to market industrial enzymes as liquid products, solid enzyme is needed. Examples of this are enzymes for solid detergents, animal feed, and flour improvement.

Several different methods have been used for the granulation of enzymes. In general, the development of granulation methods focuses on parameters such as the cost of the process and solubility of the granulate. The focus for detergent enzymes is directed onto a single parameter, ie, a dust-free product. It should not contain particles that can become airborne, and the particles must be strong and resilient in order to avoid creating dust during handling of the enzyme in the detergent factory (39).

To reduce the enzyme dust level in detergent factories to an absolute minimum, the majority of detergent enzyme granulates are coated with a layer of inert material. This coating can also be used for coloring purposes. The color of the enzyme granulate itself is often brown. Titanium dioxide [13463-67-7] can be added to the coating medium to make the product whiter; alternatively, another attractive color can be added.

For granulation of enzymes for purposes other than incorporating into detergents, the same methods can be used as for the detergent enzymes, although some of the additives needed for the production of a rigid granulate may not be accepted, ie, in certain enzymes for the food industry. Fortunately for such applications the handling of the enzyme is more gentle, and the requirement for physical stability less. For these enzymes, a fluid-bed granulation performed as an agglomeration of powder with a liquid binder, or as a coating of the enzyme onto inert carrier particles of selected size, often gives the desired quality of product. These enzyme preparations may also be coated.

Various methods are used for evaluating the quality, ie, physical strength and enzyme dust formation, of the granulate. In the elutriation process, a sample of product is fluidized in a glass tube with a perforated bottom plate for 40 minutes. Dust from the sample is collected on a filter and the enzyme activity measured. An acceptable dust level is when less than 5–10 ppm of the activity of the sample has been collected. In the so-called Heubach method, 20 g of granulate is elutriated. During the elutriation, four steel balls are rotated in the bed in order

to evaluate the impact of attrition on the dust release of the enzyme. The dust is collected on a filter and measured. The acceptable dust level is very low.

Solid Preparation Methods. *Prilling.* A suspension of the enzyme is spray cooled in waxy material, eg, an ethoxylated fatty alcohol, with a melting point over or about 50°C, to form small beads. Bead size may be varied and surface coatings may be applied.

Extrusion. The enzyme is mixed with binders, diluting agents such as inert salts, and the appropriate amount of water. The resulting thick paste is then extruded to small rods having a diameter of about 0.6 mm. Preferably the extrudate is then further spheronized to a product with a more rounded form.

Granulation. The enzyme is diluted with inert material and binders, as described above, but with the option of adding fibers which act to reinforce the granules. It is then granulated in a medium or high shear granulator.

Industrial Applications

Detergent Enzymes. Detergents, from Latin *detergere,* to clean, is here understood to mean cleaning products in a broad sense, ie, not only products for household laundering, including soaking and topical spot removal, but also automatic dishwashing detergents (ADDs) and products for a wide range of industrial and institutional (I&I) cleaning functions.

Penetration of enzymes in laundry detergent products is close to 95% in Europe, Japan, and the United States. Enzymes have become an important ingredient in detergents, along with surfactants, bleaching systems, and builders (see DETERGENCY). Several changes in washing habits and detergent formulations underlie this development.

Lower washing temperatures, required by modern textiles made from synthetic fibers and for reasons of energy conservation, have caused a demand for new, more effective, detergent ingredients. Compacted laundry detergent products compensate for reduced levels of other detergent ingredients by increasing enzyme dosages, resulting in concentrations of up to several mg enzyme protein per liter of washing liquor, as opposed to usually not more than 1 mg/L for noncompact detergents. ADDs with high alkalinity and chlorine bleach are replaced by less aggressive and environmentally more friendly detergent products containing an active oxygen bleach system plus an enzyme system. Finally, the detergent enzyme producers are able to offer products with a more favorable price/performance ratio than previously.

Laundry Soilings. Important classes of soilings to be removed by laundry detergents include water-soluble soilings, eg, soluble inorganic salts, sugar, urea, and perspiration; proteinaceous soilings, eg, blood, egg, milk, cutaneous scales, grass, and spinach; starch; fats/oils, eg, animal fats, vegetable fats, sebum, waxes, and mineral oil; particulate matter, eg, insoluble metal carbonates, oxides, silicates (clay), carbon black, dust, and humus; and bleachable stains, eg, fruit and vegetable juices, wine, tea, and grass. Stains with good water solubility are easily removed during the washing process. All other stains are partially removed by the surfactant/builder system of a detergent, although the result is often unsatisfactory, depending on the conditions. In most cases a suitable detergent enzyme

may help. Contrary to the purely physical action of the surfactant system, enzymes work by degrading the dirt into smaller and more soluble fragments. However, to remove a stain totally requires the joint effects of the enzyme, surfactant system, and mechanical agitation.

Protein and starch stains are removed by proteases and amylases, respectively. Fats and oils are generally difficult to remove at low wash temperatures by conventional detergents. By using lipases, it is possible to improve the removal of fats/oils of animal and vegetable origin even at temperatures where the fatty material is in a solid form. Particulate soils can be difficult to remove, especially if the particle size is small. Removal of particulate soil from cotton fabric can be improved by use of a cellulase which removes cellulose fibrils from the surface of the yarn.

Various kinds of dirt may adhere to textile surfaces via a glue of proteinaceous, starchy, or fatty material. In such cases of anchored dirt, an enzyme may assist in removing the dirt even though it does not attack the dirt directly. Compounds from several of these classes are intimately mixed in combined soilings, eg, human sebum on shirt collars, cocoa milk, gravy, or chocolate. Bleachable stains are the only group of stains for which no enzyme product has been marketed (ca 1993). However, the patent literature indicates that efforts are being made to develop an enzymatic bleaching system.

Laundering Conditions Around the World. Any laundering process is an interplay between the equipment used; the materials entering the process, ie, detergent, additional bleach, fabric softener, or water; and the procedure followed. Equipment and procedures in three principal geographical areas are summarized in Table 3.

Table 3. Washing Equipment and Procedures, Worldwide[a]

Conditions	U.S./Canada[b]	Japan	Western Europe
machine type[c]	agitator	impeller	drum[d]
fabric load/kg	2–3	1–1.5	3–4
wash liquor/L	35–80	30–45	18–25
wash temperature, °C	50 (hot)		
	27–43 (warm)	10–40	90
	10–27 (cold)		60
			40
			30
water hardness	relatively low	very low	relatively high
$CaCO_3$, ppm	100	50	250
recommended detergent dosage[e], g/L	1–5	1–3	8–10

[a]Information mainly extracted from Ref. 40.
[b]Chlorine bleach commonly added to wash.
[c]Drying process by automatic dryer.
[d]Heating coils used.
[e]In the United States and Japan usually without bleaching components.

Detergent compositions also vary from country to country. The world market for household detergents can be divided into four segments according to the physicochemical properties of the wash solutions prepared from the detergents.

Low pH, low ionic strength detergents are liquid detergents having pH from 7.5 to 9. They contain no bleach and only low levels of salts. Medium pH, medium ionic strength detergents are typically compact powder detergents from Japan and regular powder detergents from the United States. Their pH is about 9 and they contain no bleach. High pH, high ionic strength detergents with bleach have a pH from 9.5 to 10.5, and contain an activated bleaching systems, eg, European regular powder detergents. They also contain sodium sulfate as a filler, and builder systems, eg, sodium triphosphate, zeolite, and sodium carbonate. High dosages are used, which give a wash liquor with a high ionic strength. High pH, medium ionic strength detergents with bleach is a new group of detergents, eg, European compact powder detergents which have gained a huge market share during the early 1990s. They are compact as a result of removal of most or all of the sodium sulfate; pH is from 9.5 to 10.5.

Detergent Enzymes Performance Evaluation. Not all enzymes with a potential for stain degradation and/or removal are suitable for inclusion in detergent products. A detergent enzyme must have good activity at the pH of detergent solutions (between 7 and 11) and at the relevant wash temperatures (20 to 60°C), and must be compatible with detergent components during storage as well as during the wash process, eg, surfactants, builders, bleaches, and other enzymes. In particular, such an enzyme must be resistant toward protease degradation under these conditions. With enzymes like proteases and lipases, for which the average load of dirty laundry contains a multitude of different substrates, a broad substrate specificity is demanded.

A given enzyme may be assayed by its action on soluble substrates under chemical and physical conditions different from those encountered in a real-life wash. Such experiments indicate the enzyme's performance with respect to pH and temperature variations, or in conjunction with other soluble substances, etc. The analytical data thus obtained are not necessarily representative of the wash performance of the enzyme, and real wash trials are necessary to evaluate wash performance of detergent enzymes.

Wash trials are carried out by the use of soiled test pieces, eg, commonly used stains for protease evaluation are milk, blood, and grass. Commercial pre-soiled test pieces also may contain particulate matter, eg, carbon black, as part of the stain matrix. Test materials are available ready-to-use from a number of research and testing institutes in Europe and the United States, eg, Center for Testmaterials, Vlaardingen, Holland; Wascherei Forschungs Institut, Krefeld, Germany; EMPA, St. Gallen, Switzerland; Instituut voor Reinigingstechnieken TNO, Delft, Holland; and Testfabrics, Middlesex, New Jersey. Alternatively, enzyme manufacturers can supply preparation procedures.

Laboratory wash trials are usually conducted in small-scale models of washing machines. The Terg-o-tometer simulates the top-loaded U.S. type of washing machine, and the Launder-ometer simulates the European drum-type machine (41). The evaluation of the effects on the test pieces can be made visually or by measuring the reflectance of light under specified conditions. Typically, the intensity of light remitted at 460 nm when illuminating the test pieces with a stan-

dardized daylight source is expressed as a percentage, R, of the intensity of incident light at the same wavelength. The ΔR value is then a measure of the enzyme effect; it is defined as the difference in R between fabric washed with and without enzyme. The R value is known to correlate well with the visual impression of whiteness of the fabric, and depending on type and degree of soiling, differences in R of 2–3 units are recognizable to the human eye.

Figure 5 records protease performance as a function of enzyme dosage. The performance is better at 60°C than at 40°C until a dosage of approximately 0.05% (w/w) of the enzyme granulate in the detergent is reached. At this enzyme level, the fabric in both cases is clean, ie, the measured R value is identical to that of an unsoiled test piece washed under the same conditions.
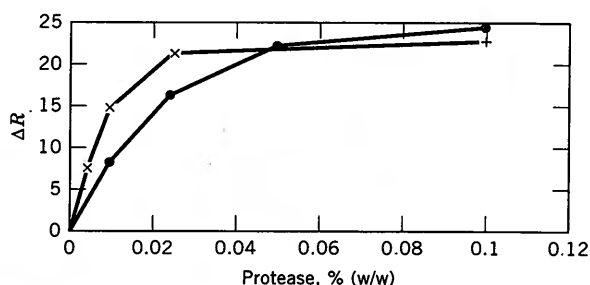
Fig. 5. Protease performance of Savinase 6.0 T (Novo) in powder detergent, 4 g/L in a Launder-ometer test. (—●—) 40°C for 47 min; (—×—) 60°C for 40 min.

