ENZYME APPLICATIONS, INDUSTRIAL

1. Introduction

Enzymes are Nature's own catalysts. They are designed to accelerate reactions taking place in the cell and its immediate surroundings, and are essential for the development and maintenance of life. These biomolecules are proteins composed of up to 20 different amino acids. By varying the structure and, for some enzymes, by incorporating cofactors, such as metal ions and organic molecules like flavins, and porphyrins a surprising diversity of activities is displayed among the several thousand enzymes known.

The effective catalytic properties of enzymes have opened the way for introducing them into a number of products and processes and applied enzymology forms an important branch of industrial biotechnology (also referred to as

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"a white biotechnology"). The most important current areas of application for industrial enzymes are summarized in Table 1 (1). In all areas of application, enzymes are used to effectively facilitate transformations that are not technically feasible by other means or to replace traditional chemical processes as enzyme-based operations inherently offer cleaner processes with less waste and impact on the environment.

The present industrial applications of enzymes are the result of a rapid development seen primarily over the past four decades thanks, first of all,

Table 1. **Enzymes Used in Various Industrial Segments and Their Application**[a]

Industry	Enzyme class	Application
detergent	protease	protein stain removal
	amylase	starch stain removal
	lipase	lipid stain removal
	cellulase	cleaning, color clarification, antiredeposition (cotton)
	mannanase	mannanan stain removal (reappearing stains)
starch and fuel	amylase	starch liquefaction and saccharification
	amyloglucosidase	saccharification
	pullulanase	saccharification
	glucose isomerase	glucose to fructose conversion
	cyclodextrin-glycosyl-transferase	cyclodextrin production
	xylanase	viscosity reduction (fuel and starch)
	protease	protease (yeast nutrition – fuel)
food (including dairy)	protease	milk clotting, infant formulas (low allergenic), flavor
	lipase	cheese flavor
	lactase	lactose removal (milk)
	pectin methyl esterase	firming fruit based products
	pectinase	fruit based products
	transglutaminase	modify viscoelastic properties
baking	amylase	bread softness and volume, flour adjustment
	xylanase	dough conditioning
	lipase	dough stability and conditioning (*in situ* emulsifier)
	phospholipase	dough stability and conditioning (*in situ* emulsifier)
	glucose oxidase	dough strengthening
	lipoxygenase	dough strengthening, bread whitening
	protease	biscuits, cookies
	transglutaminase	laminated dough strengths
animal feed	Phytase	phytate digestibility—phosphorous release
	xylanase	digestibility
	β-glucanase	digestibility
beverage	pectinase	depectinization, mashing
	amylase	juice treatment, low calorie beer
	β-glucanase	mashing
	acetolactate decarboxylase	maturation (beer)
	laccase	clarification (juice), flavor (beer), cork stopper treatment

Table 1 (*Continued*)

Industry	Enzyme class	Application
textile	cellulase	denim finishing, cotton softening
	amylase	desizing
	pectate lyase	scouring
	catalase	bleach termination
	laccase	bleaching
	peroxidase	excess dye removal
pulp and paper	lipase	pitch control, contaminant control
	protease	bio-film removal
	amylase	starch coating, deinking, drainage improvement
	xylanase	bleach boosting
	cellulase	deinking, drainage improvement, fiber modification
fats and oils	lipase	transesterification
	phospholipase	degumming, lyso-lecithin production
organic synthesis	lipase	resolution of chiral alcohols and amides
	acylase	synthesis of semisynthetic penicillin
	nitrilase	synthesis of enantiopure carboxylic acids
leather	protease	unhearing, bating
	lipase	depickling
personal care	amyloglucosidase	antimicrobial (combination with glucose oxidase)
	glucose oxidase	bleaching, antimicrobial
	peroxidase	antimicrobial

Ref. (1).

to the evolution of modern biotechnology. The latest developments in this technology introducing protein engineering and directed evolution have further revolutionized the development of industrial enzymes and made it possible to tailor-make enzymes to display new activities and adapt to new process conditions enabling a further expansion of their use. The result is a highly diversified industry that is still growing both in terms of size and complexity.

2. History

In 1833, an amylase from germinating barley was recovered and called diastase (2). Like malt itself, this product converted gelatinized starch into sugars, primarily maltose. Shortly thereafter, Berzelius proclaimed the existence of nonliving catalysts, and Schwann (3) reported on his observation and purification of pepsin (the predominant protein-degrading enzyme in gastric juice of vertebrates).

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 that could not take place without the presence of viable organisms (4). Another school (5) was convinced that 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 (6-8).

In 1878, the term enzyme, Greek for "in yeast", was proposed (9). It was reasoned that chemical compounds capable of catalysis, eg, 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.

2.1. Early Industrial Enzymes. Enzymes were used in ancient Greece for the production of cheese (10). Early references to this are found in Greek epic poems dating from ~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 thread 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 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 (11); trypsin was subsequently added to the wash (12). 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. 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 Industri A/S) 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.

Since the early successful introduction of proteases to the detergent industry several new detergent enzymes have, as indicated in Table 1 and as described in section 6.1, been introduced on the market and enzymes have added many different functionalities that play a key role to the performance of modern detergents.

Starch Industry. Enzymes have been of great value to the starch industry since the 1960s. In the 1950s, fungal amylases were used in the manufacturing 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 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.

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 amylase made it possible to use starch as a raw material for production of a sweetening agent with almost the same composition, calorie content, and sweetening effect

as ordinary cane or beet sugar. The new product, high fructose corn syrup (HFCS) or isosyrup, still dominates the market for sweeteners.

3. Catalytic Activity

3.1. Enzymatic Catalysis in General. Enzymes are catalysts, ie, they increase the rate of chemical reactions without undergoing permanent change themselves by doing so and without affecting the reaction equilibrium. The thermodynamic approach to the study of a chemical reaction is concerned with the equilibrium concentrations of the reactants and products. This involves 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.

The characteristics of enzymes are their catalytic efficiency and their specificity. Enzymes increase reaction rates by factors of often 10^6 or more compared to the uncatalyzed reaction. Many enzymes are also highly specific, ie, they catalyze only one reaction or reaction type involving only certain substrates, and consequently a vast number exists in view of the many enzyme-catalyzed reactions taking place in Nature. For example, most hydrolytic enzymes acting on carbohydrates are so specific that even the slightest change in the stereochemical configuration of the substrate is sufficient to make the enzyme unable to catalyze hydrolysis.

Usually the degree of specificity of an enzyme is related to its biological role. For example, 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 amino acids with 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. On the other hand, trypsin, also a digestive enzyme, 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* or *prosthetic groups*. A cofactor is a nonprotein compound that combines with the otherwise inactive enzyme to give the active enzyme. Examples of cofactors are ions of metals such as Mg, Ca, Fe, Co, and Cu, and organic molecules such as nicotinamide adenine dinucleotide (NAD) and flavin adenine dinucleotide (FAD). Some metal ions act by binding to specific sites in the enzyme molecule, thus stabilizing the active conformation of the enzyme molecule. Others are required for exchanging electrons in redox enzymes, such as copper ions in laccases. Tightly bound cofactors are called prosthetic groups. An example of a prosthetic group is the heme group (iron-porphyrin complex) contained in a number of enzymes such as catalases and peroxidases.

According to common theory, enzymes accelerate reactions by stabilizing the transition state, the highest energy species on the reaction pathway, 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. Compounds that mimic the structure of the transition state often bind strongly to the active site of the enzyme and may be potent inhibitors of the enzyme-catalyzed reaction (cf. section 2.3).

3.2. Enzyme Kinetics. *A Simple Model: Michaelis-Menten Kinetics.* A simple enzyme-catalyzed reaction with a single substrate can often be described by equation (1):

$$\mathrm{E} + \mathrm{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \xrightarrow{k_{\mathrm{cat}}} \mathrm{E} + \mathrm{P}$$

The enzyme, E and the substrate, S initially combine to form an enzyme–substrate complex, ES. In the second step the catalytical process occurs, whereby the enzyme is released again and the product or products, here denoted P, are formed. 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 rate V of formation of products, ie, the rate of formation of the first few percent of the products. Under these conditions the products have not accumulated, the substrates have not been depleted, and the reaction rate is generally constant over time. It is often found experimentally that V is directly proportional to the concentration of enzyme $[\mathrm{E}]_0$ and varies with the substrate concentration [S] as illustrated in Figure 1. At low [S], V is 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), which can be derived from the reaction scheme (1) assuming quasi-steady state for the enzyme–substrate complex ES (ie, slow change of [ES] compared to changes of other concentrations in the system) expresses this relation quantitatively.

$$V = \frac{k_{\mathrm{cat}}[E]_0}{1 + K_{\mathrm{M}}/[\mathrm{S}]} \quad \text{where} \quad V_{\max} = k_{\mathrm{cat}}[\mathrm{E}]_0 \quad \text{and} \quad K_{\mathrm{M}} = (k_{-1} + k_{\mathrm{cat}})/k_1$$

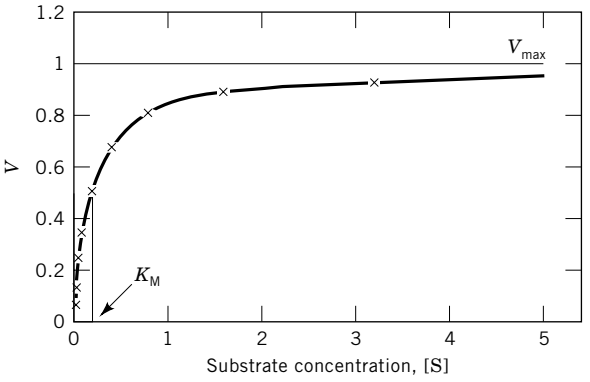

Fig. 1. Reaction rate V as a function of substrate concentration for a reaction obeying Michaelis-Menten kinetics.

The turnover number k_{cat} represents the maximum number of substrate molecules that a single enzyme molecule can convert to products per unit of time; for most enzymes, this lies in the range $1–10^4$ s^{-1}. The constant K_M is the *Michaelis constant*, and is equal to the substrate concentration at which $V = \frac{1}{2}V_{max}$; K_M for most enzymes lies in the range $10^{-6}–10^{-1}$ M. The parameter k_{cat}/K_M is called the *specificity constant* because it is a measure of the difference in specificity between competing substrates. At low substrate concentration, k_{cat}/K_M is an apparent second-order rate constant. In order to obtain an accurate determination of k_{cat} and of K_M, it is necessary to measure V at $[S] < K_M$ and at $[S] > K_M$. In cases where low 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.

More Complex Kinetics. Variation of reaction rate with substrate concentration cannot always be described by the Michaelis-Menten equation.

For example, hydrolytic rates observed with the α-amylase product Novamyl (Novozymes A/S) acting on α–1,4-linked glucose oligomers (maltodextrins) *decrease* at high substrate concentrations (13). This is a case of uncompetitive substrate inhibition (cf. section 2.3) and is probably due to the binding of a substrate molecule to a site different from the active site (an *allosteric site*), resulting in a conformational change that prevents hydrolysis of the substrate molecule in the active site.

Furthermore, real substrates of endoenzymes (enzymes cleaving polymeric substrates at internal rather than terminal sites of the chain) do often not have a uniform structure. Binding clefts of endoglycosidases, hydrolytic enzymes degrading polysaccharides, will often span 5–8 monosaccharide units, and variations in the monosaccharide units, glycosidic linkages or side chains of the substrate present inside this cleft at any given time of the degradation reaction will affect the kinetic constants. Similar arguments are valid for proteases. This kind of variation may result in an apparent non-Michaelis-Menten behavior.

Despite these shortcomings, the Michaelis-Menten equation is often useful. Even with enzymes acting on more than one substrate (eg, oxidoreductases, cf. section 6.1.4.6 apparent Michaelis-Menten behavior will normally be observed if all substrate concentrations but one are kept constant.

Heterogeneous Systems. Enzyme catalysis in a variety of applications takes place in heterogeneous environments. An example is hydrolytic enzymes such as proteases and lipases acting in a laundry wash process (14–18; see also section 6.1). In this application, enzymes and surfactants, among other components, adsorb to the soil–water and fabric–water interfaces as a function of their overall charge (ie, isoelectrical point of the enzyme vs. pH of the solution) and local charge distributions and hydrophobicity–hydrophilicity characteristics of the enzyme molecules and of the surfaces. The initially immobile soil is converted to more water soluble fragments by virtue of the enzymes in combination with the surfactant system, providing a highly dynamic environment at the interfaces. In order to describe the kinetics of such instances of interfacial catalysis, it is important to take into account the surface-to-solution exchange dynamics of enzymes and hydrolysis products from the enzymatic reaction as well as surfactants and other surface-active constituents of the system.

No universal model exists for wash systems; however, in the case of interfacial catalysis by phospholipases on vesicles of phospholipids, a system related to lipases in a wash system, experiments and kinetic theory have been described in detail (eg, 19). In lipase kinetics, activation of the enzyme by interaction with the surface is a key factor.

3.3. 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 or change its molecular structure by an irreversible reaction (forming a covalent bond), and the inhibition process consequently cannot be reverted by, eg, dilution or dialysis. Examples of irreversible inhibitors are nerve gases such as diisopropylphosphorofluoridate [55-91-4] (DFP) and the inhibitor E-64 [4-methyl-7-[(*N*-succinyl-Leu)-Leu-Val-tyrosylamido]-2*H*-chromen-2-one] of cysteine proteases.

Reversible inhibition is characterized by equilibrium between enzyme (E), inhibitor (I) and enzyme–inhibitor complex:

$$\mathrm{E} + \mathrm{I} \rightleftharpoons \mathrm{EI}$$

Many reversible inhibitors are substrate analogues and bear a close relationship to the normal substrate or to the transition state (cf. section 2.1).