Detergent enzyme performance is often reported in the form of such dose-response curves. The performance increases dramatically at the beginning, but reaches a maximum level at higher enzyme concentrations. The extent to which the enzyme is able to remove stains from the fabric depends on the detergent system, temperature, pH, washing time, wash load, etc. Enzyme wash performance varies between liquid and powder detergents and with the composition of the soiling (Fig. 6).
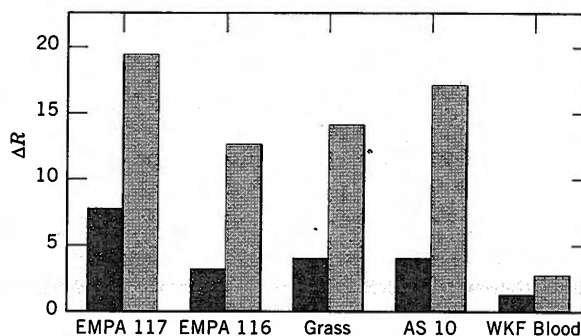
Fig. 6. Washing performance on different soilings of a U.S. liquid detergent (■) and a U.S. powder detergent (▦) in a Terg-o-tometer operating at 20°C for 10 min; one enzyme dosage. EMPA 117 (milk, blood, and ink on polyester/cotton); EMPA 116 (milk, blood, and ink on 100% cotton); grass (on 100% cotton); AS 10 (milk, oil, and pigments on 100% cotton); blood soilings (on 100% cotton).

Enzyme Types.   Proteases. A protease is an enzyme that hydrolyzes proteins into smaller fragments, ie, peptides or amino acids. A detergent protease degrades the insoluble protein in stains into fragments that can be removed or dissolved by the detergent. All detergent proteases of the early 1990s are produced by *Bacillus* strains. Some are highly alkaline, ie, have a maximum activity in the high pH range, such as Maxacal and Savinase. Others are low alkaline, eg, Maxatase, Alcalase, and Subtilisin Novo (BPN'). They all have a molecular weight between 20,000 and 30,000, and a serine in the active site of the enzyme.

Protease performance is strongly influenced by detergent pH and ionic strength. Surfactants influence both protease performance and stability in the wash solution. In general, anionic surfactants are more aggressive than amphoteric surfactants, which again are more aggressive than nonionic surfactants.

All detergent proteases are destabilized by linear alkylbenzenesulfonate (LAS), the most common type of anionic surfactant in detergents. The higher the LAS concentration and wash temperature, the greater the inactivation of the enzyme. The presence of nonionic surfactants, however, counteracts the negative effect of LAS. Almost all detergents contain some nonionic surfactant; therefore, the stability of proteases in a washing context is not problematic.

Builders, eg, sodium triphosphate and nonphosphate builders such as zeolite and citrate, remove free calcium from the washing solution. Co-builders such as nitrilotriacetic acid or polycarboxylates also may be incorporated into the detergent formulation. Wash performance of detergents decreases with increasing calcium concentration. Protease performance varies, but high calcium concentrations tend to reduce protease performance. Therefore it is an advantage to add a builder system to the detergent. Proteases need a small amount of calcium for the sake of stability, but even with the most efficient builder systems, stability during wash is not a problem.

Bleach systems oxidize proteinaceous stains on fabric, often making the stains more difficult to remove. Detergent proteases can counteract this negative effect of the bleach system. The most commonly used bleach systems in detergents consist of sodium perborate [*7632-04-4*] plus an activator such as tetraacetylethylenediamine [*10543-57-4*] (TAED). Perborate releases hydrogen peroxide [*7722-84-1*], H_2O_2, which combines with the activator to form a peroxycarboxylic acid. Most detergent proteases are stable during the wash cycle in the presence of such active-oxygen bleach systems. However, storage stability in detergents containing bleach may be a problem with the established detergent proteases. New protein-engineered proteases introduced onto the market replace the most bleach-sensitive amino acid, ie, methionine close to the active site, with other amino acids not sensitive toward oxidation. This slight change in the molecular structure significantly increases the storage stability in detergents containing bleach (42) (Fig. 7). Chlorine bleach [*7681-52-9*] (sodium hypochlorite), NaOCl, is not incorporated into laundry detergents themselves, but is used separately in some parts of the world as an additive. In normal wash concentrations above 200 ppm it quickly oxidizes the enzymes, resulting in loss of protease activity.

Most ingredients in a detergent formulation contribute to the ionic strength of the wash solution. The effect of ionic strength on protease performance depends
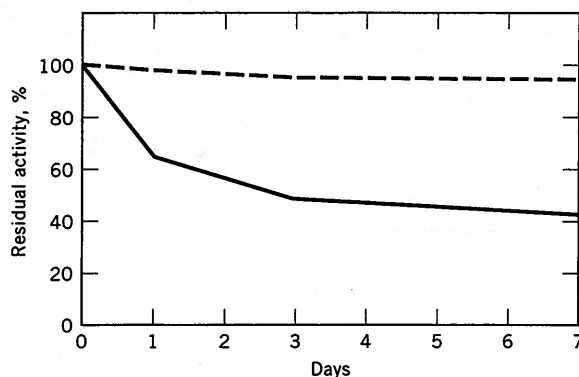
Fig. 7. Storage stability of proteases in European powder detergent with activated bleach system. (——) Traditional protease; (— — —) protein-engineered protease.

on pH and enzyme identity. The pH of wash solutions also affects protease performance (Fig. 8).

Amylases. Commercial laundry amylases comprise the α-amylase from *Bacillus amyloliquefaciens* and the heat-stable α-amylase from *Bacillus lichenifor-mis*. Alpha-amylases are characterized by attacking the starch polymer in an endo fashion, randomly cleaving internal 1,4-bonds to yield shorter, water-soluble dextrins. They are the preferred type of amylase for laundry detergents, and are included in both powder and liquid formulations in many countries. Alpha-amylases boost overall detergent performance at lower wash temperatures and with milder detergent chemical systems. They catalyze the degradation of starch stains, and improve cleaning by hydrolyzing the starch glue that binds other dirt

(a)   **(b)**
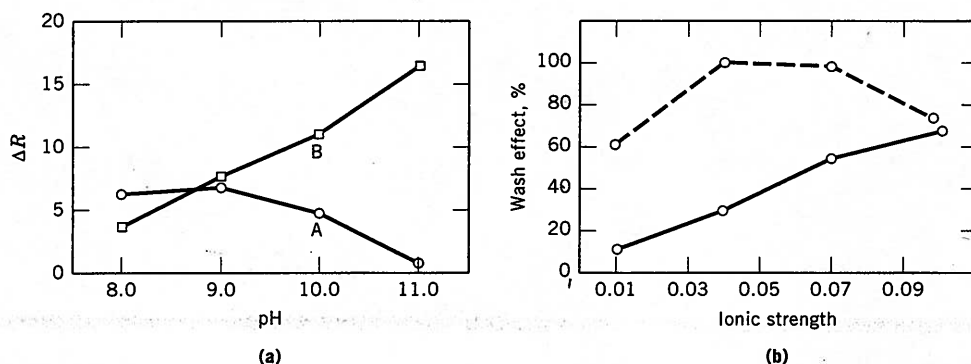
Fig. 8. Protease washing performance in a U.S. liquid detergent. Grass soiling in a 10 min wash at 30°C with one enzyme dosage. (a) pH profile of commercial proteases A and B. (b) Effect of increasing ionic strength, adjusted with Na_2SO_4, of commercial protease B at (—o—) pH 8 and (-·o·-) pH 11.

and stains to fabric. A noticeable amylase effect is obtained in the main wash, as well as with prespotting, ie, dramatically increased enzyme concentration, and presoaking, ie, prolonged reaction time.

Examples of artificially soiled test pieces used to test the performance of amylases include cocoa/milk/sugar, cocoa/sugar/potato starch, cocoa/milk/sugar/ potato starch, and starch/carbon black, all on cotton or polyester/cotton.

Bacterial α-amylases used in laundry detergents are fully compatible with detergent proteases, ie, the two enzymes work together in the wash process. During storage in both powder and liquid detergents, the amylases are very stable in the presence of proteases.

Lipases. The idea of using lipases in the wash process dates back to 1913 when O. Röhm suggested adding pancreatin [8049-47-6] to detergent formulations. Many patents have demonstrated that lipases can improve the removal of fatty stains when used in powder and liquid detergents, special presoakers, or other cleaning agents. Intense research activity is also reflected in the literature (43–45).

Only one detergent lipase, ie, Lipolase introduced by Novo in 1988, has been marketed. The first household powder detergent containing lipase was introduced in Japan in the same year; in Europe and the United States in 1990–1991. Lipase is often incorporated into the new compact powder formulations.

The slow development of a commercial detergent lipase was due to low fermentation yields and to difficulties in finding lipases with the appropriate characteristics for application in household detergent products. Patent literature indicates the only feasible way to produce lipases at an acceptable cost/performance ratio is by genetic engineering (46–48). For example, Lipolase, mol wt 32,000, was originally isolated from the fungus *Humicola lanuginosa* with low levels of enzyme expression; using rDNA techniques the lipase is expressed in acceptable yields in the harmless host microorganism *Aspergillus oryzae* (46,49).

The natural substrates for lipases are triglycerides, the main constituents of stains originating from animal fat and vegetable oil. A triglyceride molecule is composed of three fatty acid moieties linked to a glycerol backbone by ester bonds. Lipases catalyze the hydrolysis of the ester bonds, giving a mix of free fatty acids, diglycerides, monoglycerides, and glycerol. The more degraded the fatty stain becomes, the easier it is to remove from the fabric due to its increased hydrophilicity.

Because of the presence of free fatty acids in the mix of hydrolysis products, pH strongly influences the removal of decomposed stains. The best rate of removal requires a pH value above 8 (50).

Lipases are also active during a certain period of the drying step (51), eg, Lipolase displays maximum activity when the moisture content on the fabric is 20–30% by weight. This means that significant decomposition of any residual fatty matter will take place while the laundry is drying. This hydrolytic activity does not result in an immediate advantage in terms of fat removal; however, next time the stained fabric is washed the stain will be removed more effectively.

Lipases have proven to be effective in prespotters and other liquid detergent formulations when used in undiluted form for pretreatment of tough fatty stains. The low water content on the fabric in this situation is believed to be responsible for the high catalytic activity (50).

Cellulases. A 1970 patent application (52) covered the idea of using cellulases to prevent fabrics from becoming harsh as a result of repeated washings (see TEXTILES, FINISHING). Low yields and expensive production methods hindered the market development of cellulases. Very few cellulases that are stable and active at high pH were identified before the late 1980s. Celluzyme from Novo Nordisk and Bio-Beeds from Kao, originating from fungal and bacterial species, respectively, are the only alkaline detergent cellulases available in the early 1990s.

Cellulases cleave β-1,4-glycosidic bonds in cellulose and work on natural textile fibers such as cotton and blends containing cotton. Cleaning by removal of particulate soils, softening, and improved color brightness are the three basic benefits obtained from cellulases. When a textile is exposed to shear stress, either during wear or washing and tumble-drying, the surface becomes slightly damaged. A close look at the yarn reveals the presence of fibers and fibrils ranging in size from a few μm to a few mm on the surface. The damaged textile surface scatters light, giving the fabric a grayish or dull appearance, and affecting color brightness. Dust particles also tend to stick to the damaged areas, adding to the gray appearance. If the material features a number of colors, the contrast between adjacent areas of different colors will be reduced. Damaged fibers are also thought to be responsible for making the fabric surface more rigid, thereby reducing softness. Cellulases hydrolyze exposed β-1,4 bonds in the cellulose, which leads to removal of the fibrils. This is believed to be the mechanism behind the softening and color-brightening effects. Published literature indicates that little is known about the mechanism behind the cleaning action of cellulases. Possible explanations for the cleaning effect are that by removing the fibrils, the soil attached to them is released, and that the enzymatic action facilitates cleaning by exposing dirt trapped in the fiber matrix to the washing solution (53,54).

Softening effects, similar to those obtained with cellulases, can also be achieved by using claylike compounds in the detergent or cationic surfactants in fabric softening additives used in the rinse. These materials are thought to achieve their effect by coating the fibers with a lubricating layer, thereby lowering the friction and reducing the tendency of the fibrils to bond together (52). The use of cationic surfactants is not restricted to cotton or mixed cotton fabrics. However, coating fibrils by cations may lower the water absorption properties of the fabric. In some cases, eg, towels, this is undesirable and cellulases are considered the superior softening agents. Cellulases also may be preferable from an environmental point of view.

There is concern that enzyme activity of cellulases may cause loss in textile strength and/or weight loss. Removal of fibers/fibrils lowers the weight to some extent, but since the damaged fibers contribute only little to textile strength it should be possible to have negligible loss of strength by choosing a suitable enzyme concentration. Damage to the fabric itself should only occur at higher concentrations. Appropriate enzyme levels must be determined for any specific formulated product under the intended conditions of use.

Industrial and Institutional Cleaning. The application of enzymes has grown substantially within the industrial and institutional (I&I) sector. The primary field is the use of detergent enzymes for laundry purposes, but a broad range of other applications has been investigated, eg, I&I dishwashing, membrane clean-

ing, drain and bowl cleaning, cleaning of septic tanks and sewage plants, hard surface cleaning of walls or machinery parts where a cleaning-in-place procedure can be used, and cleaning of apparatus parts like endoscopes and electrodes. The choice of the relevant enzyme type is directly related to the composition of the soils, waste, or deposit that has to be dissolved and removed. Thus, as for household laundering, proteolytic, amylolytic, cellulytic, or lipolytic enzymes can be considered. Proteases are suitable for use on fabrics heavily soiled with blood and/ or meat residues, eg, from hospitals and the food industry, in particular slaughterhouses; fatty stains are removed efficiently from restaurant tablecloths and napkins by the addition of a lipase to the detergent; residues of starchy foods such as mashed potatoes, spaghetti, hot oatmeal, and chocolate are cleaned with the use of amylases.

The I&I cleaning procedures as a whole, compared with household laundering, are characterized by huge variations in the composition of the soils, types of surface to which they adhere, cleaning time available, etc. The optimum choice of enzyme type and dosage level normally has to be established through a cooperation between the customer (end user), manufacturer of the detergent, and enzyme producer.

Automatic Dishwashing. There are many differences between laundering and automatic dishwashing. The hard surfaces present in the latter process differ from textiles because they are impermeable to soils; therefore, cleaning fluids have better access to the soils.

During the 1970s, manufacturers tried to add enzymes to automatic dishwashing detergents (ADDs), which required that they leave out chlorine bleach, ie, hypochlorite precursors. Because there was no adequate substitute for chlorine bleach, tea and coffee stains were left behind. In the early 1990s the availability of active-oxygen bleaches, compatible with enzymes, and increasing concern about safety and environmental issues, are causing extensive changes in ADD formulations, especially in Europe.

Earlier formulations contained mainly chlorine bleach, metasilicates, triphosphate, and nonionic surfactants. Modern manufacturers have switched to more complicated formulations with disilicates, phosphates or citrate, phosphonates, polycarboxylates, nonionic surfactants, oxygen bleach, bleach activator, and enzymes. The replacement of metasilicates by disilicates lowers pH from approximately 12 to 10.5, at 1 g ADD/L water. The combined effect of decreased pH, the absence of hypochlorite, and the trend toward lower wash temperatures has paved the way for the introduction of enzymes into ADDs. Most ADD brands in Europe are part of the new generation of ADD products with enzymes. The new formulations are described in the patent literature (55–57).

ADD enzymes used are heat-stable proteases and α-amylases. The actions of the enzymes in ADDs are similar to those in laundering. The performance of enzymes in automatic dishwashers can be evaluated on selected stains (58) or according to standard procedures described for the testing of dishwashers involving a series of standard soils (59,60). However, some soils in the standard procedures, such as food residues cross-linked by heat, are difficult to remove (61) and are not obvious substrates for enzymes. Starch soils are considered the most stubborn kind of soil on kitchenware. This applies to freshly formed deposits as well as to the starchy film that tends to build up on plates leaving them with a dull

appearance. Several α-amylases exist with a high temperature optimum, approximately 70°C (62), that are efficient at removing starch film even under the harsh conditions existing in a dishwasher (63).

Protein residues, eg, soft-boiled egg yolk, are difficult stains to handle. If the stains are not totally denatured, proteases can decompose them. There are commercial proteases with a high temperature optimum (60°C) that can remove most protein soils in a dishwasher (63). Patents on the use of lipases in ADDs have claimed that lipases can reduce the formation of spots and films on glasses (62,64–66); however, no commercial application of lipases in ADDs has been implemented.

Starch Conversion. In the early 1960s the first amyloglucosidase type of enzyme was launched. This enabled starch to be broken down completely into glucose. Shortly afterward, almost all glucose production was reorganized for enzymatic hydrolysis instead of acid hydrolysis; advantages included greater yields, higher degree of purity, and facilitated crystallization. In 1973 an immobilized glucose isomerase was developed that made the industrial production of fructose syrup feasible. In the 1990s the starch processing industry is the second largest consumer of enzymes. Because of the many different products made from starch, significant research efforts have been devoted to this area of application.

The primary steps in the conversion of starch are liquefaction, saccharification, and isomerization. By controlling the enzymatic reactions, sugars of different sweetness can be produced to suit the various needs of manufacturers of food and nonalcoholic beverages.

A slurry of the starch is cooked in the presence of a heat-stable bacterial endo-α-amylase. The enzyme hydrolyzes the α-1,4-glycosidic bonds in pregelatinized starch, the viscosity of the gel rapidly decreases, and maltodextrins are produced. The process may be terminated at this point. The solution is purified and dried, and the maltodextrins are utilized as blandtasting functional ingredients in dry soup mixes, infant foods, sauces and gravy mixes, etc.

Further hydrolysis using an amyloglucosidase leads to the formation of sweet-tasting, fermentable sugars. Sweet starch hydrolyzates with special functional properties may be obtained by using a fungal α-amylase either on its own or in combination with an amyloglucosidase. Alternatively a vegetable β-amylase can be used. The sweet syrups and dextrose are used in beverages, confectionery, canned fruit, bakery products, and ice cream.

Many products made by fermentation are also based on the conversion of starch. Some examples of the use of enzymatically hydrolyzed starches are the production of alcohol, ascorbic acid, enzymes, lysine, and penicillin.

Starch Liquefaction. Starch in its natural state is only degraded slowly by α-amylases. To make the starch susceptible to enzymatic breakdown, it is necessary to gelatinize and liquefy a slurry with a 30–40% dry matter content. Gelatinization temperature depends on the type of starch (67); corn is the most common source of industrial starches followed by wheat, tapioca, and potatoes. Liquefaction is achieved by adding a heat-stable α-amylase to the starch slurry. The equipment used for liquefaction may be stirred tank reactors, continuous stirred tank reactors (CSTR), or a jet cooker. Most starch processing plants liquefy the starch with a single enzyme dose in a process using a jet cooker (Fig. 9).
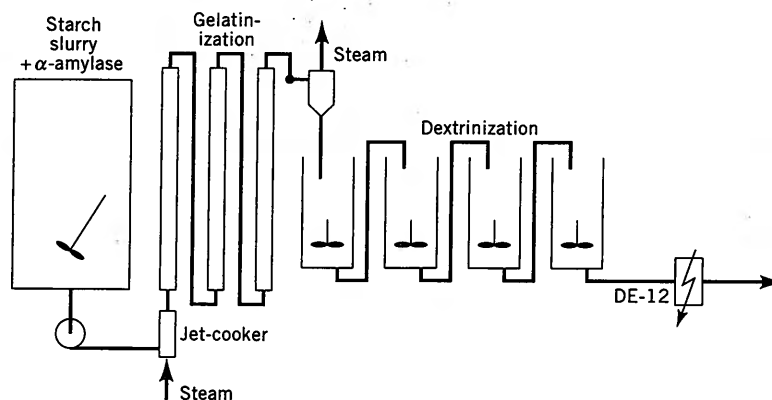
Fig. 9. Starch liquefaction process. 35% dry substance at pH 6.3, 40 ppm Ca^{2+}. Single enzyme dose of 0.5 kg α-amylase/120 L starch slurry, at 105°C for 5 min (gelatinization), followed by 95°C for 2 hours (dextrinization) (68).

In the alcohol industry, grain or potato raw materials are milled and water added to form a slurry or mash which is heated either batchwise or continuously. Traditionally, the mash is heated to 150°C by the injection of live steam. To reduce viscosity, α-amylases are added both during heating to 150°C and during cooling. Thermostable α-amylases from *Bacillus licheniformis* are the most commonly used enzymes for these processes (68).