When the inhibitor and the substrate compete for the same site on the enzyme, the inhibition is called *competitive*. If the uninhibited enzyme obeys the Michaelis-Menten equation, the observed reaction rate with inhibitor added will be described by the equation:

$$V = \frac{k_{\mathrm{cat}}[\mathrm{E}]_0}{1 + \dfrac{K_{\mathrm{M}}(1 + [\mathrm{I}]/K_{\mathrm{i}})}{[\mathrm{S}]}}$$

where K_{i} is the equilibrium constant of the reaction in equation 3, ie, the formation constant of the complex EI. From this it is seen that at high [S], where the number of substrate molecules greatly outnumbers 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 2.

With a reversible inhibitor binding site different from the active site, the inhibition can be either *noncompetitive* or *uncompetitive*, depending on whether or not the inhibitor can bind to the enzyme without the substrate being bound to the active site. In noncompetitive inhibition, only the apparent V_{max} is affected [$V_{\mathrm{max,\ app}} = V_{\mathrm{max}}/(1+[\mathrm{I}]/K_{\mathrm{i}})$], whereas an uncompetitive inhibitor affects both apparent V_{max} [$V_{\mathrm{max,\ app}} = V_{\mathrm{max}}/(1+[\mathrm{I}]/K_{\mathrm{i}})$] and K_{M} [$K_{\mathrm{M,\ app}} = K_{\mathrm{M}}(1+[\mathrm{I}]/K_{\mathrm{i}})$]. Finally, inhibition is called *mixed* when K_{M} and V_{max} are both affected, but differently than in the uncompetitive model.

3.4. Effect of Temperature and pH. The temperature dependence of enzymes often follows the rule that a 10°C increase in temperature approximately doubles the activity. However, this is only true as long as the enzyme is not deactivated by the thermal denaturation characteristic for enzymes and

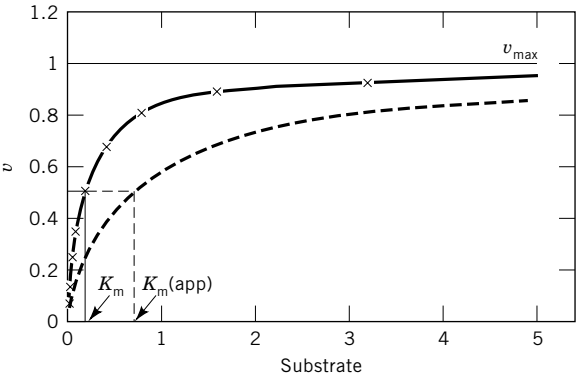

Fig. 2. The double-reciprocal plots of $1/V$ as a function of 1/[S]. Two determinations of V for each [S] are shown. $1/V_{max} = 0.87$ and $-1/K_M = -4.05$ (arbitrary units). This figure is a nonlinear regression plot of the same data where $V_{max} = 1.00$ and $K_M = 0.20$ (arbitrary units).

other proteins. The three-dimensional 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, enzymes with optima near 100°C and with optima at low temperatures exist as well.

The pH dependency of an enzyme-catalyzed reaction also typically exhibits an optimum. The subtilisins mentioned above, eg, have a broad pH optimum in the alkaline range. Other enzymes have a narrow pH optimum. The nature of the pH profile often gives clues to the mechanism of the enzyme-catalyzed reaction. Generally speaking, the pH optima for different enzymes span a wide range on the pH scale.

Temperature profiles may vary with varying pH, and pH profiles may vary with varying temperature. Both types of profile may also vary with other reaction conditions such as identity of the substrate, substrate concentration and buffer composition.

3.5. Enzyme Assays. An enzyme assay determines the amount of active enzyme present in a sample. Typically, enzymes are present at very low levels in application situations (eg, in the nanomolar range in detergent solutions for laundering or dishwashing). Therefore, it is not practical to determine enzyme content on a stoichiometric basis. Instead, enzyme activity in the sample 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 rate. These parameters must, therefore, be carefully controlled in order to achieve reproducible results when using the assay.

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 ultraviolet (uv) or the visible (vis) region. In other cases, a nonabsorbing system can be coupled to a system in which the substrate or product absorbs in the uv or vis region. Spectrofluorometry is also increasingly used. This technique may be applied if the substrate or product fluoresces or if it is possible to tag them with fluorescing groups.

An example of direct spectrophotometrical assays is the use of synthetic peptide *p*NA (4-nitroanilide) substrates for determining protease activity. If the protease cleaves the amide bond to 4-nitroaniline, release of the latter may be monitored spectrophotometrically at ~410 nm. An example of an indirect (coupled) spectrophotometric assay is the determination of α-amylase using a (4-nitrophenyl)maltoheptaoside in which the nonreducing end has been chemically modified (capped with an ethane-1,1-diyl group). Initially, the substrate is cleaved by the α-amylase, and subsequently one of the reaction products, (4-nitrophenyl)maltotrioside, is cleaved by an added auxiliary enzyme, α-glucosidase, liberating 4-nitrophenol, a chromophore that can be measured at 405 nm. The ethane-1,1-diyl group protects the maltoheptaoside from being directly attacked by the α-glucosidase.

A variation on the theme of spectrophotometric assays consists in providing insoluble substrates such as dyed cellulose, dyed and cross-linked starch, proteins labeled with fluorophores, etc. Progress of the enzyme-catalyzed degradation of the insoluble substrate is monitored via spectrophotometry or spectrofluorometry of the dye or fluorophore released. A point here is that such heterogeneous systems in some cases (cf. section 2.2.3) mimic practical application conditions more closely than an entirely soluble system. Also, the systems decribed may often be made into chemically and physically robust kits with substrate tablets that do not require special storage conditions and may be easily used for field work, in schools, etc, where instrumentation is limited.

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 triacylglycerols and the fatty acids formed are neutralized with NaOH. The rate of consumption of NaOH is a measure of the catalytic activity (16). The method can even be used under real application conditions (15).

4. Enzyme Classification and Nomenclature

4.1. Early Nomenclature and the EC Classification. During the nineteenth century, it was common practice to identify enzymes or extracts containing enzymes by adding the suffix '*in*' to the name of their source (a plant, a microorganism, etc). This led to names such as *papain, notatin, pancreatin, pepsin, subtilisin*, etc. However, this system does not give any indication of the nature of the reaction catalyzed by the enzyme or the type of substrate involved, nor does it convey information about the structure of the enzyme. Furthermore, not all source-derived names ending in *in* denote enzymes (eg, penicillin). By the end of the nineteenth century, a somewhat more informative system was in use.

The suffix '*ase*' was appended to the name of the substrate involved in the reaction, eg, amylase acting on starch (Latin *amylum*); *cellulase* acting on cellulose; *protease* on proteins; *lipase* on lipids; *urease* on urea; etc. Names that reflected the function of the enzyme, but still with the ending *ase*, were also used, but did not necessarily carry much information about the specific reaction being catalyzed (eg, *invertase, synthetase, catalase*). Some names combined the two principles, however: *glucose isomerase, galactose oxidase*.

In the long run, with the number of known enzymes increasing rapidly, a system of names based partly on sources, partly on substrates, and partly on reactions, was not satisfactory. In 1956, the International Union of Biochemistry (IUB) set up the *International Commission on Enzymes*, and in 1961 the Commission presented a report in which recommendations were given for classifying and naming enzymes. At that time, the list of enzymes totaled 712 entries.

According to the Enzyme Commission (EC) proposal, enzymes are classified, as the main principle, on the basis of the reactions they catalyze. Six main reaction types are recognized, leading to six main classes of enzymes (Table 2).

Each class is divided into subclasses and subsubclasses according to details of the reaction catalyzed. For example, in class 3, the subclass 3.1 consists of esterases and the subclass 3.2 of glycosidases, and within 3.1 the subsubclasses are characterized by the nature of the substrate, eg, 3.1.1 comprises carboxylic ester hydrolases, 3.1.2 thiolester hydrolases, etc. To the three digits are added serial numbers, and the resulting four digits are prefixed by "EC"; thus one arrives at classification numbers such as EC 3.1.1.3 for esterases acting on triacylglycerols (triglycerides). A systematic name is given to reflect the described classification, in this case "triacylglycerol acylhydrolase". In recognition of the fact that the systematic name may be too cumbersome for daily use, the enzyme list often explicitly includes "other names", in this case "lipase".

Another example is lactase (trivial name) catalyzing the conversion of lactose to galactose and glucose and given the systematic name "β-D-galactoside galactohydrolase" and the number EC 3.2.1.23 (class 3 for hydrolases; 3.2 for glycosidases; and 3.2.1 for enzymes hydrolyzing *O*-glycosyl bonds).

During the next decades, the enzyme list was revised and expanded by various bodies under the auspices of IUB in collaboration with International

Table 2. **The Six Main Classes of Enzymes in The EC Nomenclature**

Number	Class	Type of reaction catalyzed
1	oxidoreductases	transfer of electrons
2	transferases	group-transfer reactions
3	hydrolases	hydrolysis, i.e. cleavage of substrate with the addition of water
4	lyases	addition of groups to double bonds or elimination of groups to leave double bonds or rings
5	isomerases	transfer of groups within substrate molecules to yield isomers
6	ligases	formation of C–C, C–S, C–O, and C–N bonds by condensation reactions coupled to ATP cleavage

Union of Pure and Applied Chemistry (IUPAC). The last printed version of the enzyme list appeared in 1992 (20), featuring 3196 entries and some 5700 literature references.

Today (2004), the number of enzymes identified is almost 4000. The responsibility for the enzyme list is now held by the Nomenclature Committee of International Union of Biochemistry and Molecular Biology (IUBMB), which still has contact to IUPAC in the Joint Committee on Biochemical Nomenclature. The list with annotations and literature references is specifically maintained by a working group at Trinity College, Dublin, Ireland. A master relational database (*IntEnz*) containing all the information thus gathered is administered by the European Bioinformatics Institute in Cambridge, U.K. (www.ebi.ac.uk/intenz).

Three main web sites offer access to the enzyme list and the systematic names: the IUBMB home page (www.chem.qmul.ac.uk/iubmb), the Swiss Institute of Bioinformatics' site *SwissProt ENZYME* (21; http://au.expasy.org/enzyme/), and *BRENDA* (www.brenda.uni-koeln.de). The latter is an update of the printed *Enzyme Handbook* series (22) and contains a large amount of information on substrates, products, and inhibitors.

There are some inherent deficiencies in the system. Any EC number may cover more than one specific enzyme, considered as a chemical compound (and many do). Thus, enzymes are not uniquely identified in the system. Also, the classification says nothing about the chemical structure of the enzymes. Sometimes the annotations for a given EC number contain statements specifying some chemical aspect such as "a group of multi-copper enzymes" or "flavin-containing". However, in the early days, too little information was available in general to enable the use of chemical structure as the main basis for classification. This has changed dramatically now.

4.2. Linking Amino Acid Sequences to Enzyme Properties. Today, it has become almost a routine matter to determine the amino acid sequences of proteins, and in particular enzymes. The three-dimensional structure of an enzyme determines the biochemical properties and the structure is determined by the amino acid sequence, which itself is encoded by the gene sequence. In recent years, many enzymes have in fact been discovered as gene sequences instead of via their catalytic activities.

Since many enzymes having related activities share the same three-dimensional molecular fold, structure-based classifications have recently been used to group enzymes into distinct families. These structure-based families are usually updated with new protein sequences by sequence comparison and structure modeling programs. In some enzyme databases, new entries are finally checked manually before they are added to an enzyme family.

Several enzyme family classifications are hierarchical, eg,

Class
"glycosyl hydrolase"

Clan
"GH-B" = beta-jelly roll-fold

Family
"7", comprising endoglucanase (EC 3.2.1.4); cellobiohydrolase (EC 3.2.1.91).

Subfamily
none

In many cases, the enzyme families do not correspond to the EC number system. Often one family comprises several activities as in the example above. A challenge for the future is to divide enzyme families into subfamilies that correspond to one activity only. This would increase the predictive power for new discovered gene sequences.

Examples of well-established enzyme classification databases are

The Pfam Protein Families Database

A large collection of multiple sequence alignments covering many common protein domains and families (23): URL: http://www.sanger.ac.uk/Software/Pfam/

The InterPro Database
A database of protein families, domains, and functional sites in which identifiable features found in known proteins can be applied to unknown protein sequences (24):
URL: http://www.ebi.ac.uk/interpro/

The CAZy database
Describes families of structurally related catalytic and carbohydrate-binding modules (or functional domains) of enzymes that degrade, modify, or create glycosidic bonds (25):
URL: http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html

The MEROPS database
An information resource for peptidases and the proteins that inhibit them (26):
URL: http://merops.sanger.ac.uk/

5. Enzyme Discovery

The biochemical and biophysical properties of an enzyme such as activity, stability, and specificity are naturally important characteristics for its usefulness in an industrial application. The cost of the enzyme is, however, equally important due to the fact that, in most cases, the enzyme has to replace a cheap but less environmentally sustainable chemical-based reaction or process. Consequently, high level expression is an absolute requirement in order for the enzyme to provide a real alternative to the traditional process relying on chemicals.

Beginning with the demonstrated usefulness of the first industrial enzymes in use in the beginning of the last century, scientists and companies began to look for sources of new and better enzymes. Originally, attention was focused on animal and plant material, eg, the pancreas and malt. Although these served well as cheap raw materials, it became clear by 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. This was the first revolution in the field of industrial enzymes, and a dedicated microbial screening effort resulted in a number of new microbial enzymes reaching the market.

The second revolution in enzyme discovery came with the developments in genetic engineering/molecular biology in the 1980s. These new tools provide the ability to isolate a gene encoding an interesting protein from one organism and introduce it into a microorganism that has been adapted as a dedicated host cell. The new introduced DNA becomes a stable and integrated part of the genetic material of this cell and produces an enzyme product encoded by the introduced DNA. In this way, it is possible to combine a set of optimized production hosts with a wealth of natural diversity, not necessarily present in microorganisms, suitable for protein production under industrial conditions.