In German batch processes, raw materials are not milled; gelatinization is achieved by cooking with live steam in a Henze cooker. No addition of enzyme or mechanical agitation is necessary during the cooking stage. The cooked mash is blown through a counter pressure valve into the mash tub where liquefaction takes place either at high (80°C) or low (55–60°C) temperature.

Cooking extruders have been studied for the liquefaction of starch, but the high temperature inactivation of the enzymes in the extruder demands doses 5–10 times higher than under conditions in a jet cooker (69). For example, continuous nonpressure cooking of wheat for the production of ethanol is carried out at 85°C in two continuous stirred tank reactors (CSTR) connected in series; plug-flow tube reactors may be included if only one CSTR is used (70).

During liquefaction, the α-1,4 linkages are hydrolyzed at random. This reduces the viscosity of the gelatinized starch, and increases the dextrose equivalent (DE), ie, a measure of the degree of hydrolysis of the starch. The liquefaction is carried out in such a way as to give the required DE for the subsequent process. For saccharification to dextrose, a DE of 8–12 is normal. Higher DE values are often necessary in maltodextrin production. The maximum DE obtainable is approximately 40. The important α-amylases used in industry originate from *Bacillus licheniformis, Bacillus subtilis,* and *Aspergillus oryzae.* Table 4 lists application conditions and key characteristics of enzymes available for starch processing.

β-amylases are exoenzymes that attack amylose chains and result in the successive removal of maltose units from the nonreducing end. In the case of amylopectin, the cleaving stops two to three glucose units from the α-1,6-branch-

Table 4. Starch-Degrading Enzymes of Industrial Importance

| Enzyme | Origin | Application | | Ca^{2+}, ppm^a |
		Temp., °C	pH	
α-amylase				
bacterial, mesophilic	*Bacillus subtilis*	80–85	6–7	150
bacterial, thermophilic	*Bacillus licheniformis*	95–105	6–7	20
fungal	*Aspergillus oryzae*	55–70	4–5	50
pullulanase	*Bacillus acidopulluly-ticus*	55–65	3.5–5	0
amyloglucosidase	*Aspergillus niger*	55–65	3.5–5	0

^{a}Minimum dosage.

ing points. β-amylase [*9000-91-3*] is used for the production of maltose syrups and for adjunct processing in breweries. The most important commercial products are made from barley or soybeans.

Isoamylase [*9067-73-6*] (glycogen-6-glucanohydrolase) and pullulanase [*9012-47-9*] (pullulan-6-glucanohydrolase) hydrolyzes α-1,6-glucosidic bonds of starch. When amylopectin is treated with a pullulanase, linear amylose fragments are obtained. Using a heat- and acid-stable pullulanase in combination with saccharifying enzymes makes the starch conversion reactions more efficient (71).

Malted barley contains α- and β-amylases along with proteases and phytases. Most standardized microbial enzyme preparations for industrial starch conversion contain approximately 100 times more amylase activity than malt. In beermaking, malt is not just valuable for its enzymes but also for flavor compounds.
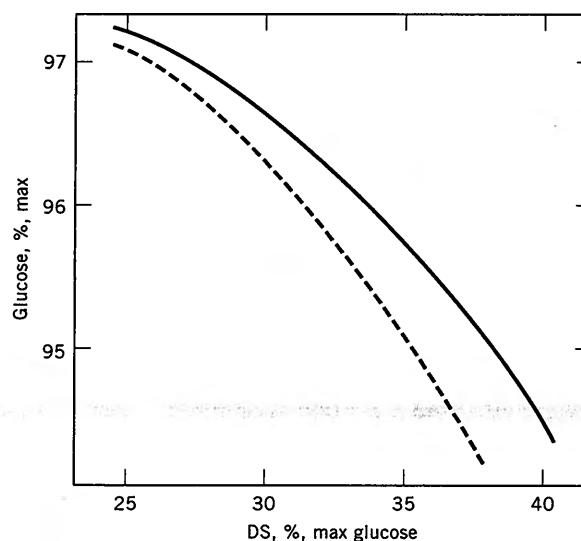
Saccharification of Liquefied Starch. Maltodextrin [*9050-36-6*], DE of 15–25, produced from liquefied starch is commercially valuable for its rheological properties. Maltodextrins are used in the food industry as fillers, stabilizers, thickeners, pastes, and glues. When maltodextrins are saccharified by further hydrolysis using an amyloglucosidase or fungal α-amylase, a variety of sweeteners can be produced with DE values of 40–45 (maltose), 50–55 (high maltose syrups), and 55–70 (high conversion syrup) (72). By applying a series of enzymes including β-amylases, glucoamylases, and pullulanases for debranching, it is possible to produce conversion syrups with maltose contents close to 80% (71). A syrup with a glucose content of 95–97% can be produced from most starch raw materials, eg, corn, wheat, potatoes, tapioca, barley, and rice.

An obstacle in the saccharification of starch has been the α-1,6 bonds, ie, the branch point barriers. Amyloglucosidases, introduced in the early 1960s, hydrolyze the α-1,4 bonds rapidly, but the α-1,6 bonds are hydrolyzed much more slowly. By using a pullulanase in conjunction with the amyloglucosidase (AMG) at the start of the saccharification, the α-1,6 bonds of the branched dextrins can also be hydrolyzed rapidly. As a result, fewer branched oligosaccharides accumulate toward the end of the saccharification. This reduces the tendency for glucose molecules to condense to mostly isomaltose. The point at which the reverse reaction outweighs glucose formation is thus shifted toward a higher glucose concentration

(Fig. 10). Figure 11 indicates that AMG and pullulanase together yield higher glucose than AMG alone. It also illustrates the influence of the dry substance (DS) on the maximum yield of glucose. Carrying out the saccharification at lower dry substance levels results in higher glucose yields.
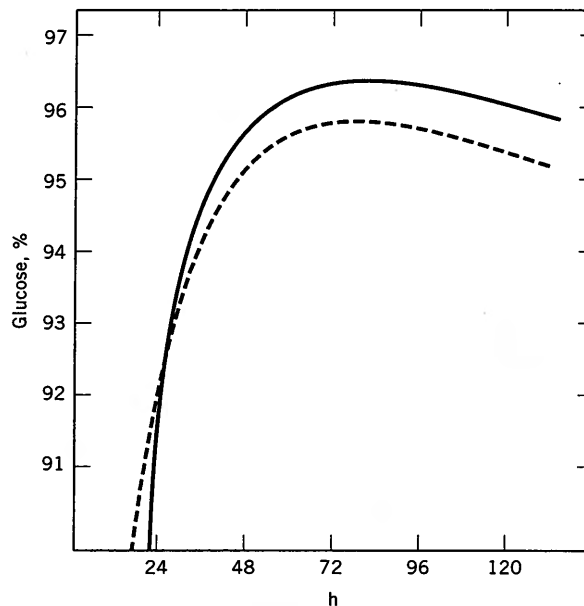
Fig. 10. Saccharification of starch using amyloglucoside (AMG); (———) with pullulanase; (———) without pullulanase. Initial dry substance (DS) of 28% at 60°C, pH 4.3.

Fig. 11. Effect of dry substance (DS) on maximal obtainable % glucose; (———) AMG and pullulanase; (———) AMG.

The purification of saccharified starch depends on the raw material used, and may be different from plant to plant. When the starch slurry is liquefied in a jet cooker the saccharification process is carried out at 55–65°C, pH 4–4.5, for 24–72 hours. The subsequent steps consist of filtration or centrifugation, ion exchange, isomerization, treatment with activated carbon, and evaporation to form a storage-stable product.

The minor content of impurities found in the starch slurry are connected to the starch granules themselves. To facilitate the purification of the starch during filtration, cellulases, pentosanases, glucanases, proteases, and pectinases are sometimes used. Wheat starch is known to form precipitates or hazes that are difficult to filter. Arabinoxylan, pentosanes, and lysophospholipids are claimed to be responsible for this problem (73).

Glucose Isomerization. Enzymatic isomerization of glucose to fructose provides a real alternative to sugar (sucrose) derived from sugarcane or sugarbeets. The commercial product obtained is known as high fructose corn syrup (HFCS). Two grades of the syrup have become established on the world market, HFCS-42 and HFCS-55, which contain 42 and 55% fructose on dry substance basis. These products account for over one-third of the caloric sweetener market in the United States.

Glucose [*50-99-7*] can be isomerized to fructose [*57-48-7*] by a reversible reaction. The equilibrium point for glucose $\rightleftarrows$ fructose is 50% under industrial conditions, and the reaction is slightly endothermic (74). The isomerization reaction can only be conducted economically by using immobilized enzymes, and reaction parameters in this system have to be adjusted carefully in order to obtain the optimal yield of fructose; eg, pH approximately 7.5 or higher, and temperatures of between 55 and 60°C to ensure high enzyme activity and stability. Under these conditions glucose and fructose are rather unstable and decompose easily to organic acids and dark-colored by-products. To overcome these problems, the reaction time is kept short by passing glucose continuously through a column of immobilized glucose isomerase.

The Immobilized Enzyme System. The glucose isomerases used are immobilized and granulated to a particle size between 0.3 and 1.0 mm. The enzyme granulates must be rigid enough to withstand compaction when they are packed into the column. Ca^{2+} acts as an inhibitor in the system, and therefore calcium salts need to be removed from the feed syrup. Conversely, Mg^{2+} acts as an activator, and magnesium salts are added to the feed syrup.

When selecting a suitable feed syrup, the main criteria are optimization of enzyme productivity and minimization of the formation of by-products. Typical feed syrup specifications are shown in Table 5. Higher syrup concentration and higher viscosity results in a reduced isomerization rate due to diffusion resistance in the pores of the immobilized enzyme. A deaeration step is desirable to remove dissolved oxygen that would otherwise increase the formation of by-products. The pH is adjusted to the optimum level for the productivity of the enzyme.

During operation, the immobilized enzyme loses activity. Most commercial enzymes show decay as a function of time (Fig. 12). The glucose isomerase in a reactor is usually replaced after three half-lives, ie, when the activity has dropped to around 12.5% of the initial value. The most stable commercial glucose isomerases have half-lives of around 200 days in practical use. To maintain the same

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Table 5. Feed Syrup Specifications

Specification	Value
temperature, °C	55–60
pH	7.5–8.0
dry substance by weight, %	40–50
glucose content, %	≥95
SO_2, ppm	0–100
calcium ion, ppm	≤1
$MgSO_4 \cdot 7\ H_2O$ (activator); g/L	0.15–0.75
conductivity, μS/cm	≤100
uv absorbance, 280 nm	≤0.5

fructose content in the finished syrup, the feed-flow rate is adjusted according to the actual activity of the enzyme. With only one isomerization reactor in operation, the result would be excessive variations in the rate of syrup production. To avoid this, several reactors at different stages in the cycle of enzyme decay are operated in combination.