What can be considered the third revolution in enzyme discovery came as a spin-off from the second revolution. Developments in deoxyribonucleic acid (DNA) manipulation technologies also provide the opportunity to introduce desired mutations at any location in a gene. Thereby one has the possibility of inserting, deleting, or changing any amino acid in a protein and evaluating the consequences of the alteration, thus producing tailor-made improvements of the naturally occurring enzymes. With the continuous improvements in understanding of the relationship between protein structure and function, protein optimization is becoming an extremely valuable field for enzyme discovery.

Figure 3 compares the flow in a state-of the-art enzyme development to the traditional process used before the genetic engineering era (2). Note that not all steps are necessarily carried out for the development of a new industrial enzyme product. A recent review provides a more detailed description of the various elements of enzyme discovery (27).

5.1. Obtaining New Enzymes from Nature. Microorganisms can be found in all ecological niches on Earth and are thus able to exist under an incredibly broad span of growth conditions. With respect to temperature (from $<0^{\circ}C$ to above the boiling point of water), pH (from pH 1 to >10), and salinity, the enzymes from such organisms often show the ability to carry out their catalytic function under rather extreme conditions. Nature can be considered a treasure trove of enzymes able to function under many and extremely diverse conditions. The task for the industrial microbiologist is to find and isolate the enzyme capable of carrying out the desired reaction under the desired conditions.

Traditional enzyme screening is based on cultivation in the laboratory of a broad diversity of microorganisms isolated from a wealth of ecological niches. These organisms are then screened for the ability to perform the desired catalytic reaction. Based on an understanding of the intended industrial process, screening criteria are defined that include the substrate to be converted, pH, tempera-

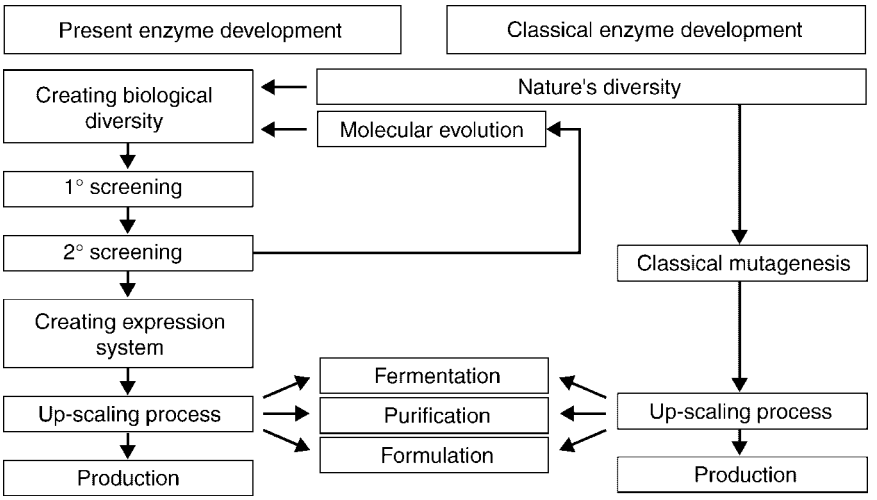

Fig. 3. The steps involved in classical versus state-of-the-art development of enzymes. Reproduced with permission from Ref. (01).

ture, and compatibility with other chemical compounds. Microbiologists have intensively collected microbial isolates and generated large and diverse culture collections. Often, knowledge about the physiology, taxonomy, ecological, or geographical origin of a microorganism will guide the microbiologist to its relevance for a particular screening.

The gene that encodes the interesting enzymatic property is subsequently cloned and expressed in a more suitable host in order to obtain higher yields of enzyme and enzyme of higher purity. Gene isolation can be carried out as "expression cloning" in which a library of fragments of genomic DNA or cDNA is generated in a host microorganism and screened for the encoded activity. The enzyme responsible for the conversion might be purified from a culture of the identified organism and be biochemically characterized. Information in the form of, eg, N-terminal, C-terminal, or internal amino acid sequences of the identified enzyme can assist the isolation of the corresponding gene, eg, by generating DNA primers for a PCR-based isolation procedure.

Bioinformatics has become a preferred tool for enzyme discovery due to the increasing information about sequences of interesting enzymes. Homologous genes encoding, eg, amylases, are aligned and used to identify conserved regions. DNA primers directed toward such regions are used in a PCR-based approach on genomic DNA preparation from a number of microorganisms. The results of such work are DNA fragments encoding parts of hitherto unknown amylases from these organisms. With the rapidly growing number of sequences being available, this approach becomes a convenient method for expansion of the known and characterized natural diversity.

The latest approach is the so-called "metagenomic" screening. It is recognized that only a limited fraction of naturally occurring microorganisms can be propagated under laboratory conditions and their diversity has therefore not

been available for studies. DNA is extracted from environmental samples, eg, soil and used for an "expression cloning" experiment. By this method, necessity of cultivating the donor organism is by-passed, which is an exciting but also a challenging approach to enzyme discovery (28).

5.2. Protein Engineering and Directed Evolution. Initial protein optimization was based on the understanding of the three-dimensional structure of the protein. The trained protein engineer should be able to use the combination of the protein structure, the biochemical and biophysical properties of the native protein, and the specific requirements for function in the desired application as a guide in choosing which residues should be altered in order to achieve the desired enzymatic properties. The enzyme containing the designed alterations is characterized and the result is compared to the expected and desired characteristics. This process is denoted "rational protein engineering", but is still a trial-and-error process where the chance of success depends on the intended improvements to the function of the molecule. Years of experiments with protein stability have resulted in a substantial knowledge-base often sufficient for successful optimization of properties related to stability at high temperature and extreme pH or stability toward harsh chemicals. Optimizing the enzyme toward optimal activity, eg, catalytic efficiency or a general performance in a complicated process, is more demanding and often the understanding of the protein or the industrial process limits the success.

Since rational protein engineering is subjected to limitations, it has been a desire to utilize the Darwinian principles of evolution in protein optimization, the so-called "Directed Evolution". This laboratory process consists of repeated rounds of three steps. Diversity is created by random mutagenesis either over the complete gene or in selected regions of the gene. The ensemble of mutated genes is transformed into a host microorganism, such that a library of cells is generated, each cell containing a mutated gene and expressing a different variant protein. The last step is to identify and isolate the fittest molecules in this population, which is carried out in a screening procedure able to distinguish the individuals based on the properties relevant for the desired reaction. The fittest molecule is typically isolated and used as the starting point for the next round of mutagenesis, library generation, and screening.

Pim Stemmer pioneered work in the early 1990s on DNA shuffling. The basic principle is that new, random hybrid molecules are generated from the genes encoding improved enzymes originating from the first round of screening (29). By this technology, mutations found in different gene are shuffled and combined in new patterns. This technology ensures that harmful mutations are deselected and beneficial mutations accumulate during the rounds of directed evolution.

5.3. The Host Microorganism. The vast majority of industrial enzymes are produced recombinantly in a rather limited range of microorganisms. Few products are still produced from mutated nonrecombinant microbial strains. Such products exist either for applications where customer or public concern about the use of GMM (genetically modified microorganism) technology results in a requirement for such products or in cases where the product constitutes a mixture of enzymes produced by one natural microorganism; eg, mixtures of cellulytic enzymes used for cellulose degradation. Typically, it is impossible to

obtain the same, high levels of protein expression and product purity from the traditional non-GMM strains resulting from chemical or physical mutation programs. The choice of host microorganism for production of industrial enzymes is often critical for the commercial success of the product.

The important parameters for a good production host for industrial enzyme production include an extended history of safe use of the microorganism and good growth characteristics in submerged fermentation on relatively simple and cheap substrates. In addition, efficient protein expression, secretion, low background of contaminating host cell proteins, and no or low production of unwanted metabolites are considered essential. Crucial to the entire process is the use of good molecular biology tools for DNA transformation to give genetically stable transformants or rational modification of the host strain as required.

The host microorganisms mostly used are either filamentous fungi or gram-positive bacteria. The filamentous fungi include *A. oryzae, Aspergillus niger, Fusarium* sp. and *Trichoderma* sp. The most frequently used bacterial hosts are found amongst *Bacillus* sp., *e.g. Bacillus licheniformis* and *Bacillus subtilis*. Other examples of commonly used microbial hosts are *Streptomyces* sp.; yeast species such as *Pichia* and *Hansenula* and even the gram-negative bacterium *Eschericia coli*. A future solution is production of recombinant proteins in plants although currently only insignificant amounts of industrial enzymes are produced from such hosts.

The host microorganisms might be mutationally or genetically manipulated in order to further improve their characteristics. It is desirable to remove side activities that might interfere with the function of the intended product. This could relate to enzymatic reactions that have a negative impact on the intended reaction, eg, by interacting with the substrate or the product thereby reducing yields. Another issue relates to the fact that some secreted enzymes are prone to degradation due to proteases from the host organism. To maintain product stability, it might be necessary to remove or reduce some of the proteolytic activity of the host.

Successful manipulation of a host microorganism for industrial enzymes production becomes more realistic with the availability of the sequenced genome of the organism. With the speed of genome sequencing these days, most of the popular production organisms will have their genome sequence available in the near future. For a number of organisms, the sequenced and annotated genomes are available: eg, *B. subtilis* (30) and *Aspergillus sp*. (http://www.cadre.man.ac.uk).

By coupling genomic information with techniques such as DNA array and proteomics studies, in principle, any property of the cell can be addressed. The primary targets likely will include enhanced protein secretion, improved protein expression and folding, and fermentation characteristics of the host.

6. Production of Industrial Enzymes

Today almost all industrial enzymes are produced by microorganisms by submerged fermentation. A tiny fraction is still made by surface culture fermentation.

Finally, a handful of enzymes are extracted from animal or plant sources, primarily trypsin, chymosin, papain [9001-73-2], and a few others (31).

Enzymes are usually sensitive to harsh physical and chemical conditions, and care must be taken during the whole production process to avoid inactivation of the enzyme. This demands careful selection of production processes and conditions for each individual enzyme. Different formulation methods are subsequently applied to assure the stability and activity of the enzymes during storage and application. Basically, the production process of industrial enzymes can be divided into three steps: fermentation, recovery, and formulation of the enzymes. Optimization of not only the individual unit operations, but also the interaction, is critical for the overall economy of the process (32).

6.1. Fermentation. The volume of industrial fermentors ranges from 20 to several hundred m^3, in a few cases, exceeding 1000 m^3. A conventional fermentor (33) is shown in Figure 4. Almost all fermentations are aerobic. Thus oxygen is required by the microorganism. In many cases, oxygen supply may become the limiting factor; therefore design of the fermentor to obtain a maximal transport of oxygen is necessary. This is achieved by a combination of agitation and injection of compressed air (34). Heat caused by microbial metabolism and agitation is removed through a cooling jacket or coil. Baffles are placed near the wall to increase the mixing efficiency and to prevent vortex formation. For

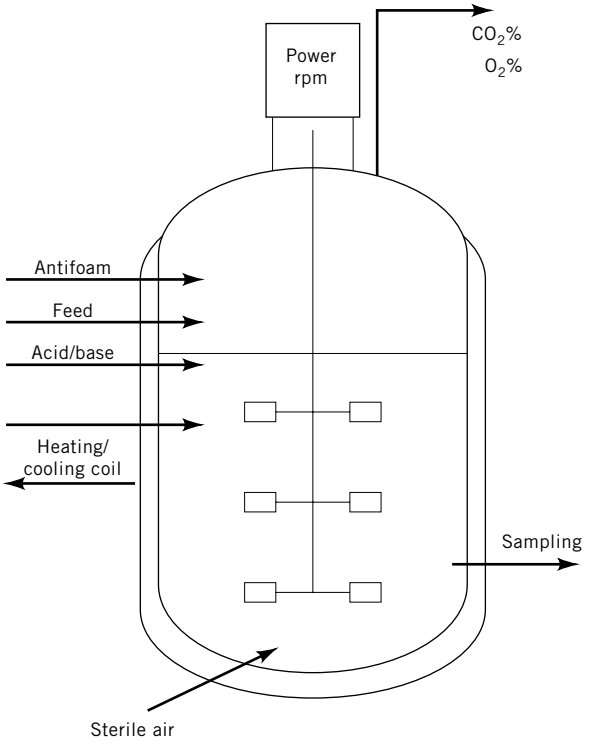

Fig. 4. A conventional fermentor. Foam, total pressure, PO_2, pH, temperature, and weight must be monitored and controlled.

microorganisms that are very sensitive to shear stress, air lift fermentors (35) 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 (36,37) and propagating microorganisms to obtain reproducible fermentations. The stock culture is stored frozen (typically at −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, plant protein, and sugars. 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 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 (38).

Temperature, pH, foam level, 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; however, simulation of quantitative models may provide more efficient optimization of fermentation.

The type of microorganism chosen will also influence the strategy and time for the fermentation. It has traditionally been either a bacteria or a fungus. In the first case, the typical species is *Bacillus* (39), whereas in the case of fungus, *Aspergillus* or *Trichoderma*, have been the preferred ones (40).

6.2. Recovery and Purification. The principal purpose of recovery is to remove non-proteinaceous material from the enzyme preparation. Most industrial enzymes are produced as extra cellular enzymes, ie, released into the medium by the microorganism with only a few other components. The first recovery step is often the removal of whole cells and other particulate matter (41) by centrifugation (42) or filtration (43). In the case of centrifugation, different versions of centrifuges or decanters are used. Filtration can either be done by pressure, vacuum, or cross-flow filtration. Flocculation of cell suspensions has been reported to facilitate the separation both by centrifugation and filtration (44). In the rare cases 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 (45). Enzymes can be extracted from disrupted microbial cells, and ground animal (trypsin) or plant (papain) material by dilute salt solutions or aqueous two-phase systems (46).