Reactor design for glucose isomerization in the United States has been documented (75). The diameter of the reactor is normally between 0.6 and 1.5 m. Typical bed height is 2–5 m. The ratio between the bed height and diameter of a reactor should be at least 3:1 to ensure good flow distribution. Plants that produce more than 1000 t of HFCS per day, based on dry matter, use at least 20 separate reactors.
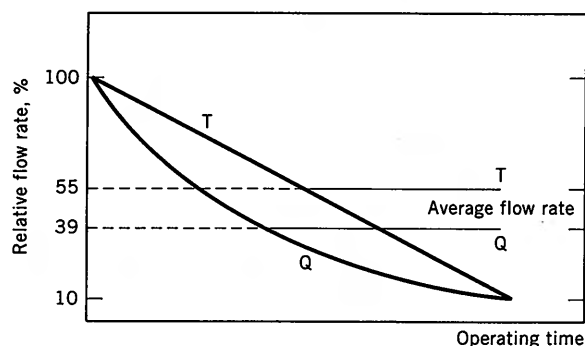
Fig. 12. Activity (syrup flow rate) vs operating time for typical immobilized isomerases, Sweetzyme T and Q.

Enzymes in Textile Finishing. To prevent the warp threads, ie, those running along the length of the fabric, from breaking during weaving, the thread is coated with an adhesive substance called a "size." Many different compounds have been used, but since the 1900s starch has been the most common sizing agent. After weaving, the size must be removed to prepare the fabric for bleaching or dyeing, ie, finishing.

Enzymatic desizing is one of the oldest nonfood applications of commercial amylases. Another type of enzyme, microbial cellulases, has developed within the

textile industry as a tool for fabric finishing, in particular for denim garment finishing. Cellulases can achieve the fashionable worn look traditionally obtained by the abrasive action of pumice stones, ie, stone-washing.

Cotton Textiles. Starch-splitting enzymes are used for desizing cotton textiles due to their high efficiency and specific way of desizing woven fabric without harmful effects on the yarn. Recommendations on how to desize textiles depend on the type of equipment used, and exact recommendations do not exist. Some general guidance has been published, and a brief guide is given here.

Desizing on a jig is a simple method where the fabric is transferred from one roll to another through a bath. The sized fabric is prewashed in boiling water, whereby the starch is gelatinized. The fabric then goes through an impregnation stage before a thermostable amylase is added. Before adding the enzyme, the desizing liquor is adjusted to pH 6.5–7.0 and a temperature in the range 60–80°C. At the end of the process, the temperature is raised to 90–95°C. The dextrins formed are then removed by washing at 90–95°C for 120 seconds. The jig process is not suitable for continuous high speed operation, where the enzyme reaction time can be reduced to between 15 and 120 seconds.

Desizing on pad rolls is a continuous process with regard to the passage of the fabric, but a holding time of 2–16 h at 20–60°C is required. A mesophilic α-amylase is used before the size is removed in a wash chamber.

Desizing in a steam chamber is a fully continuous process. The desizing reaction is performed in a steam chamber at 95–100°C. This demands the most temperature-stable amylase available.

Denim Finishing. Most denim garments are treated in laundries before reaching consumers. The garments are given a fashionable appearance such as the stone-wash or acid-wash finishes.

Stone-washing is carried out by lightweight pumice stones that are put into industrial laundry machines with the jeans. The stones rub against the denim and remove some of the dye. However, too much abrasion from stones can damage the fabric, particularly hems and waistbands.

Enzymatic stone-washing is performed either entirely without stones or sometimes by a combination of stones and enzymes. Cellulases are used to attack the surface of the cellulose fiber, but leave the interior intact. Denim garments are dyed with indigo blue, which stays on the surface of the yarn. The cellulase partly hydrolyzes the surface of the fiber, and the indigo blue is partly removed. Either neutral-type cellulases acting at pH 6–8 or acid-type cellulases acting at pH 4–5 are used for these processes.

A typical enzymatic stone-washing process (76) is as follows: load garments into industrial laundry machine, add water, and heat to 50–60°C. Adjust pH to 6.0 with acetic acid or buffer. Desize garment with α-amylase for 10–15 min, and drain water. Add new water, heat to 55–60°C, adjust pH to 6.5–7.0, and add cellulase. Tumble for 20–90 min, drain, rinse twice, and dry.

Backstaining occurs when indigo blue dye that has been lifted off the garment is redeposited onto it. In denim finishing, this effect has to be taken into account carefully. Backstaining depends, among other things, on the pH of the wash liquor. At low (4–6) pH values backstaining is relatively high. However, it is significantly lower in the pH range around neutral. Therefore, neutral cellulases result in a minimum of backstaining and a better stone-wash finish is ob-

tained. The possibility exists to obtain variations in the color and contrast of the denim by using acid cellulases, neutral cellulases, or a combination of both.

Compared with pumice stones, cellulases work without damaging washing machines or garments, there is no need for disposal of the used stones, and the quality of the wastewater is improved. In addition, the labor-intensive job of removing the dust and small stones from the finished garment is eliminated. The overall economics of the process, the higher number of denim garments in each wash load, shorter treatment time, and environmental advantages have made enzymatic stone-washing, or biostoning, a process with great future potential (76).

Biopolishing of Cotton Fabrics.   The prevention of pilling, ie, the accumulation of balls of fluff on fabric, and improvement of the smoothness and softness of cotton fabrics are of interest in the textile industry.

In the case of softness, conventional softeners are inclined to be washed out and often make fabrics feel greasy. These problems can be overcome using mixtures of cellulases. The process is referred to as biopolishing. The softness obtained through biopolishing is washproof and nongreasy. Cellulases also have a permanent effect on reducing the tendency to pilling. The enzymatic action only affects the cellulose part of mixed fibers and yarns. The enzyme hydrolyzes the microfibrils protruding from the surface of yarn because these are most accessible. As a result, the microfibrils become weakened and tend to break off. This gives a smoother surface. The improvements in the fabric are obtained with a limited reduction in the bursting strength and no detrimental effect on the fabric's ability to absorb water (77).

Enzymes for Silk Degumming.   Raw silk thread and raw silk fabrics must be degummed to remove sericin, a protein substance that covers the fibroin, ie, the silk fiber. Traditionally, degumming is performed using alkaline soap. However, proteolytic enzymes provide an alternative. With enzymes, there is no risk of damaging the fibroin, of excessive degumming, or of uneven dyeing resulting from residues of soap. The cost of treating the wastewater is reduced as well.

Enzymes in the Tannery.   The processing of skins and hides for leather (qv) has been based on enzymes ever since 1908 when Otto Röhm patented the first standardized bate based on pancreatic enzymes (78). Leather chemistry research helped to improve understanding of the bating process, and at the same time spurred on developments to improve leather processing (79).

Stages involved in the processing of hides to leather include curing, soaking, liming/unhairing, deliming, bating, pickling, and tanning. The main benefits of using enzymes during the different stages of leather manufacturing are reduced process time, increased opening up of fibrous structure, cleaner surface, increased softness, improved area yield, and reduced need for chemicals.

The animal hide is composed of three main layers. The grain is the outer layer where the hairs are embedded. This layer also contains the small glands producing oils and sweat. The corium represents the principal part of the hide. To convert raw hide into leather, a great deal of interfibrillar material must be removed from this layer. The proteoglycan dermatan sulfate is important because it can be regarded as a kind of cement binding collagen fibrils. The connective tissue on the underside of the hide contains the blood vessels, fat tissue, and some meat proteins. The proteins to take into consideration are collagen, keratin, and

some glycoproteins. Acidic polysaccharides attached to a protein core are another important group of substrates.

Cured hides must be properly soaked to obtain satisfactory rehydration and removal of unwanted material. Interfibrillar proteins should be degraded in order to increase water uptake. Bacterial proteases and pancreatic proteases are normally preferred, and are compatible with most tannery chemicals used in soaking, ie, most surfactants and preservatives containing sodium chlorite.

Lipids have been studied more intensively for applications in tanneries. Industrial lipases can be used for degreasing (80).

Enzymes for Liming and Bating. An important discovery (79) for the leather industry was the close relationship between the amount of dermatan sulfate removed and the degree of opening up. Until this discovery, the use of enzymes during the liming and unhairing steps was not considered worthwhile.

During liming, the highly charged dermatan sulfate glycosaminoglycan side chains are split from the proteoglycan back-bone because of the strongly alkaline conditions created by the lime. Thereby a sheath of high ion-density is removed from nearly every collagen fibril. This noncollagenous protein core of the proteoglycan can also be degraded by alkaline-stable proteolytic enzyme preparations. This application is known as enzyme-assisted liming. One example is the use of NUE 0.6 MPX (81) (Novo Nordisk A/S) which provides a number of options, ie, accelerating the unhairing process, improving the opening up of grain leather, reducing the requirement for sulfides by up to 40% (79), reducing the growth and mottle of pelt, increasing the strength of grain leather, and increasing the area yield.

Prior to the bating process, the hides are delimed with ammonium sulfate and/or ammonium chloride. Proteases are then applied. The early preparation proposed by Röhm was pancreatic trypsin. The use of a bating enzyme makes the hides soft and supple to prepare them for tanning. A new microbial protease, Pyrase 250 MP (82) (Novo Nordisk A/S) has been found to be a promising substitute for pancreatic trypsin [9002-07-7], which is more expensive because it must be extracted from pancreatic glands.

Future Developments. Leathermaking is a labor-intensive industry with an eye on costs and improved productivity. Enzymes increase output without requiring investments in new equipment. A large amount of organic waste originates from the beamhouse of a tannery (83), and as environmental awareness grows and regulations become tougher, enzymatic processes may provide ways of treating tannery waste such as chrome shavings and fleshings. Enzymes may also be used to replace chemicals, eg, a degreasing process based on solvents may be substituted by an enzymatic process that can be performed in water.

Enzymes in Pulp and Paper Production. Enzyme-modified starch has been used for adhesives to strengthen paper base and for surface coating. Developments since the late 1980s of further uses of enzymes in papermaking include pitch control and bleach boosting. (see PAPER; PULP).

Pitch Control. Resinous constituents of wood cause problems in paper machines by sticking to the rollers and causing spots or holes in the paper; the worst cases cause paper webs to rupture. Costly stoppages, wastages, and quality problems because of these resinous substances can be avoided by using lipases (84).