Ultrafiltration is increasingly used to remove water, salts, and other low molecular-weight impurities after the first recovery step (43,47). 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. Precipitation of the enzymes by use of salts, polymers, or organic solvents is another simple method for recovery of enzymes. 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 high 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.

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. A key purification method is crystallization, which is controlled by the combination of pH, salt, concentration, etc. The method has to be carefully developed in order to obtain both desired purity, yield, and acceptable processing times (48). Other techniques have been described to purify proteins (49), but they are often too costly for industrial enzymes and are only used in situations where the application demands a high purity. These include chromatography, aqueous two-phase extraction (50), and ion exchange.

6.3. Formulation. When an enzyme is recovered from the fermentation broth, it is usually present in an aqueous solution or processed to a dried state. Both types of preparation have to be formulated into a final product to comply with requirements appropriate to their final application. In general, formulation issues can be divided into liquid formulation or solid formulation, where immobilization can be considered a subgroup of the latter.

A very important requirement is related to the storage of enzymes from the time of manufacture to the time of application, ie, the catalytic activity of the enzyme must be maintained after prolonged storage at relevant temperatures and humidity. In the case of a liquid formulation, other stability issues like microbial stability and physical stability are important as well. As industrially recovered enzymes in aqueous solution are potentially excellent growth media for microorganisms, it is usually necessary to prevent microbial growth; and it is also usually required that the enzyme must remain in solution to avoid inhomogeneous dosage. As industrial enzymes are used in a range of very different industries, these applications may have special requirements. These can be of a technical nature such as having no precipitate and specifications regarding color and odor, or the product should not give rise to any unwanted effects in the application, eg, when mixed into a liquid detergent. Side activities are often also a problem to address, 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 can be dust level, particle size, and flow ability

in solid formulations. Finally, certain requirements derive from approval considerations, eg, only food-grade ingredients, absence of certain microorganisms, and kosher/halal restrictions on enzymes for food applications.

Any formulation is a compromise between the previously mentioned requirements and the necessary cost associated with formulation of the final industrial enzyme product. 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.

Enzyme Stability of Liquid Formulations. Loss of enzyme-catalytic activity may be caused by physical denaturation, eg, high temperature, drying/freezing or by chemical denaturation, eg, acidic or alkaline hydrolysis, proteolysis, oxidation, denaturants (surfactants or solvents). The 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 overview of additional examples is available (51).

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 (52). 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, borate and derivatives (53).

Microbial Stability of Liquid Formulations. Microbial growth is hindered by reducing water activity and adding preservatives. An overview is available (54). Reduction in water activity is typically obtained by including up to 50% of a polyalcohol such as sorbitol, glycerol, or propylene glycol. Furthermore, high concentrations of salt like NaCl or Na_2SO_4 or a combination of salts and polyalcohols have 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 ones

are benzoic acid and sorbic acid, but formic, acetic, and propionic acid also have this effect. 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 (55).

Physical Stability of Liquid Formulations. 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. It is in general more optimal to solve these issues early in the process, ie, in the fermentation or early recovery steps.

Other Liquid Formulations. It is sometimes possible to add properties in liquid formulations that provide additional functions. This includes microencapsulation (qv) of enzymes for protection against autoproteolysis, bleach, and other harmful components when dispersed in a liquid detergent (56); addition of certain polymers to protect the enzyme after it has been added to liquid detergents (57), or to boost activity in the final application. It has further been tested to use non-water liquid slurry formulation of enzymes to improve stability. Finally it is known to use addition of surfactants or wetting agents, eg, in α-amylases for textile desizing, in order to improve their effect; and addition of buffers to keep the application pH under control.

6.4. 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 multienzyme 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. 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, and enzyme inactivation. Enzyme stability factors, eg, temperature, pH, proteases, oxidation, and solvents/organics, are also 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 reuse of enzymes. Further, the advantage associated with a continuous process is achieved, eg, better process control, optimizing product yield, and quality. A significant advantage of immobilized enzymes is the total absence of catalytic activity in the product.

Immobilization Methods. Numerous enzyme immobilization techniques have been described in the literature; comprehensive books on this and related subjects, including industrial applications, are available (58–63).

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. 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. Cross-linking of enzyme crystals with glutaraldehyde has been described.

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 like silica, may be adequate for use in nonaqueous media. Entrapment in polysaccharide/polymer gels is used for both cells and isolated enzymes. A granulation process using silica to make cheaper macroporous immobilized product has been described.

Membrane reactors, where the enzyme is adsorbed or kept in solution on one side of an ultrafiltration membrane, provide 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 (64).

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. In general, for large scale industrial applications, the cost of immobilization method, pressure resistant and productivity are very critical parameters, demonstrating the preferred methods to be glutaraldehyde cross-linking or adsorption.

Industrial-Scale Applications of Immobilized Products. When immobilized glucose isomerase, based on cross-linking with glutaraldehyde of cells, was introduced in the early 1970's, it was believed that other industrial applications of immobilized enzymes would soon be found; however, this has turned out not to be true. One of the reasons is the high and expensive demands to the purity and solubility of the substrates. In the case of glucose isomerase, the cost of purifying the syrup before entering it into the enzyme reactor is substantially higher than the enzyme cost. Further, molecular biology techniques have improved dramatically in efficiency over the last decades resulting in significant reduction of enzyme production costs, omitting the need for immobilization. So instead of initiating an era of immobilized products, immobilized glucose isomerase today stands as a special case of efficient use of an intracellular enzyme. A few other immobilized enzymes have been commercialized, however, so far not to the same scale as glucose isomerase. They include aminoacylases for production of amino acids, penicillin acylase for production of 6-aminopenicillanic acid (65), and lipases for both interesterification of fats and oil as well as for synthesis of esters (60). They have demonstrated the feasibility of using immobilized enzyme technology in nonaqueous or low water systems.

6.5. Solid Formulations—Granulation. Although the trend is to market industrial enzymes as liquid products, solid enzymes still account for a significant part of the total volume of industrial enzymes. Especially in the segment of enzymes for solid detergents, animal feed, textile, and flour improvement. Granulation is the generic term for a particle size enlargement process. A granulate is preferred to powders, especially in order to secure a dust free product, ie, 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 and other places (66). Further technical advantages of granulates to powders is improved flow properties, a reduced risk of segregation, and improved product homogeneity. Like liquid formulation, a list of important quality parameters exists, which needs to be considered when making a granulate, including stability (both on storage and processing at customer), dust levels, release rate of enzyme, solubility, color, particle size and distribution, segregation, as well as restraints from registration or safety issues.

Several different methods have been used for the granulation of enzymes (67). In general, the development of granulation methods focuses on parameters such as the cost of the process and additives as well as the solubility of the granulate. The mechanism of granulation is very complex and difficult to model, making it very empirically based (68).

For many years, the focus on enzymes for powder detergent has been directed toward reducing airborne dust during handling, by making enzyme granulates increasingly resistant to physical breakage. The two dominating processes to obtain attrition-resistant enzyme granulates for the detergent industry are either high shear mixer granulation or fluidized-bed spray coating; in some special cases extrusion is used as well. High shear mixer granulation is typically a continuous process, making it a very efficient process for large volumes. The enzyme is mixed with inert material, eg, salt; binders, eg, dextrin; and fibers, eg, cellulose, added to reinforce the granulates. Fluidized-bed granulation is typically operated in batch mode. In this case the enzyme, optional together with a binder, is sprayed on to a core, eg, sugar/starch type of core in

a fluid bed. To reduce the enzyme dust level in the detergent factories to an absolute minimum, the majority of detergent enzyme granulates are further coated with a layer of inert material, typically an organic polymer and/or salt. This coating layer can also be used for coloring purposes. Titanium dioxide [13463-67-7] can be added to the coating medium to make the product whiter; alternatively, another attractive color can be added.

Another important segment for solid formulated enzymes is the feed industry. The same granulation methods are used as for the detergent enzymes, however, in this case stability of the enzyme during high temperature pellet formation of the final feed is a very critical parameter. A good pelleting stability can, eg, be achieved by applying to the granulate an extra coating of vegetable oil or other materials. In the case of enzymes for food application, some of the additives needed for the production of a rigid granulate may not be accepted. 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, integrated spray drying and fluidized-bed granulation, or a simple fluidized-bed granulation 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 parameters of the solid product. Of special importance are the physical strength and the ability of the products to generate airborne dust. In the elutriation test, a sample of product is fluidized in a glass tube with a perforated bottom plate. Dust from the sample is collected on a filter and the enzyme activity measured. In the so-called Heubach 3 method, the granulate is elutriated as well; however, 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 levels are in both cases very low, with enzyme dust levels on the pasts per million (ppm) level.

Besides high shear mixing granulation and fluid-bed granulation, a few other techniques are used for making solid enzyme formulations. These include prilling, where a suspension of the enzyme is spray cooled in waxy material, eg, an ethoxylated fatty alcohol with a melting point $>$or $\sim 50^{\circ}$C, to form small beads. Although the process is simple, it is not much in use, primarily because of the relative high cost of the wax, combined with difficulties to coat afterward and thus reduce the dust levels. Another method in use is extrusion. The enzyme is mixed with binders, diluting agents such as inert salts or starch, and the appropriate amount of water. The resulting thick paste is then extruded to small rods with a diameter of $\sim$0.6 mm. Preferably, the extrudate is then further spheronized to a product with a more rounded form. Like prilling it is a very simple process, however, for some industries it can be a challenge to achieve a sufficiently small particle size at an acceptable capacity.

7. Industrial Applications of Enzymes

7.1. Detergent Enzymes. The term *detergents*, from the Latin *detergere*, to clean, is understood here 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. Recent reviews are available that describe the composition and formulation of modern detergents (15,69–72) and highlight current developments and problems (73).

Penetration of enzymes into laundry detergent products is close to 95% in Europe and Japan and 75% in the United States (72). It is increasing rapidly in other regions, including Latin America and China. Thus enzymes have become an important ingredient, along with surfactants, builders, and bleaching systems (see Detergency). This has happened in concert with changes in washing habits and detergent formulations:

- Lower laundering temperatures, required by modern textiles made from synthetic fibers and for reasons of energy conservation, led to a demand in the 1970s and 1980s for new and more effective detergent ingredients.
- Modern-day compacted laundry detergent products (liquids, powders, tablets and various liquid- and powder-containing pouches) have compensated for reduced levels of other detergent ingredients by increasing enzyme dosages, resulting in concentrations in products that give up to several mg enzyme protein per liter of washing liquor.
- ADDs with high alkalinity and chlorine bleach have to a large extent been replaced by less aggressive and environmentally friendlier detergent products containing an hydrogen peroxide-based bleach system plus an enzyme system.

In addition, the detergent enzyme producers are able to offer products with a more favorable price/performance ratio than previously. This is to a large extent due to the dominating use today of genetically engineered production strains that give high yields and thus economical and also environmentally sound production processes.

Functions of Enzymes in Detergents. An important task for any detergent is to *remove visible soilings*. In household laundering and dishwashing, important classes of soilings to be removed by 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 and other carbohydrates; fats/oils*, eg, animal fats, vegetable fats, sebum, waxes, and mineral oil; *particulate matter* such as insoluble metal carbonates, oxides, silicates (clay), carbon black, dust, and humus; and *bleachable stains*, eg, fruit and vegetable juices, wine, tea, and grass. Materials or compounds from several of these classes may be intimately mixed in *combined soilings*, eg, human body soil on shirt collars, cocoa milk, tea with milk or cream, gravy, or chocolate.

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 many cases, a suitable detergent enzyme may help, as will be described below for the individual enzyme classes. Contrary to the purely physical action of the surfactant system, enzymes work by degrading the dirt into

smaller and more soluble fragments. However, total removal of a stain requires the joint effects of the enzyme, surfactant system, and mechanical agitation.

Removal of the above soilings when they are attached directly to the textile or kitchenware surfaces may be described as a *primary or direct cleaning effect* of the (enzymatic) detergent. However, various kinds of dirt may also adhere to textile surfaces via residues (usually invisible) of proteinaceous, carbohydrate, or fatty material. In such cases of "anchored dirt" in the laundry, an enzyme may assist in removing the dirt even though it does not attack it directly.

A still more sophisticated effect of the degradation of such invisible films of sticky materials is the prevention of *redeposition* of dirt and colorants. Some items in a wash load may release particulate soils or colorants (whether originating in food stains or excess dye in dyed fabrics) to the wash liquor and these may then bind to the film-covered areas. Such redeposition may range from the dramatic, visible after one "disaster" wash, to incremental effects that only gradually make themselves noticeable as slight graying or yellowing of garments or a dull look of plates, glasses, etc, after repeated dishwashing. Inclusion of enzymes that keep away the sticky residues prevents this kind of dingy build-up. This may be termed an *antiredeposition effect*. Other ingredients in the detergent matrix may have antiredeposition effects as well, in particular bleaching systems that bleach dyes in solution and polymers that bind to the redepositing soil materials and keep them in solution.

All-round reviews of enzymatic detergency and specific detergent enzymes are available (15,74–77). Enzymes have traditionally been regarded as a minor additive to or "auxiliary ingredient" in laundry detergents (71). It has recently been proposed, however, that enzymes may replace a larger part of conventional detergent compositions (78).

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, water and of course the soiled laundry.
- The procedure followed.

Equipment and procedures in three principal geographical areas are summarized in Table 3 (71,79).

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

- Low pH, low ionic strength: liquid detergents giving wash liquors of pH from 7.5 to 9. They contain no bleach and only low levels of salts.
- High pH, high ionic strength: detergents with bleach have a wash liquor pH from 9.5 to 10.5 and contain an activated bleaching system, eg, European regular powder detergents. They also contain sodium sulfate as a filler, and builder systems based on sodium triphosphate, zeolite,

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

Conditions	United States/Canada[b]	Japan	Western Europe
machine type	agitator[c]	impeller[c]	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) 10–27 (cold)	10–30	(90) 60 40 30
typical water hardness[e]	relatively low	very low	relatively high
$CaCO_3$, ppm	100–150	<100	250
recommended detergent dosage, g/L	1–3	1–2	5–10

[a]Information mainly extracted from Refs. 71 and 79.
[b]Chlorine bleach often added separately to wash.
[c]Top-loading.
[d]Heating coil used. Front-loading.
[e]Large variations throughout the region.

and sodium carbonate. High dosages are used, which give wash liquor with a high ionic strength.

- High pH, medium ionic: European and Japan compact powder detergents and probably North American powders in general. In the compact powders, most or all of the sodium sulfate has been removed; pH in the wash liquor is from 9.5 to 10.5.

This kind of classification becomes important when considering enzymes that depend heavily on pH and ionic strength for their performance (see, eg, section 6.1.4.1).

Performance Evaluation of Laundry Detergent Enzymes. 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 (20–60°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, broad substrate specificity is demanded.

A given enzyme may be assayed analytically by its action on soluble or insoluble substrates under chemical and physical conditions different from those encountered in a real-life wash. Such experiments may give information on the enzyme's relative activity as a function of pH and temperature or its compatibility with other soluble substances, etc. The analytical data thus obtained, however, do not necessarily correlate with the wash performance of the enzyme, which must be evaluated in more realistic wash trials (80).

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 presoiled test pieces may also contain particulate matter, eg, carbon black, as part

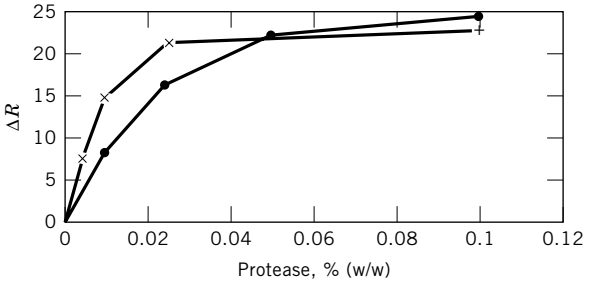

Fig. 5. Protease performance of a commercial protease (Savinase, Novozymes A/S) in a powder detergent dosed at 4 g/L in a Launder-ometer trial using a particular test soiling (-●-) 40°C for 47 min; (-x-) 60°C for 40 min.

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.

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. 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 standardized 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.

If one relates the observed ΔR values to the full difference in R between the soiled test fabric and a corresponding clean fabric, one obtains a measure of the *wash effect* or *degree of stain removal*, often expressed as a percentage.

As an example, 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 ~0.05% (w/w) of the enzyme granulate in the detergent is reached.

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 in particular varies between liquid and powder detergents and with the composition of the soiling (Fig. 6).

It was mentioned above (in section 6.1.1) that enzymes are effective for preventing redeposition of various types of soiling. Assays have been proposed recently that may be used to assess an enzyme's effects to this end (81,82). The main idea is to deposit the enzyme substrate in question (protein, gelatinized starch, fat, etc) on fabric and then subject it to the wash with/without the enzyme and then to a second wash where the depositing soil is introduced either via test fabrics (such as commercial EMPA 101 swatches that will release carbon black)

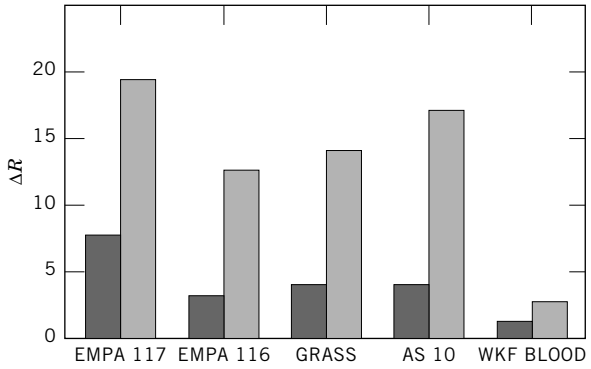

Fig. 6. Washing performance on different soilings of a U.S. liquid detergent (■) and a U.S. powder detergent (□) in a Terg-o-tometer operating at 20°C for 10 min; one enzyme (protease) dosage. EMPA 117 (milk, blood, and ink on polyester/cotton); EMPA 116 (milk, blood, and ink on 100% cotton); grass on (100% cotton); AS 10 (milk, oil, and pigments on 100% cotton); blood soiling (on 100% cotton).

or directly added to the wash liquor. It is also possible to add both the gluey or fatty material and the dye or particulate colorant simultaneously and directly to the wash liquor.

Enzyme Types. Proteases. A protease is an enzyme that hydrolyzes proteins into smaller fragments, ie, peptides or even amino acids. A detergent protease must degrade the insoluble protein in stains into fragments that can be removed or dissolved by the detergent. All currently used detergent proteases belong to the class of serine proteases (named for the serine residue invariably present in the active site) and are based on enzymes naturally produced by *Bacillus* strains. The selection of serine proteases available today is the result of intensive protein engineering programs (77,83–85). Some are highly alkaline, ie, have a maximum activity in the high pH range (~9.5–11), others are low alkaline (work best at pH <9.5). They all have a molecular weight between 20,000 and 30,000.

Protease performance is thus strongly influenced by detergent pH and also by ionic strength, and the two parameters are not independent. This is illustrated by the example in Figure 7. Most ingredients in a detergent formulation contribute to the ionic strength of the wash solution, but the ionic strength may be adjusted with few other consequences by using sodium sulfate, a traditional filler and formulation aid in powdered detergents.

In general, surfactants influence both enzyme performance and stability in the wash solution, and this is particularly true for proteases. On one hand, surfactants are often needed for a protease to exert its cleaning effect to a measurable degree. On the other hand, surfactants may be aggressive toward the enzyme molecules. For example, detergent proteases are typically destabilized by linear alkylbenzenesulfonate (LAS), a very common type of anionic surfactant in detergents. The presence of nonionic surfactants, however, counteracts the negative effect of LAS. Adsorption of the enzyme to substrates and fabric surfaces most probably also stabilizes the enzyme. All in all, the stability of proteases in a washing context is not problematic in practice.

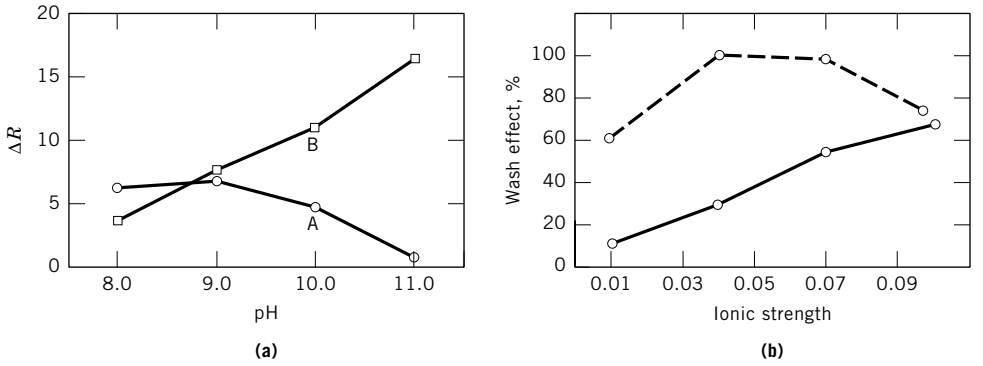

Fig. 7. Protease washing performance in a U.S. liquid detergent. Grass soiling in a 10 min wash at 30°C with one enzyme dosage. (**a**) pH profile of commercial proteases A and B. (**b**) Effect of increasing ionic strength, adjusted with Na_2SO_4, of commercial protease B at (−○−) pH 8 and (--○--) pH 11.

Builders such as sodium carbonate, sodium triphosphate, zeolite, and citrate, as well as cobuilders such as nitrilotriacetic acid and polycarboxylates, all have as their main function the removal of free calcium ions from the washing solution in one way or other. They are added to detergent formulations because wash performance in general decreases with increasing concentration of free calcium. High calcium concentrations also tend to reduce protease performance, although this effect is variable. Conversely, proteases need a small amount of calcium for the sake of stability, but even with the most efficient builder systems, stability during the wash is not seen as 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 today (86,87) consist of sodium perborate [7632-04-4] or sodium percarbonate plus an activator such as tetraacetylethylenediamine [10543-57-4] (TAED). The persalt 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. In new protein-engineered proteases introduced onto the market, a bleach-sensitive methionine residue close to the active site has been replaced by other amino acids not sensitive toward oxidation. This slight change in the molecular structure significantly increases the storage stability in detergents containing bleach (88), as exemplified in Figure 8.

Chlorine bleach [7681-52-9] (sodium hypochlorite, NaOCl), is not incorporated into laundry detergents but is used separately in some parts of the world as an additive. Added in this way, it quickly oxidizes most enzymes, and certainly proteases, resulting in complete loss of activity. In fact, such inactivation may happen already at the much lower levels of hypochlorite encountered in areas with tap water chlorination. Addition of low levels of a hydrogen peroxide source to the detergent may counteract this by reducing hypochlorite to chloride.

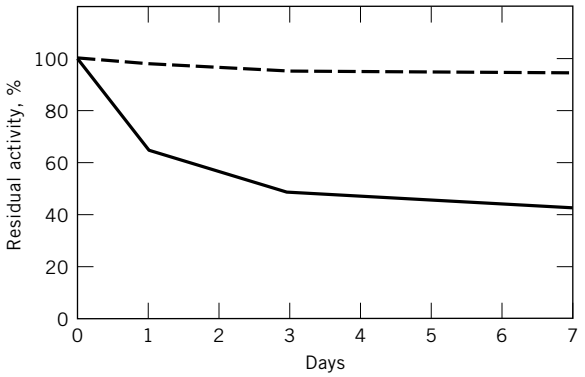

Fig. 8. Storage stability of proteases in European powder detergent with activated bleach system. (—) Traditional protease; (- - -) protein-engineered protease.

Amylases. Commercial laundry amylases such as the amylase from *B. amyloliquefaciens* and the heat-stable amylase from *B. licheniformis* are α-amylases, ie, enzymes characterized by attacking the amylose part of starch in an endo fashion, randomly cleaving internal glycosidic α-1,4-bonds to yield shorter, water-soluble dextrins. Both have molecular weights of ~ 55,000. By now, α-amylases have become a common ingredient in both powder and liquid formulations in many countries. They boost overall detergent performance at lower wash temperatures and with milder detergent chemical systems. They improve the removal of stains containing starch, such as a wide range of food stains [curry sauces, gravy, gelatinized rice and pasta starch, etc, see (89)], and contribute to general whiteness maintenance by preventing redeposition (81). 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). It is important to note that starch find its way onto garments in other ways than by soiling with foodstuff. First, many textiles have been treated with starch as a size at the textile plant, and this may not have been completely removed in the desizing step (although this process may be greatly aided by amylases, cf. section 6.5.1). Second, in many places starch is deliberately applied to shirts, lab coats, etc, so as to get a stiffening of the fabric.

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. In fact, on combination soilings like the cocoa test materials mentioned above, synergy may be observed between amylase and protease action (90). Also, during storage in both powder and liquid detergents, the amylases are very stable in the presence of proteases.

The main problems with amylases are dependency on calcium ions (for structural stability—calcium can be regarded as a cofactor, cf. section 2.1) and sensitivity toward bleaching agents; the latter has shown to be a problem during storage in powder detergents containing a bleaching system. A development

parallel to the one discussed above leading to a bleach-stable protease has led to the protein engineering of a bleach-stable variant of the traditional *B. licheniformis* amylase by replacement of a critical methionine residue (77,91). Other perspectives for amylase development are outlined by Aehle (92).

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. As was the case with the other enzyme classes, an opportunity to test the idea seriously came only much later when enzymes active at high pH were identified. The first detergent on the market to contain a lipase was a compact powdered detergent launched by Lion in Japan in 1988. The enzyme was Lipolase, introduced by Novo, and incidentally was the first industrial enzyme to be produced using a genetically modified host organism. The enzyme, mol wt 32,000, was originally isolated from the fungus *Humicola lanuginosa* at low levels of enzyme expression; the gene was then inserted in the host microorganism *A. oryzae* (93), leading to much higher yields and thus to a cost-effective and more environmentally friendly (less resource-demanding) process.

Since then, the development of lipases for detergents has continued (14,74,77,94,95). Lipolase and other early lipases had some deficiencies (96) that for a while were invariably associated with lipases in general, but recent developments have shown that they can be overcome. This has been accomplished on the basis of a detailed understanding of some of the mechanistic aspects of lipase detergency that will now be summarized.

Animal and vegetable fats consist mainly of triglycerides or tri-*O*-acylglycerols (TAGs), which are natural lipase substrates. The enzymes catalyze hydrolysis of the ester bonds in TAGs to give a mix of free fatty acids, diacylglycerols, monoacylglycerols, 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. Thus, for optimum removal, a pH value >8 is required (97). Conversely, the liberation of acid makes it possible to monitor the lipolytic process at constant pH by using the technique of pH-stat titrations, cf. section 2.5 (16).

Other factors that influence the hydrolytic process strongly are calcium ion presence (due to calcium-fatty acid interaction; this may lead to calcium soap formation, which however, also represents a removal of reaction products from the degradation zone) and the nature of the surfactant mix used in the detergent. Surfactant intervention is multifacetted: assistance in modifying the surface of the fatty substrate prior to hydrolysis; competition for vacant sites on the substrate surface; possibly inhibition of the enzyme; assistance in removal of products of the reaction; diversion of lipase molecules from substrate surface to micelle surfaces. The lipase itself needs to be well suited for approaching the hydrophobic substrate that often is a solid at the wash temperature and needs to have good activity when located at the interface. Many lipases in their resting conformation in an aqueous environment have a lid or flap that covers the active site and that needs to be opened by appropriate interaction of the enzyme molecule with the surface. Much of the directed evolution work has been devoted to modification of the hydrophobic contact zone of the lipase molecules (94).