Triglycerides are important constituents of resin. In softwood, the triglycerides account for 20–40% of total resin content, and in hardwood, 40–50%. The paper industry uses the term pitch for resins that create problems in paper machines. Traditionally, pitch is controlled or reduced by aging the wood, by use of chemicals to avoid deposits on the rolls, or by intensive washing of the pulp. All these methods add to the cost of paper production. An alternative is to add a lipase to the pulp in a reaction lasting about one hour with the help of agitation. Results from Japanese paper mills show substantial reductions in pitch-related problems when using a lipase. The lipase is now used regularly to treat the groundwood pulp for the production of newsprint (85).

Bleach Boosting.   The lignin residues in chemical pulp have a strong tendency to turn yellow or brown when exposed to light and heat. Residual lignin is traditionally removed in a series of bleaching steps using a combination of chlorination and extraction. The effluent from bleaching plants is of great environmental concern because of the content of chlorinated organic substances. Residual lignin in pulp binds to hemicellulose; enzymes capable of opening up the hemicellulose structure of the pulp have been shown to facilitate the removal of lignin. During the bleaching stage, bleaching chemicals can attack any residual lignin much more effectively if the pulp has been treated with enzymes first. The first experimental industrial enzyme preparations for bleach boosting are xylanases; when used in dosages of approximately 1 kg/t of pulp, the desired brightness level of the finished paper can be obtained with one-third less chlorine (86).

Enzymes in the Animal Feed Industry.   Many feed components are not fully digested by animals. The direct addition of enzymes to feed can enhance the digestibility of these components. Enzymes have proven to be a successful tool in allowing feed compounders to develop feeds with unconventional and inexpensive formulations. Enzymatic processing of low cost raw materials such as cereals, beans, or seeds allow them to act as substitutes for high quality feed components for young animals. Another area of application is silage production, which can be improved significantly by the combined action of industrial enzymes and lactic acid bacteria.

Enzymes should be added to the feed together with the pre-mix. Granulated enzyme products may readily be mixed with feed components, as they are based on normal feed components such as wheat or soy grits. A wide range of enzyme products are available. Enzyme products should contain specific enzyme activities necessary to degrade specific substances such as glucans, starch, protein, pectin-like polysaccharides, phytic acid, raffinose, stachyose, hemicellulose, and cellulose.

The effect of enzymes, recorded in many feed trials, includes increased final weight of the animal, better feed utilization, improved feed conversion ratio (FCR), more homogeneous production, reduced mortality, and a reduced amount of sticky droppings (in chickens) (87).
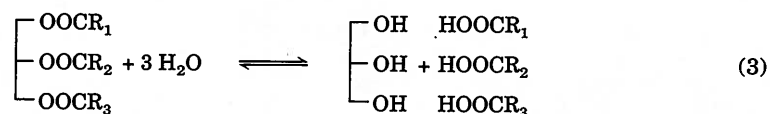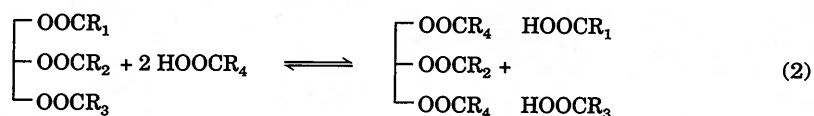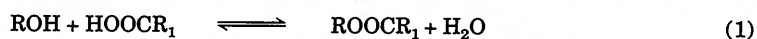
The earlier a piglet or calf can be weaned, the better from the breeder's point of view. Great amounts of milk powder are being used in feed milk replacers; with high milk prices, there is an interest in replacing at least part of the milk powder with vegetable protein. Using enzymatic modification, it is possible to make soy protein or rape seed protein perform similarly to milk with regard to nutritional

properties and functional properties like solubilization and emulsification. Such modifications are made by using plant cell-wall degrading enzymes, proteases, and specific carbohydrases.

Enzymatic Modification of Lipids. The value of enzymes as biosynthetic agents has been recognized for many years, particularly in the field of lipids. Because of the highly selective mode of action and the ability of specific enzymes to catalyze reactions in organic–aqueous interfaces, enzymes are useful in synthetic organic chemistry. To enhance stability and the rates of reaction, immobilized enzymes are often used.

Concentrated efforts to introduce immobilized lipase technology in the fats and oleochemicals industry have been made since the mid-1980s (88).

A number of specific lipases are used for ester synthesis (eq. 1); transesterification, eg, acidolysis with 1,3 specific lipase (eq. 2); and hydrolysis reactions, eg, with nonspecific lipase (eq. 3).

$$ROH + HOOCR_1 \rightleftharpoons ROOCR_1 + H_2O \tag{1}$$

$$\begin{bmatrix} OOCR_1 \\ OOCR_2 \\ OOCR_3 \end{bmatrix} + 2\ HOOCR_4 \rightleftharpoons \begin{bmatrix} OOCR_4 & HOOCR_1 \\ OOCR_2 + \\ OOCR_4 & HOOCR_3 \end{bmatrix} \tag{2}$$

$$\begin{bmatrix} OOCR_1 \\ OOCR_2 \\ OOCR_3 \end{bmatrix} + 3\ H_2O \rightleftharpoons \begin{bmatrix} OH & HOOCR_1 \\ OH + HOOCR_2 \\ OH & HOOCR_3 \end{bmatrix} \tag{3}$$

The components involved in the reactions are oils (triglycerides), glycerol, free fatty acids, esters, and alcohols. Lipases provide an opportunity for the oils and fats industry to produce new types of triglycerides, esters, and fatty acids, and also to make existing products of a higher quality than when using conventional technology. A few examples of products are edible oil that is nutritionally balanced with respect to saturated and unsaturated fatty acids, cocoa butter equivalents, esters for lubricants and cosmetics, monoglycerides as emulsifiers, etc.

Food Applications. A number of features make enzymes ideal catalysts for the food industry. They are all natural, efficient, and specific; work under mild conditions; have a high degree of purity; and are available as standardized preparations. Because enzymatic reactions can be conducted at moderate temperatures and pH values, simple equipment can be used, and only few by-products are formed. Furthermore, enzymatic reactions are easily controlled and can be stopped when the desired degree of conversion is reached.

Dairy Products. Milk is processed into a variety of products. Even in ancient times calf rennet was used for coagulation during cheese production. The milk clotting effect of rennet is due to a specific and limited hydrolysis of the κ-casein surrounding the protein micelles. As a result, the micelles lose their electrostatic charge and are able to aggregate with the help of calcium and phosphate ions to

form a network which traps the fat micelles. A gel structure is thus formed. The enzyme present in rennet, chymosin [9001-98-5] (rennin), is extracted from the gastric mucosa of young mammals such as calves and lambs and is a highly specific endoproteinase.

Microbial rennets from a number of producers, eg, Novo Nordisk, Gist Brocades, and Miles, have been available since the 1970s and have proved satisfactory for the production of different kinds of cheese. Their price is considerably lower than that of chymosin. Their properties have proven very similar to those of chymosin (89,90), and only slight modifications of the traditional cheesemaking technique are required in practice.

Microbial rennet may be produced by submerged fermentation of selected strains, eg, the fungus *Rhizomucor miehei*. Various versions of such enzymes have been developed; the principal differences are the thermolability of the enzyme itself. This helps cheesemakers develop their particular type of cheese under local conditions. Products made by recombinant DNA techniques inducing microorganisms to produce chymosin are now being introduced onto the cheese market by Pfizer and Gist Brocades.

In some parts of the world, pepsin is also used to clot milk, but it is much less specific and can give rise to a number of degradation products that tend to taste bitter.

Lactase [9031-11-2] (β-galactosidase) is used to manufacture milk products with a reduced content of lactose, ie, milk sugar, by hydrolyzing it to glucose and galactose. Many people are lactose intolerant and do not have sufficient lactase to digest lactose. By using lactase, lactose can be broken down, and a whole range of lactose-free milk products made. Manufacturers of ice cream, yogurt, and frozen desserts use lactase to improve scoop, creaminess, sweetness, digestibility, and texture of the products.

Two other practical applications of enzyme technology used in dairy industry are the modification of proteins with proteases to reduce possible allergens in cow milk products fed to infants, and the hydrolysis of milk with lipases for the development of lipolytic flavors in speciality cheeses.

Baking.   Flour contains enzymes, the most important of which are amylases and proteases. However, the quantities of these enzymes are not always ideal for baking purposes, and supplementary enzymes are often added. Many potential applications of enzymes are being investigated to improve the properties of bread. Traditional applications of enzymes are for improvement of the dough, loaf volume, crumb structure, and shelf-life. The enzyme products used are free-flowing microgranulates that are easy to handle and freely mixed with flour.

To standardize the α-amylase content of flour, a fungal α-amylase is used. Amyloglucosidase [9032-08-0] is used to break down starch, oligosaccharides, and dextrins into glucose to develop crust coloring and, together with fungal α-amylase, for stable chilled or frozen doughs. Fungal α-amylase also improves dough-handling, crumb structure, and loaf volume.

A pentosanase that works on the gluten–pentosan fraction of flour results in easier dough-handling and improved crumb structure. Pentosanases are able to replace 50–100% of the emulsifiers used, ie, an additive is replaced by a more natural ingredient.

A neutral bacterial endoprotease can be used to weaken the gluten in wheat flour, if necessary, or to provide the plastic properties required in a dough used for biscuits.

A bacterial maltogenic amylase preparation has unique qualities as an antistaling agent. Additionally, it cannot be overdosed, which means that there is no risk of obtaining sticky doughs and a gummy crumb structure.

Brewing. The malting of barley to produce enzymes is one of the central steps in the brewing process (see BEER; BEVERAGE SPIRITS, DISTILLED). If too little enzyme activity is present during malting or mashing the extract yield is low, wort separation takes longer, the fermentation process is slower with less alcohol being produced, beer filtration rate is reduced, and beer flavor and stability is inferior. Industrial enzymes are used to supplement malt enzymes in order to prevent these problems. Industrial enzymes are also used to give a better liquefaction of adjuncts, produce low carbohydrate beer, shorten beer maturation time, and produce beer from cheaper raw materials.

Malt is the traditional source of α-amylase for the liquefaction of adjuncts. The action of α-amylase ensures a simpler liquefaction stage and a reduced process time. There is a strong trend to use heat-stable α-amylase preparations, eg, Termamyl, that are much more stable than malt's own α-amylase. This allows the various malt enzymes to be preserved for the saccharification process where they are used more fully and they are not otherwise inactivated during liquefaction and go to waste; this rationalizes brewhouse operations, gives a better wort, and ultimately better beer. The use of heat-stable α-amylase also eliminates the malt from the adjunct cooker, giving a smaller adjunct mash, and the brewer has greater freedom to balance volumes and temperatures in the mashing program, problems for many brewers who use a high adjunct ratio.