Lipolase in particular and other early detergent lipases had low activity in the water-rich environment of the wash, but attained maximum activity during the drying-phase when water activity dropped (97). The benefit from hydrolysis of fatty stains appeared only after the next wash when the degradation products could be washed away. The lipase concept thus became associated with the need for multiple wash cycles (96). This picture has changed with the advent of true first-wash lipases (18,98).

In conclusion, effective decomposition of fatty soils is now possible in a single wash cycle and at low temperature thanks to the application of lipases. Detailed electron microscopy and radiotracer studies have shown that lipases may even contribute to the removal of fatty residues in the interior parts of cotton fibers where the surfactants that remove the fat from fiber surfaces apparently do not work effectively. Also, antiredeposition effects were described above; such effects can be very pronounced with lipases and hydrophobic colorants that get absorbed into fatty residues.

Cellulases. The term *cellulases* covers a broad class of enzymes produced by microorganisms (both fungi and bacteria) with the purpose of degrading cellulose to low-molecular saccharides.

Cellulose is a β-glucan, ie, a polymer consisting of glucose units linked together via glycosidic β-1,4-bonds. Enzymes hydrolyzing glycosides in general are termed glycoside hydrolases (GHs), and those specifically hydrolysing β-glucans are β-glucanases. Typically, cellulases are secreted by the organisms in question as complexes comprising several specialist GHs that each contribute in their way to the overall degradation process by either attacking internal positions of the polymer chain (endoglucanase activity) or cutting off terminal oligosachharides (eg, cellobiohydrolases that produce cellobiose, the β-1,4-linked glucose dimer). One example that has been intensively studied is the cellulase complex from *Humicola insolens* (99). The complex as such was marketed in the 1980s as a cellulase preparation for detergent applications, and several individual components of the complex have later been isolated and marketed for particular purposes.

A special feature of many—but not all—cellulases is their cellulose-binding module (CBM), a part of the enzyme molecule that has the function of binding to the initially insoluble substrate. The rest of the molecule, including the active site region, is thus tethered to the substrate surface via a flexible linker region. Information on families of GHs with cellulose-related activities and on CBMs can be found, eg, in the CAZy database (cf. section 3.2).

Cellulases may be assayed in the laboratory and classified according to their effects across a series of cellulosic substrates. A favorite water-soluble substrate is CMC (carboxymethylcellulose). Insoluble ones include cross-linked and dyed, and acid-swollen preparations of real cellulose.

Taken as a class, cellulases provide a range of benefits when washing cotton and cotton blends, especially over several wash cycles (100):

- Color care (retainment of bright colors, rejuvenation of colors that have become dull through wear).
- Fabric care (providing softness).

- Cleaning (removal of soilings, particulate matter as well as other materials).
- Antiredeposition (preventing the redeposition of soils from the wash liquor).

The mechanism behind the color care effect is believed to be the following: The surface of cotton-based textile fabrics during wear tends to develop a layer of protruding microfibrils with lengths in the μm range. The microfibrils easily get damaged and entangled and eventually will create "fuzz" and "pills" that scatter incident light, resulting in a grayish or dull appearance. If the material features a number of colors, the contrast between adjacent areas of different colors will be reduced. The microfibrils that are sufficiently well exposed and filled with defects to be accessible to the cellulase are shaved off, restoring the undisturbed view of the colored textile. The same mechanism can explain the softening effect. The entangled fuzz layer is hard and tends to attract particulate matter such as clay, dust, soot, zeolite particles from the detergent matrix and depositing calcium salts (soaps, carbonates, etc), leading eventually to a harsh feel.

In principle, the mechanism behind the effects of cellulase on removal of certain stains as well as fatty materials (such as sebum or olive oil) (101,102) could be the same. For particulate matter, one may easily imagine how removing the fuzz layer that had captured the particles will also remove the stains themselves. Also, the surface will be less likely to capture new particles, so the cellulase treatment also has an effect toward preventing deposition of new soil while the garment is worn and preventing redeposition of soil released into the wash liquor from the wash load as such (*antiredeposition effect*; cf. (82,100)).

Interestingly, however, different cellulases provide the above benefits to quite different degrees. This has to some extent been rationalized by assuming different reactivities of the various enzymes to various more or less crystalline regions of the cellulose substrate (100).

Softening effects, similar to those obtained with cellulases, can also be achieved by using clay-like compounds in the detergent or cationic surfactants in fabric softening additives used in the rinse. These materials are believed 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 (103). 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 has been concern that cellulase action on cellulosic textiles 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 much higher concentrations. Appropriate enzyme levels must be determined for any specific formulated product and any given cellulase under the intended conditions of use.

Perspectives for future cellulase developments are described by Maurer (104).

Mannanases. A great number of household products, foodstuffs (eg, ice cream and barbeque sauces) and cosmetics contain natural gums, ie, complex carbohydrates that play a role as formulation aids to provide the right texture or consistency of products. Guar gum, consisting of galactomannan, a polysaccharide, is one such example. Mannanases, enzymes that degrade mannans, are further examples of GHs, in addition to amylases and cellulases. The inclusion of a mannanase in a detergent may provide both stain-removal and antiredeposition effects just as with proteases, amylases, and lipases (75,105). Announcements have been made of the commercial use of such enzymes (106).

Oxidoreductases (redox enzymes). Bleaching systems in modern detergents are "oxygen-based", ie, they consist of or produce hydrogen peroxide and peroxycarboxylic acids that then bleach stains and certain colorants in solution, in some cases assisted by a metal complex as a catalyst (86,87,107). There are enzymes that produce hydrogen peroxide by reduction of dioxygen (eg, glucose oxidase), and there are enzymes that use hydrogen peroxide to oxidize a number of organic substrates (peroxidases) or use dioxygen directly to oxidize substrates (laccases). It is therefore a natural thought that one could construct a detergent bleaching system using some of these types of enzymes.

The mere generation of hydrogen peroxide using an oxidase and a suitable substrate has never materialized in detergents, although many patents describe such systems. The main reason is probably that it is difficult to compete against the traditional hydrogen peroxide sources (sodium perborates and sodium percarbonate), both cost-wise and in terms of space taken up in the detergent formulation. Also, the substrate itself and its oxidation product would have to be in all ways compatible with the wash process. An alcohol oxidase using methanol as substrate and producing formaldehyde would hardly be acceptable, even though the substrate would be very weight-effective.

Peroxidase systems should be useful for bleaching fugitive dyes in solution. Some textile dyes are substrates themselves for peroxidases, and by introducing a mediator, an auxiliary compound that becomes oxidized by a peroxidase (or laccase) and then itself oxidizes dye molecules, a broad range of dyes can be bleached in this way. A *dye-transfer inhibition system* based on a fungal peroxidase and a phenothiazine derivative as a mediator has been described (74,108). No such system has been implemented in commercial detergents, however.

Stain bleaching is a complicated task for any bleaching system because there needs to be a good effect on the unwanted stains combined with a low risk of bleaching textile dyes on garments. Peroxycarboxylic acids satisfy these criteria and are tough to compete with, and although many patent applications have proposed the use of peroxidase and laccase systems for this purpose, no such system has been commercialized. A laccase system that bleaches dyes located in the textile fibers has been put to good use in the denim industry, cf. section 6.5.3.

Enzymatic systems for producing the technically attractive peroxycarboxylic acids have been proposed, but there are good arguments why hydrolytic systems have not succeeded in producing these acids (90,109).

Automatic Dishwashing. There are many differences between laundering and automatic dishwashing (ADW). 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. The ADW detergent compositions are quite different with only very little surfactant, present primarily to prevent excessive foaming. Recent developments in ADW have been reviewed in the literature (eg, 110,111).

During the 1970s, manufacturers tried to add enzymes to automatic dishwashing detergents (ADDs), which required that they left out the chlorine bleach used at the time, ie, hypochlorite precursors, because the available proteases and amylases were inactivated very fast by hypochlorite. Since there was no adequate substitute for chlorine bleach, tea and coffee stains were left behind. Then, in the early 1990s the availability of active-oxygen bleaches, compatible with enzymes, and the increasing concern about safety and environmental issues, led to extensive changes in ADD formulations, especially in Europe.

Earlier formulations in addition to chlorine bleach contained mainly metasilicates, triphosphate, and nonionic surfactants. The new and more complicated formulations featured disilicates, phosphates or citrate, phosphonates, polycarboxylates, nonionic surfactants, a hydrogen peroxide source, bleach activator, and enzymes. The replacement of metasilicates by disilicates lowers pH in the wash liquor from ~12 to 10.5. The combined effect of decreased pH, the absence of hypochlorite, and the trend toward lower wash temperatures paved the way for the introduction of enzymes into ADDs. Today, most ADD brands in Europe are part of the new generation of products with enzymes. It is an important fact for the application of enzymes in ADDs that a very large part of the market is tablet products, many of them with two or three layers. This means that enzyme granulates have to be suited for the mechanical processing involved, but also that advantage may be taken of the possibility of separating enzymes and peroxide source in separate tablet layers, ensuring better storage stability, and enabling different dissolution rates for different parts of the product.

The ADD enzymes in current use are heat-stable proteases and α-amylases. The actions of the enzymes in ADDs are similar to those in laundering. The performance of these enzymes can be evaluated according to standard procedures described for the testing of dishwashers and involving a range of standard soils.

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, ~70°C (112), which are efficient at removing starch film even under the harsh conditions existing in a dishwasher (112).

There are also commercial proteases with a high temperature optimum (~60°C) that can remove most protein soils in a dishwasher if the latter are not totally denatured (112). Some protein residues present particular challenges, most notably eggs that contain, particularly in the egg-white, inhibitors that reduce the performance of many serine proteases (cf. section 6.1.4.1).

Some soils in the above-mentioned standard procedures, such as food residues cross-linked by heat, are difficult to remove (113) and are not obvious substrates at all for enzymes.

Industrial and institutional cleaning. The application of enzymes has grown substantially within the I&I sector (15). The primary field here is the use of detergent enzymes for laundry purposes, but a broad range of other applications has been investigated, eg, I&I dishwashing, membrane cleaning, 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, proteases, lipases, and various types of glycoside hydrolases 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.

An interesting situation occurs with burnt milk residues on heat exchangers and ultrafiltration modules in the dairy industry. Here, by applying a combination of a protease and a lipase, the milk-based substrate itself upon hydrolysis provides both emulsifiers and foaming agents for the cleaning process in the form of peptides and fatty acids.

As a whole, the I&I cleaning procedures, 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 cooperation between the customer (end user), manufacturer of the detergent, and enzyme producer.

7.2. Enzymes for Grain and Biomass Processing. Enzymes are used by grain-based industries in the manufacturing of a variety of products. The primary substrates are starch-containing grains, such as corn, wheat, and tapioca that are converted into value-added food and industrial ingredients. In the brewing industry, enzymes are added to supplement the natural enzymes in malt to provide a number of benefits. Furthermore, enzymes are used to generate fermentable sugars in the production of ethanol from various starch containing crops. Currently, intense efforts are devoted to the development of enzyme-based processes for the production of ethanol based on lignocellulosic materials to provide a bio-fuel that is competitive with fossil fuels.

Enzymatic Sweetener Production. In the 1960s, almost all starch-based glucose production changed from processes based on acid-catalyzed hydrolysis to processes based on enzymatic hydrolysis as a number of benefits could be obtained; most importantly greater yields, a higher degree of purity, and easier crystallization. Later, immobilized glucose isomerase made the industrial production of high fructose syrup feasible. As a consequence, the starch industry has become one of the most important users of industrial enzymes.

By controlling the enzymatic reactions, valuable syrups and modified starches with different compositions and physical properties can be obtained. These tailor-made glucose syrups are used in a wide variety of foodstuffs such as soft drinks, confectionery, meat products, baked products, ice cream, sauces,

baby food, and canned fruit. Depending on the degree of degradation or modification desired, the processes liquefaction, saccharification, and isomerization may be applied. Syrups and breakdown products of starches are usually characterized by the terms, DE = Dextrose Equivalent and DX = the true dextrose (= D-glucose) content. The number of glucose molecules may also be characterized by the reducing value.

Starch Liquefaction. As native starch is only slowly degradable by enzymes a gelatinization, typically of a 30–40% dry matter suspension, is needed to make the starch susceptible for breakdown. The gelatinization temperature depends of the type of starch (114). 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 enzyme hydrolyses the α-1,4-glycosidic bonds in pregelatinized starch, whereby the viscosity of the gel rapidly decreases and maltodextrins are produced. The process may be terminated at this point, the solution purified and dried, and the maltodextrins utilized as bland tasting functional ingredients in products like dry soup mixes, infant foods, sauces, and gravy mixes.

The equipment used for liquefaction may be stirred tank reactors, continuous stirred tank reactors (CSTR), or a jet cooker. However, most starch processing plants liquefy the starch with a single enzyme dose in a process using a jet cooker (Fig. 9) (115). Cooking extruders have also been studied for the liquefaction of starch, but due to the high temperature in the extruder inactivation of the enzyme activities demands 5–10 times higher dosages than in a jet cooker (116).

When cornstarch is used, the sweetener industry wants to perform the entire process from wet milling through liquefaction to saccharification to glucose at a pH 4.5. Several process benefits such as less formation of unwanted side products, less color formation, and fewer unit operations can be obtained. The characteristics for optimally performing liquefying α-amylases in these processes are calcium independent stability, a high heat stability, a high specificity, and a high activity at pH 4.5.