Traditionally, the use of barley has been limited to 10–20% of the grist when using a good-quality malt. When going to higher levels of replacement or when using a lower quality malt, processing becomes more difficult. In these cases, the mash will need to be supplemented with extra enzyme activity in order to be able to maintain performance. The enzymes are added individually according to need or at mashing-in as a malt-equivalent blend of α-amylase, β-glucanase, and protease.

Wort separation and beer filtration are two common bottlenecks in the brewing process. Poor lautering not only causes a loss in production capacity, but can also lead to losses in extract yield. Furthermore, a slow lautering negatively affects the quality of the wort, which may give beer filtration problems and problems with the flavor and stability of the beer.

A thorough breakdown of β-glucans and pentosans during mashing is essential for fast wort separation. Undegraded β-glucans and pentosans carried into the fermenter reduce the beer filter capacity and increase the consumption of kieselguhr. A wide selection of β-glucanase–pentosanase preparations for use during mashing or fermentation/maturation are available to solve these problems.

Small adjustments in fermentability can be obtained by adding a debranching enzyme or fungal α-amylase at mashing-in, or by adding a fungal α-amylase at the start of fermentation. Beer types with a very high attenuation can be made with saccharifying enzymes, eg, fungal α-amylase to produce maltose and dextrins

for the main part, and amyloglucosidase to produce glucose from both linear and branched dextrins.

If the yeast does not get enough free amino nitrogen, the fermentation will be poor and the beer quality inferior. A neutral bacterial protease added at mashing-in can be used to raise the level of free amino nitrogen. This is useful when working with poorly modified malt or with high adjunct ratios.

Diacetyl is formed by a nonenzymatic oxidative decarboxylation of α-acetolactate, produced by yeast during primary fermentation. The diacetyl is removed again by yeast during the maturation stage by conversion to acetoin, which has a much higher flavor threshold value. By adding α-acetolactate decarboxylase [9025-02-9] at the beginning of the primary fermentation, it is possible to bypass the diacetyl stage and bring about the formation of acetoin during primary fermentation. This makes it possible to shorten or completely eliminate the maturation period (Fig. 13).
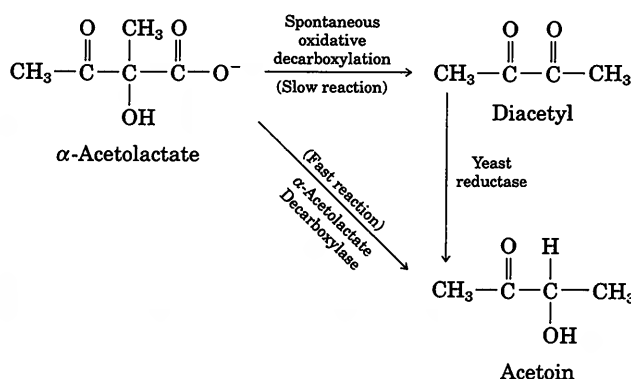

Fig. 13. Removal of α-acetolactate during fermentation.

Protein Modification. The hydrolysis of proteins with enzymes is often an attractive means of obtaining better functional and nutritional properties in food proteins. Enzymes are applied to food proteins for manufacturing new and valuable products from vegetable origin or from animal proteins that are present in by-products, eg, from slaughterhouses. Many different protein raw materials are used with different purposes in mind. Extraction processes with enhanced yields include soya milk, scrap meat recovery, bone cleaning, gelatin, fish/meat stickwater, rendering of fat, and deskinning of fish roe. Processes for producing new and promising food ingredients include isoelectric soluble soya protein, egg white substitute from soya protein; emulsifier of soya protein, soluble wheat gluten, foaming wheat gluten, blood cell hydrolyzate, whey protein hydrolyzates, casein hydrolyzates, soluble meat proteins, and gelatin hydrolyzates. The characteristics of some commercial enzyme products used for the industrial conversion of food protein products are shown in Table 6.

The enzymes used on proteins are proteases that cleave peptide bonds by a hydrolysis reaction. The hydrolysis parameters are a percent protein (S), enzyme–substrate ratio in activity units per kg protein (E/S), pH, and temperature. In addition, the specificity and properties of the enzyme itself are taken into ac-

Table 6. Commercial Proteolytic Enzymes[a]

| Product name[b]	Origin	Activity, Anson units/g[c]	Practical application	
			pH	°C
Alcalase	*Bacillus licheniformis*	2.4	6–10	10–80
Esperase	*Bacillus lentus*	2.4	7–12	10–80
Neutrase	*Bacillus amyloliquefacus*	0.5	6–8	10–65
Rennilase[d]	*Mucor meihei*		3–6	10–50
Trypsin[e]	pancreatic	3.3	7–9	10–55

[a]Liquid form unless noted.
[b]Novo Nordisk trade names.
[c]One Anson unit is the amount of enzyme that, under standard conditions, digests hemoglobin at an initial rate, liberating per minute an amount of TCA-soluble product which produces the same color with phenol reagent as one milliequivalent of tyrosine (91).
[d]Liquid or granulate form; Anson units standardized in milk clotting activities.
[e]Granulate form.

count to determine the course of a reaction on a given protein. The degree of hydrolysis is an important quantitative measure used to assess a proteolytic reaction. This is calculated by determining the number of peptide bonds cleaved and the total number of peptide bonds in the intact protein. The degree of hydrolysis of enzymatically treated proteins can be used to indicate properties of relevance to food applications. It is therefore of the utmost importance that the degree of hydrolysis be measured while the reaction is occurring. Only in this way is it possible to stop the reaction at a definite point, when the desired property of the product has been obtained. The whole subject of methods for monitoring protease reactions has been reviewed (92). Relatively simple analytical tools are often used, eg, pH-stat/pH-drops, osmometry, viscosimetry, and chemical determination of free amino groups.

As a result of the enzymatic degradation of proteins, key indexes change, ie, protein solubility indexes (PSI), peptide chain length (PCL), and protein solubility in 0.8 M TCA (TCA-index) (Fig. 14). Unpleasant bitterness was once a problem for some protein hydrolyzates. This problem can now be overcome by proper selection of the reaction parameters and the enzymes used.

Bone Cleaning. As an alternative to rendering, an enzymatic process can be used to upgrade fresh bones to valuable products, eg, cleaned bone suitable for gelatin production and a meat protein hydrolysate for the food industry. This process is performed as a two-step enzyme process, ie, scrap meat recovery and bone cleaning.

First, the fresh bone material is crushed and mixed with water to a dry solids content of approximately 25%. Neutrase is added and the slurry is agitated at 60–65°C, pH 6.5–7; inactivation of the enzyme is simultaneously achieved when the reaction is carried out at 60–65°C. To ensure a high yield of hydrolyzate and avoid formation of a bitter flavor, the degree of hydrolysis, according to the TNBS method (92), should be in the range of 5–10%. To defat the hydrolyzate, it is centrifuged at 90°C. The fat is a valuable by-product that is further refined for use in foods. The protein solution may be concentrated or dried, and is used as
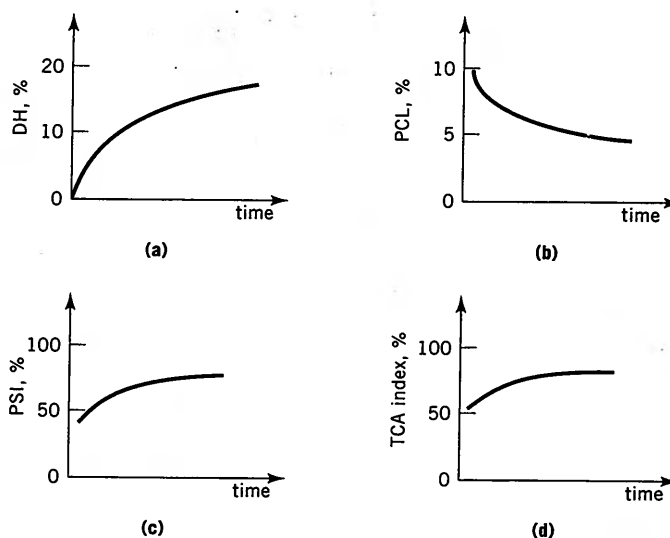
Fig. 14. Time dependence of key indexes during enzymatic hydrolysis: (**a**) degree of hydrolysis (DH); (**b**) peptide chain length (PCL) (soluble peptides); (**c**) protein solubility index (PSI); (**d**) protein solubility in 0.8 M TCA (TCA/index), %.

an ingredient in the meat industry or as a clear soluble meat extract for soups and seasonings.

Bone cleaning is the second stage of enzymatic extraction. The solid bone fraction from the first separation is mixed 1:1 with hot water (65–75°C) and treated with alkaline-type proteases. After a reaction time of one hour, the bones are separated and washed with water. The cleaned bones make an excellent raw material for the production of gelatin.

Modification of Wheat Gluten. Wheat gluten hydrolyzate can be used to make protein-enriched foods and drinks. A completely soluble hydrolyzate is desirable for this application. High protein solubility can be obtained at higher degrees of hydrolysis, as shown in Figure 15. A degree of hydrolysis of about 10% results in solubilization of more than 90% of the gluten. A 100% soluble, bland-tasting wheat gluten hydrolyzate with high yield can be recovered by centrifugation and concentration. Inactivation of the protease is carried out during hydrolysis if the reaction is carried out at a temperature above the denaturation temperature of the enzyme (93). Examples of hydrolysis curves are shown in Figure 15. They are plotted by using a pH-stat for the monitoring of the progress of the reaction.

A protein ingredient with good whipping properties can be used in baked goods and for different types of candy. The optimal whipping properties of wheat gluten are obtained at a degree of hydrolysis of 2–3%. The active whipping protein is recovered by centrifugation and drying.