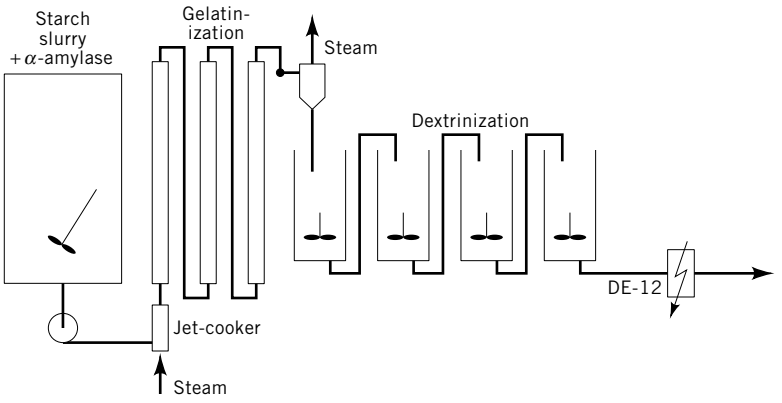

Fig. 9. Starch liquefaction process, 35% dry substance at pH 6.3, 40 ppm Ca^{2+}. A single enzyme dose of 0.5 kg α-amylase/120 L starch slurry, at 105°C for 5 min (gelatinization), followed by 95°C for 2 h (dextrinization) (115).

For wheat and potato starch, liquefaction is in general carried out at pH 5.0–6.5. Higher pH values should be avoided due to increased formation of by-products such as precursors for maltulose, a disacchanide made up of glucose and fructose. Maltulose formation is also enhanced at high temperatures and long residence times, and is more pronounced at increasing DE's.

Industrially important α-amylases are typically derived from *B. licheniformis, Bacillus stearothermophilus, B. subtilis* or *A. oryzae*. Table 4 lists selected characteristics of microbial enzymes used for starch processing and their application conditions.

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-branching 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.

Maltogenic amylases hydrolyze α-1,4-glycosidic linkages from the nonreducing end of the maltodextrin chain. Maltodextrins, oligosaccharides and maltotriose are hydrolyzed mainly to maltose.

Isoamylase [9067-73-6] (glycogen-6-glucanohydrolase) and pullulanase [9012-47-9] (pullulan-6-glucanohydrolase) hydrolyze the α-1,6-glucosidic bonds of starch. When amylopectin is treated with a pullulanase, linear amylose fragments are obtained. When a heat- and acid-stable pullulanase is used in combination with saccharifying enzymes, the starch conversion reactions become more efficient (117).

Saccharification of Liquefied Starch. Liquefied starch can be further hydrolyzed using amyloglucosidase or fungal α-amylase, thereby leading to the production of sweeteners with DE values in the range of 40–45 (maltose), 50–55 (high maltose), or 55–70 (high conversion syrup) (118). By applying a series of enzymes including β-amylase, glucoamylase, and pullulanase as debranching enzymes, it is possible to produce intermediate-level conversion syrups with maltose contents close to 80% (117). A syrup with a glucose content of 95–97% can be produced from most starch raw materials using the optimum combination of enzymes.

Table 4. **Starch-Degrading Enzymes of Industrial Importance**

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

[a]Minimum dosage.
[b]Having pH optimum at an intermediate temperature.
[c]Having pH optimum at a high temperature.

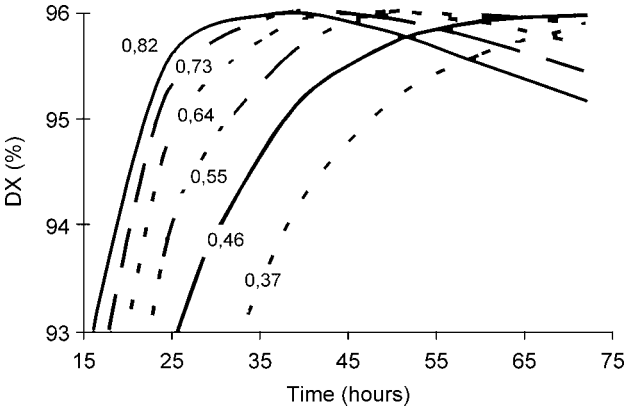

Fig. 10. DX as a function of time and dosage a commercial amyloglucosidase (AMG E, Novozymes A/S). 30% dry solids, pH 4.3, 60°C. Enzyme dosage in L/t solids.

Amyloglucosidase hydrolyzes the α-1,4-linkages rapidly, but during the saccharification process the α-1,6-linkages of the highly branched amylopectin are hydrolyzed much more slowly. Normally, the dosage of amyloglucosidase is adjusted to obtain the desired final degree of saccharification, DX, within 48–70 h as shown in Figure 10.

At the start of the saccharification, the rate of dextrose formation is high, but it gradually decreases toward the end of the process, partly due to the accumulation of branched dextrins, and partly because of the increasing concentration of dextrose accelerates reversion (repolymerization of dextrose into isomaltose and other saccharides). At a certain point, the rate of reversion outbalances that of dextrose formation, and if the saccharification is not terminated at this point (maximum DX), the dextrose level will gradually decrease toward chemical equilibrium (~85% dextrose at 30% DS, 60°C). By using a pullulanase together with the amyloglucosidase at the start of the saccharification, the α-1,6-linkages of the branched dextrins are rapidly hydrolyzed. In consequence, fewer branched oligosaccharides accumulate toward the end of the saccharification.

The processes applied to purify saccharified starch depend on the raw material used and may also vary from plant to plant. The saccharified liquid is typically filtered to remove insoluble impurities such as denatured protein and fat. Subsequent steps may involve centrifugation, ion exchange, isomerization, treatment with activated carbon, and evaporation to form a storage-stable product.

Continuous Saccharification. It is possible, in theory, to obtain the same final DX obtained in a batch process by a continuous saccharification under almost the same reaction conditions. It would, however, be necessary to use either a plug-flow (tube) reactor or an infinite series of infinitely small CSTR to obtain such a product. In practice, a limited number of saccharification tanks are used resulting in a somewhat lower final DX (119). A series with a minimum of 8 tanks is recommended.

Production of Maltose Syrups. Maltose syrups are produced by saccharification of liquefied starch with maltogenic exo-enzymes. Enzymes for this purpose are fungal α-amylase, maltogenase, pullulanase, or barley β-amylase.

The saccharification is typically conducted at a temperature of 55–65°C, pH 4.8–5.5, and at a solids level of DS 30–40%. The reaction time may be in the range of 20–40 h. The sugar spectrum obtained depends on the enzyme dosages and on the reaction time used.

Enzymes as Processing Aids in the Purification of Saccharified Wheat Starch. Wheat starch has a minor content of impurities in the starch milk that adheres to the starch granules. Thus, wheat starch is known to form precipitates or haze, which is difficult to filter. Arabinoxylan, pentosans, and lysophospholipids are claimed to be responsible for this problem (120). To facilitate the refining of the saccharified starch, enzymes such as cellulases, pentosanases, glucanases, proteases, and pectinases may be used.

Glucose Isomerization. Enzymatic isomerization of glucose to fructose provides a cost-effective alternative to white sugar (sucrose) from sugar cane or sugar beets. The commercial product obtained is termed high-fructose corn syrup (HFCS). Two grades of the syrup have established themselves in the world market, HFCS-42 and HFCS-55, containing 42% or 55% fructose based on dry substance, respectively. These products account for over one-third of the caloric sweetener market in United States. Annually, >8 million tons of HFCS are produced using glucose isomerase thereby representing the largest industrial application of an immobilized enzyme.

Glucose can reversibly be isomerized to fructose. The equilibrium conversion for glucose to fructose is 50% under industrial conditions and the reaction is slightly endothermic (121). The reaction is usually carried out at 60°C and at pH 7–8. To avoid excessive reaction time, the conversion is normally limited to ~45%.

The isomerization reaction can only be economic by using immobilized enzyme. The reaction parameters in the system have to be controlled carefully to obtain a reasonable yield of fructose. Thus, pH must be ~7.5 or higher in order to secure high activity and stability of the enzyme. Under these conditions glucose and fructose are rather unstable and decompose easily to organic acid and colored by-products. To overcome these problems, the reaction time is kept as short as possible. This is done by operating the immobilized enzyme in a fixed-bed reactor process in a column through which glucose flows continuously. To ensure optimal flow conditions, the enzyme granules must be rigid enough to prevent them from being compressed during the operation.

The feed syrup DX should be as high as possible in order to obtain the maximum isomerization rate. With low feed DX, the isomerization reaction must come closer to chemical equilibrium in order to attain a given fructose concentration, eg, the standard 42%. The rate of isomerization decreases with increasing fructose concentration as chemical equilibrium is approached. Consequently, any fructose content in the feed syrup (eg, from fructose enrichment recycle streams) should be limited to a minimum.

The immobilized glucose isomerases in industrial use are granulated to a particle size between 0.3 and 1.0 mm. The Mg^{2+} ion acts as an activator and stabilizer of the enzyme, and is therefore added to the feed syrups in the form of $MgSO_4 \cdot 7\ H_2O$. The amount necessary depends on the presence of calcium ions that act as an inhibitor in the system by displacing the magnesium ion activator from the isomerase enzyme. Therefore, the calcium ion content should be kept as

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
$MgSO_4 \cdot 7H_2O$ (activator); g/L	0.15–0.75
conductivity, μS/cm	≤ 100
uv absorbance, 280 nm	≤ 0.5

low as possible. At a Ca^{2+} ion content in the feed syrup of 1 ppm or lower, the addition of 45 ppm Mg^{2+} will be sufficient.

The main criteria for selecting the feed syrup specifications are optimization of enzyme productivity and limitation of by-product formation. Typical feed syrup specifications are shown in Table 5.

Enzymes in the Beer Brewing Industry. Traditionally, beer is produced by mixing crushed barley malt and hot water in a large circular vessel called a mash copper. Besides malt, other starchy cereals such as maize, sorghum, rice isolated starch, and barley are added to the mash. These are known as adjuncts. After mashing, the mash is filtered in a lauter tun. The resulting liquid known as sweet wort is then run off to the copper where it is boiled with hops. The hopped wort is cooled and transferred to the fermentation vessels where yeast is added. After the fermentation, the so-called "green beer" is matured before the final filtration and bottling.

If too little enzyme activity is present in the mash, it can have a number of consequences: The extract yield is too low, the wort separation takes too long, and the fermentation process is too slow. Also, too little alcohol is produced, the filtration rate is reduced and the flavor and stability of the beer is affected. Addition of enzymes can be used to supplement the malt's own enzymes in order to prevent these problems. Furthermore, industrial enzymes can be used to ensure better adjunct liquefaction, to produce low-carbohydrate beer (light beer), to shorten the beer maturation time, and to produce beer from cheaper raw materials. The most important enzymes used in the beer industry are listed in Table 6 (122,123).

Brewing with Adjuncts. As microbial amylases in general are much more stable than the native malt amylases, a simpler liquefaction, a shorter process time, and an overall increase in productivity can be achieved. It is also a safeguard of the brewhouse operation that malt enzymes are preserved for the saccharification process. This results in better wort and, ultimately, a better beer. Eliminating the malt from the adjunct cooker means less adjunct mash and thus more freedom in balancing volumes and temperatures in the mashing program—a problem for many brewers using a high adjunct ratio.

Traditionally, the use of barley has been limited to 10–20% of the grist when using high quality malts. At higher levels or with low quality malts, processing becomes more difficult. In these cases, the mash needs to be supplemented with

Table 6. **Steps in the Brewing Process Where Microbial Enzymes May Be Used**[a]

Operation	Enzymes	Enzyme action	Function
decoction vessel	α-amylases	degrade starch	adjunct liquefaction
(cereal cooker)	β-glucanases	degrade glucans	reduce viscosity and aid filtration
Mashing	α-amylases	degrade starch	malt improvement
	proteases	increase soluble protein	malt improvement
	β-glucanases	degrade glucans	improve wort separation
	pentosanase	degrade pentosans of especially wheat	improve extraction
fermentation	debranching enzyme	degrade α-1,6 branch points of starch	secures maximum fermentability of the wort
	fungal α-amylase and amyloglucosidase	increases maltose and glucose content	increase % fermentable sugar in light beer
	β-glucanases	hydrolyze glucans	reduce viscosity and aid filtration
	α-acetolactate decarboxylase	convert α-acetolactate	avoid diacetyl formation (decrease fermentation time)
conditioning tank	protease	modifies protein–polyphenolic compounds	reduce chill haze

[a]Refs. (119,124).

additional enzyme activity if the brewer is to benefit from the advantages of using unmalted barley while still maintaining brewing performance. Brewers can either add a malt-equivalent blend of α-amylase, β-glucanase, and protease at the mashing-in stage or add the enzymes separately as required.

General Filtration Problems. Wort separation and beer filtration are two common bottlenecks in brewing. Poor lautering not only reduces production capacity, but can also lead to lower extract yields. Furthermore, slow lautering negatively affects the quality of the wort, which may lead to problems when filtering the beer and ultimately affecting the flavor and stability of the beer.

A thorough breakdown of β-glucans and pentosans during mashing is essential for fast wort separation. Nondegraded β-glucans and pentosans carried over into the fermentor reduce the beer filtration capacity and increase the consumption of filter aid. A wide range of β-glucanase/pentosanase preparations for use in mashing or fermentation/maturation is available to solve these problems.

Enzymes to Improve Fermentation. Small adjustments in fermentability can be achieved by adding a fungal α-amylase at the start of the fermentation or by adding a debranching enzyme together with a glucoamylase at mashing-in. Beer types with very high attenuation (light beer) can be produced using the addition of saccharifying enzymes. Fungal α-amylases are used to produce mainly maltose and dextrins, whereas glucoamylase produces glucose from both linear and branched dextrins.

Table 7. **Effect of Enzyme Addition on the Yield of Fermentable Sugar**[a]

Operation	Enzymes used	% Yield of fermentable sugar based on raw material	% Yields of fermentable sugar based on wort carbohydrates
mashing	malt only	65	
	malt + α-amylases	75	
Fermentation	none		6
	debranching enzyme		75
	fungal α-amylase		85
	amyloglucosidase + debranching enzyme		95

[a]Ref. (124).