Extraction Processes of Material. Many ingredients used by the food and brewing industries are produced by extraction from plant matter. Examples include protein, starch, sugar, fruit juice, oil, flavor, color, coffee, and tea. These are all found in the cells of plant matter, ie, seeds, fruits, etc.
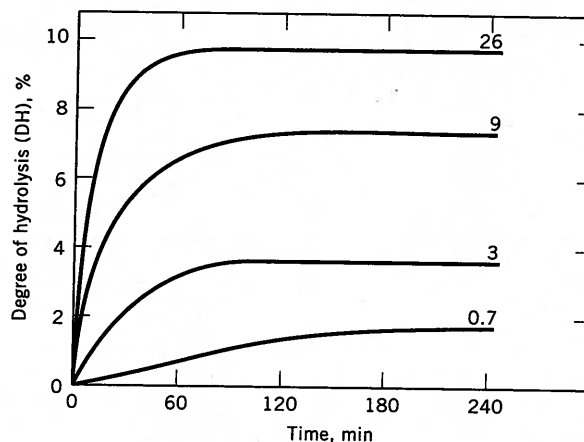
Fig. 15. Enzymatic hydrolysis of wheat gluten at 72.5°C and pH 7.5 by an alkaline protease from *Bacillus licheniformis*. The numbers on the curves are enzyme–substrate ratios (E/S) in activity units (AU)/kg of protein where S = 7.4% (N × 5.7).

An important development is the degradation of very complex polysaccharides found in the cell walls of unlignified plant matter. These cell walls are composed of cellulose fibers to which strands of hemicelluloses are attached. The fibers are embedded in a matrix of pectic substances linked to a structural protein. Enzyme preparations capable of attacking plant cell walls contain different enzyme activities, eg, pectinase, hemicellulase, and cellulase. Conventional enzyme products within these groups are, however, unable to degrade completely the rhamnogalacturonan backbone of the pectic substances. The enzyme complex SPS-ase, named after its intended commercial use as a soya polysaccharide-degrading enzyme, has been produced from a selected strain of *Aspergillus niger* (94). This enzyme preparation contains 10–15 different enzyme activities that offer the possibility of producing different varieties of commercial enzyme products designed for various applications.

Pectinases have been used in fruit juice processing since the 1930s. They are used regularly when making juice from almost all types of fruit and berries, and the use of cell-wall degrading enzymes is also found in winemaking. The enzymes are used to improve the yield of juice, liquefy the entire fruit for maximal utilization of the raw material, improve color and aroma, clarify juice, and break down all insoluble carbohydrates like pectins, hemicellulose, and starch. For the clarification of juice, a mixture of pectinases are required, ie, pectin transeliminase (PTE), polygalacturonase (PG), and pectin methylesterase (PE). Araban is a polysaccharide with a high molecular weight, which may cause haze problems in concentrates. Therefore, apart from the pectinase activities mentioned, clarification enzymes should also contain a substantial amount of arabanase side activity.

Oil from rape seed, coconut, corn germ, sunflower seed, palm kernel, and olives is traditionally produced by a combined process using pressing followed by extraction with organic solvents. Cell-wall degrading enzymes may be used to extract vegetable oil in an aqueous process. They break down the cell-wall structure and release the oil. This concept is already in commercial use in connection

with olive oil processing, and has been thoroughly investigated for rape seed oil extraction (95).

Economic Aspects

Worldwide consumption of industrial enzymes amounted to approximately $720 million in 1990; about one-third was accounted for by the U.S. market. Estimation of worldwide consumption is difficult because official production figures are scarce. A relatively large portion of the production of starch-processing enzymes is for internal consumption. Furthermore, the currency used for the estimation also influences the result considerably.

The growth in volume of the enzyme business from 1980 to 1990 is estimated to be 5–10% per year. The estimated worldwide enzyme sales per industry are shown in Table 7. The detergent and starch conversion industries are by far the most important, and account for 60% of total enzyme sales. Five principal industries account for around 85% of enzyme sales, whereas the remaining sales are spread over many different industries.

Table 7. Estimated Worldwide Enzyme Sales by Industry, 1990

Industry	Sales, 10^6 $
detergent	300
starch	125
dairy	80
textile	60
alcohol	45
other	110
Total	*720*

Table 8. Estimated Worldwide Enzyme Consumption by Product Type, 1990

Enzyme	Consumption, 10^6 t
protease[a]	309
rennet (animal and microbial)	74
glucose isomerase	41
glucoamylase	75
amylase (other than glucoamylases)	112
cellulase	55
lipase	11
papain	8
invertase	8
pectinase	7
other	20
Total	*720*

[a]Includes everything except rennet and papain; bulk share is in the detergent industry.

Three proteases account for almost all sales to the dairy industry, ie, chymosin extracted from calves' stomachs, chymosin produced by fermentation, and substitutes also produced by fermentation. Four different types of enzyme are used in the detergent industry, ie, proteases, amylases, cellulases, and lipases. Cellulases and lipases have only recently been introduced. Table 8 shows a breakdown of estimated worldwide sales consumption by product types.

Environmental and Safety Aspects

The industrial use of microbial enzymes produced by modern biotechnology is an important contribution to the development of green technology. Enzymes have a positive impact on the environment because they replace conventional chemical-based technologies and conventional energy-intensive manufacturing processes, originate from natural biological systems, are totally biodegradable, and leave no harmful residues.

The safety and environmental impact of the production of industrial enzymes can be evaluated on three different levels, ie, the potential risk if the microorganisms, their products, or both are released into the environment; the possible health hazards to staff working with the microorganisms, their products, or both; and safety when products are used by the consumer.

Enzymes are totally biodegradable, and their release into the environment does not cause problems. The release of the production organism itself is controlled by two categories of safety measures which are complementary. The first is physical containment in a fermenter system and recovery plant with a high standard of hygiene. The second is biological containment. Being specially bred, either by traditional techniques or by modern genetic engineering techniques, to produce one specific substance, the production organisms are adapted to grow optimally only under the defined conditions during fermentation. The growth of strains of production organisms in nature is handicapped in comparison with microorganisms already existing in the environment. Their chances of survival in the environment are extremely limited.

Like other proteins, enzymes are potential allergens. In addition, proteases may act as skin and eye irritants. However, during the production and handling of industrial enzymes, the occupational health risks entailed by these properties can be avoided by protective measures, and by the form in which the enzyme preparations are supplied. In order to reduce dust generation, enzymes are supplied as liquids, encapsulates, or immobilized preparations.

To guarantee that enzymes can be used safely by the consumer, microbial enzymes are obtained from nonpathogenic and nontoxinogenic microorganisms grown on raw materials that do not contain compounds hazardous to health. When a new strain is developed, it is checked for key taxonomic characteristics, and appropriate safety tests are performed. For genetically engineered strains, the new genetic properties are carefully described.

Genetically engineered microorganisms can be used under the same conditions of containment, and the same security rules apply as for equivalent, naturally occurring microorganisms. Provided an enzyme is produced by a harmless

host, the contained use of recombinant microorganisms does not warrant any special provisions concerning production conditions, worker protection, environmental assessment, field monitoring, or product approval.

Regulatory Aspects.   National authorities have preferred to use or adapt existing legislation and regulations. For the adaptation of the food additive regulations to fit the processing aids applications of enzymes, guidance has been available in the recommendations of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), and the Food Chemicals Codex (FCC). The enzyme manufacturers associations, the Association of Microbial Food Enzyme Producers (AMFEP) in Europe and the Enzyme Technical Association (ETA) in the United States, work nationally as well as internationally for a harmonization of regulation. The Codex Committee on Food Additives and Contaminants (CCFAC) plays an important role in this work. In the European Community, common guidelines are being developed for the evaluation of enzymes for food and feed uses. This is a result of the increased attention on new biotechnology rather than on enzymes *per se*. The contained use of genetically modified microorganisms in the manufacture of enzymes has been one of the first cases of a product of recombinant DNA technology reaching the industrial marketplace. A working group on food safety and biotechnology is also considering the use of food enzymes made from genetically modified organisms.

Food Enzymes.   The source of a food enzyme determines its primary regulatory status. Traditionally, enzymes from edible parts of plants and animals, eg, papain from papaya and chymosin from calf stomach, have been accepted for food use without further evaluation. Although a few plant and animal enzymes still find industrial uses, the majority of food enzymes are produced by fermentation of microorganisms. Three categories of microorganisms have been defined by JECFA for regulatory purposes: (*1*) those considered to be food stuffs, such as *Aspergillus oryzae*, the most prominent example of this category; (*2*) those considered harmless contaminants of food, such as *Aspergillus niger, Bacillus subtilis, Bacillus licheniformis,* and *Saccharomyces cerevisiae*; and (*3*) all other microorganisms.

A microbial source for a food enzyme must be nonpathogenic and nontoxicogenic. Manufacturers of microbial food enzymes have always selected their production microorganisms from the safe end of the spectrum of available sources. Consequently, a few species have acquired a record of safe use as sources of a wide variety of food enzymes.

If a food enzyme preparation did not contain any material derived from fermentation other than the enzyme protein itself, the evaluation of its safety would be simple, and no toxicological studies would be required. For enzymes from recombinant microorganisms, the primary regulatory status is determined by the host microorganism, the donor organism, and any vectors involved in the genetic transfer. It is important to note that the new technology makes it possible to establish the function of the various segments of inserted DNA in the recombinant. Whenever the function is known, the origin of that segment of DNA becomes irrelevant.

The majority of food enzymes are used as processing aids, and they have no function in the final food. For that reason, they do not need to be declared on the label, and will not be present in the final food in any significant amount. A few enzymes, however, are used both as processing aids and as food additives. When

used as additives, they must be declared on the food label using the appropriate class name, eg, preservative or antioxidant; E-number; and generic name, eg, lysozyme or glucose oxidase. AMFEP has defined Good Manufacturing Practice (GMP) for microbial food enzymes. The most important element is to ensure a pure culture of the production microorganism.

Product specifications for microbial food enzymes have been established by JECFA and FCC. They limit or prescribe the absence of certain ubiquitous contaminants such as arsenic, heavy metals, lead, coliforms, *E. coli,* and *Salmonella.* Furthermore, they prescribe the absence of antibacterial activity and, for fungal enzymes only, mycotoxins.

Enzymes are used as feed digestibility enhancers for chicken and pigs. They must comply with purity specifications comparable to food-grade enzyme specifications. European Community (EC) guidelines for the assessment of additives in animal nutrition are being revised to make them applicable for enzymes. Upon completion of these guidelines, the regulatory status of feed enzymes will be established in EC directive 70/524/EEC.

Technical Enzymes. When an enzyme is used for a technical application, ie, industrial but nonfood and nonfeed, its regulatory status is determined by its properties as a naturally occurring substance. These properties determine the classification and consequent labeling in accordance with existing schemes for chemicals. It should be noted that enzymes are not listed as dangerous chemicals.

Enzyme manufacturers have developed formulations that minimize the release of enzyme dust. In the case of liquid preparations, handling precautions recommend users to avoid the formation of aerosol sprays. In all cases, direct contact with the skin or eyes should be avoided. Enzymes have a good record of occupational health and safety.

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