The alcohol content is another parameter that brewers are interested in controlling. The amount of alcohol in a beer is limited by the amount of solids (extract) transferred from the raw materials to the wort, and by the level of fermentable sugar in the extract. In turn, the sugar content is controlled by the amount of starch degradation catalyzed by the amylases in the mash and by the saccharifying enzymes used during fermentation. The sugar content is controlled by the extent of starch degradation due to the effect of the amylases in the mash and the saccharifying enzymes used under the fermentation. The approximate percentage yields of fermentable sugars are shown in Table 7 (123).

Yeast is a living organism and it needs proteins in order to grow and multiply. If the yeast is not supplied with enough free amino nitrogen, the fermentation will be poor, and the beer quality will be inferior. A neutral bacterial protease added at mashing-in can be used to raise the level of free amino nitrogen, which is beneficial when working with poorly modified malt or with high adjunct ratios.

Diacetyl Control. When exactly is a beer mature? An important question for brewers because this determines when they can "rack" the beer to make way for the next batch. The simple answer to the above question is: When the diacetyl level drops below a certain limit (~0.07 ppm). Diacetyl(butane-2,3-dione) gives the beer an off-flavor like buttermilk, and one of the main reasons for maturing a beer is to allow the diacetyl to drop to a level where it cannot be tasted. Diacetyl is formed by the nonenzymatic oxidative decarboxylation of α-acetolactate (2-hydroxy-3-oxo-2-methylbutanoate), which is produced by the yeast during primary fermentation. The yeast removes the diacetyl again during the beer maturation stage by conversion to acetoin (3-hydroxybutan-2-one), which has a much higher flavor threshold value. In fact, acetoin is almost tasteless compared with diacetyl.

By adding the enzyme α-acetolactate decarboxylase [9025-02-9] at the beginning of the primary fermentation process, it is possible to bypass the diacetyl step (Fig. 11) and convert α-acetolactate directly into acetoin. Most of the α-acetolactate is degraded before it has a chance to oxidize and less diacetyl is, therefore, formed. This makes it possible to significantly shorten or completely eliminate the maturation period (124,125). Thus, the brewery enjoys higher fermentation and maturation capacity without investing in new equipment.

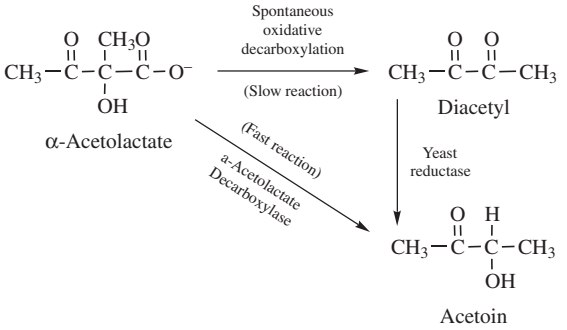

Fig. 11. Effect of dry substance (DS) on maximal obtainable % glucose; (—) AMG and pullulanase; (- - -) AMG.

Chill Proofing Enzymes. In spite of the final cold filtration of the beer before packaging, cloudiness or haze may form during chilling. The amount of haze varies with the type of beer and increases with age, exposure to oxygen, and agitation during shipping. The haze is a result of the formation of complexes of peptides with polyphenolic procyanidins (126). It was proposed in the patent literature (127) as early as 1911 to use proteases to prevent the formation of chill haze in beer. The original patents described the application of bromelin, papain, and pepsin for chill proofing. In fact, papain is superior to other enzymes used for chill proofing (123).

Enzymes for Ethanol Production. In the alcohol industry, enzymes are also well established in the production of fermentable sugars from starch. The production of ethanol from crops rich in starch has been practiced for centuries. The use of industrial enzymes for alcohol production from starch was reviewed ~1969 (128), and a presentation of various enzyme based cooking procedures was published (129-131) covering potable alcohol as well as fuel alcohol.

The main process stages in alcohol production from starch-containing crops comprise: (*1*) dry milling; (*2*) gelatinization; (*3*) dextrinization; (*4*) saccharification; (*5*) fermentation; (*6*) distillation; and (*7*) drying of the stillage. Very often a simultaneous saccharification and fermentation (SSF) is applied instead of a complete saccharification before fermentation with ordinary yeast. The starch may be liquefied and pre-saccharified using first α-amylase and then glucoamylase. The resulting fermentable sugar is cooled and transferred to the fermentor where yeast is added. The fermentation processes may be performed continuously using holding times during the fermentation step of ~24–30 h. For batch-wise SSF processes, the fermentation time may be 50–75 h.

In the so-called Dry Milling Process, a set of hammer mills grind the corn to a fine powder. By using the "Warm or hot slurry preliquefaction processes", high solids (~35% grain w/w) are processed (129). A split enzyme dose has been found to provide optimal process performance and economy. About one-third of the α-amylase is dosed at 65–85°C prior to jet-cooking and the remainder during secondary liquefaction at 80–85°C.

The saccharification may be performed before fermentation or simultaneously with the fermentation process. Amyloglucosidases with high activity

and high thermostability allows saccharification systems to be operated up to 70°C. This results in a greater flexibility in operating conditions and can be an advantage for an SSF process to follow. Acid-stable α-amylase is known to improve breakdown of partially converted starch and large oligosaccharides during fermentation, ensuring maximum yield during saccharification or SSF. They can also improve filtration and centrifugation rates by eliminating partially converted starch. Glucoamylase products containing such activities are, therefore, highly demanded.

Fermentation. The bottleneck in an alcohol plant is often the fermentation tanks. Cereals tend to be low in soluble nitrogen compounds. This results in poor yeast growth and increased fermentation time, which may be overcome by adding a small amount of protein-degrading enzyme to the mash (128). Improving yeast nutrition by addition of enzymes has proved to secure a higher intake of grain per hour in the plant without extra investments in tanks, distillation towers, etc. It is estimated that capacity increase based on corn up to 20–30% may be implemented without extra investments. A way to do this may be by reduction of yeast flocculation effects, by increase of the content of free amino acids, and yeast nutritious compounds like minerals and vitamins (132).

Ethanol for Fuel. Over the last decade, there has been an increasing interest in fuel alcohol as a result of increased environmental concern, higher crude oil prices and, more acutely, by the ban in certain regions of the gasoline additive MTBE (*tert*-butyl methyl ether), that can be interchanged directly with ethanol. Therefore, intense efforts are currently undertaken to develop improved enzymes that can enable utilization of cheaper and currently not fully utilized substrates such as lignocellulose to make bio-ethanol more competitive with fossil fuels. The cost of enzymes required to turn lignocellulose into a suitable fermentation feedstock is a major issue, and current developments focus both on the development of enzymes with increased activity and stability as well as on their efficient production. Huge governmental programs have been launched in the United States by the Department of Energy to support these developments spurred by the general emphasis on reducing pollution and the working toward fulfilling the Kyoto protocol.

Ethanol Production Using Lignocellulose-Based Raw Materials. Degradation of biomass using cellulases has been a major research area for >30 years; however, it has not yet been economically feasible, partly due to the complex substrates, cost of enzymes, and overall lack of efficiency. The current process used in most economical analyses can be briefly described as using cocurrent dilute acid prehydrolysis of the lignocellulosic biomass with simultaneous enzymatic saccharification of the remaining cellulose and fermentation of the resulting glucose to ethanol. In addition to these unit operations, the process involves feedstock handling and storage, product purification, wastewater treatment, enzyme production, lignin combustion, product storage, and other utilities (133). The most promising techniques to release the cellulose fibers have been adaptation of the pulping techniques used for preparation of cellulose fibers for paper production. Among those, the continuous steam explosion pulping at high pressures and low residence times has been shown to produce pulps with sufficient release of the cellulose for further processing to ethanol. The National Renewable Energy Laboratory (NREL) in the United States has undertaken

a complete review and update of the process design and economic model for the biomass-to-ethanol process based on cocurrent dilute acid prehydrolysis, along with simultaneous saccharification (enzymatic) and cofermentation (134). To improve process economy, the major focus areas at present are to reduce the cost of industrial cellulases significantly and to improve the activity and thermostability of the enzymes (135).

7.3. 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 byproducts are formed. Furthermore, enzymatic reactions are easily controlled and can be stopped when the desired degree of conversion is reached.

Dairy. 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 globules. 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 that 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, Chr. Hansen and DSM 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 (136,137) and only slight modifications of the traditional cheese-making 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 cheese-makers develop their particular type of cheese under local conditions. Products made by recombinant DNA techniques inducing microorganisms to produce chymosin have successfully been introduced and now cover more than two-thirds of the U.S. market for chymosin in cheese making. 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 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 milk-based drinking products, ice cream, yogurt, and frozen desserts use lactase to improve digestibility, sweetness, scoop, and texture of the products.

Another important application of enzyme technology used in the dairy industry is the modification of proteins with proteases to reduce the allergenicity of cow milk for infant formula products. Subtilisin-type proteases are used to obtain a high degree of hydrolysis and thus low or hypoallergenic formulas,

while extracted trypsin/chymotrypsin is used to generate other types of hydrolysates (138).

A smaller enzyme application within the dairy industry is the hydrolysis of milk with lipases for the development of flavors in specialty cheeses.

Baking. Wheat flour contains enzymes, the most important being amylases. However, the quantities of these enzymes are not always ideal for baking purposes, and supplementary enzymes are often added. Traditional applications of enzymes are for improvement of the dough properties during processing (especially industrial processing of bread), loaf volume, crumb structure, and shelf-life (139). The enzyme products used are either microgranulates that are easy to handle and freely mixed with flour or liquid formulated enzymes.

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. Microbially produced pentosanases, ie, xylanases, which partly degrade the pentosan (hemicellulose) fraction of flour, result in easier dough-handling and improved crumb structure. Both the α-amylase and xylanases have become standard ingredients in most bread baking formulas/bread improvers.

Another important application of the enzyme within baking is the use of a bacterial maltogenic amylase preparation (Novamyl, Novozymes A/S) for antistaling of bread, which increases the shelf life from days to weeks by maintaining the softness and elasticity of the bread. The particular enzyme has unique properties that prevent overdosing effects observed for other bacterial amylases used for providing softness, meaning that there is no risk of obtaining sticky dough and a gummy crumb structure.Recently, the availability of novel lipases has allowed for the application of such to provide emulsification properties by *in situ* degradation of the wheat lipids to "emulsifier-like" structures making it possible to remove or reduce the level of chemically based emulsifiers like DATEM and SSL otherwise used in the baking industry.

A less important application is the use of neutral bacterial endoprotease used to weaken the gluten in wheat flour, if necessary, or to provide the plastic properties required in a dough used for biscuits.

Protein Modification. Treatment of vegetable or animal protein with proteases is a way to obtain controlled hydrolysis and thus change the flavor, functional, and nutritional properties of food proteins. Different protein raw materials are used with different purposes in mind. Extraction processes with enhanced yields include soy milk, scrap meat recovery, bone cleaning, gelatin, fish/meat stick-water, and rendering of fat. Processes for producing protein hydrolysates used as ingredients in processed foods include soluble soy protein, soluble wheat gluten, foaming wheat gluten, blood cell hydrolyzate, whey protein hydrolyzates, casein hydrolyzates, soluble meat and fish proteins, and gelatin hydrolyzates. The characteristics of some commercial enzyme products used for the industrial conversion of food protein products are shown in Table 8.

Different protease specificity (preference for amino acids at point of hydrolysis) and degree of hydrolysis (%DH) determines the properties of the protein

Table 8. **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
Flavourzyme	*Aspergillus oryzae*	–	5–7	20–60
Neutrase	*Bacillus amyloliquefacus*	0.5	6–8	10–65
PTN (Trypsin)	pancreatic	3.3	7–9	10–55

[a]Liquid form unless noted.
[b]Novozymes 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).

hydrolysates, eg, the solubility, emulsification properties, and flavor. 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 importance that the degree of hydrolysis is controlled and followed during the processing. 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 (140). 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, trichloroacetic acid, (TCA-index) (Fig. 12). Unpleasant bitterness is often a problem for some protein hydrolysates. This problem can to some extent be addressed by proper selection of the reaction parameters and the enzymes used.

Proteolytic modification of milk protein is an important application in infant formula products to prevent or reduce the risk of developing nontolerance/allergenicity to bovine milk in infants (141).

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 13. A degree of hydrolysis of $\sim$10% results in solubilization of $>$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 (142). Examples of hydrolysis curves are shown in Figure 13. 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

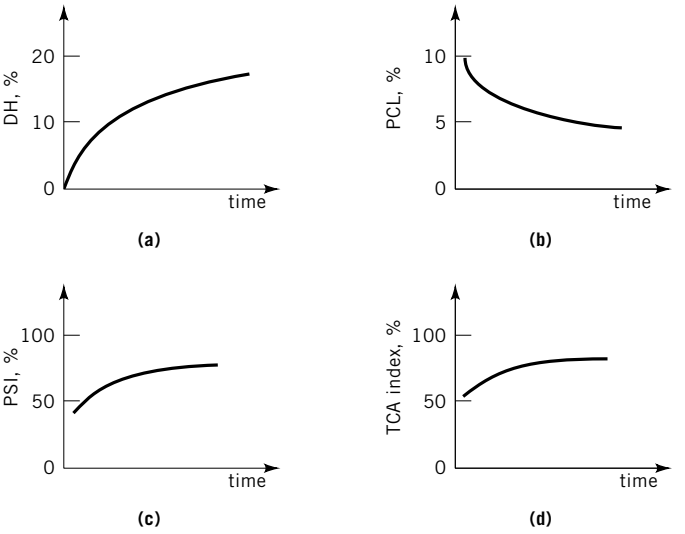

Fig. 12. Activity (syrup flow rate) versus operating time for typical immobilized isomerases, Sweetzyme T and Q.

wheat gluten are obtained at a degree of hydrolysis of 2–3%. The active whipping protein is recovered by centrifugation and drying.

Alternative modifications of protein include the use of transglutaminase to increase the cross-linking of protein to change the functionality and provide texture to reconstituted meat and dairy products and limited proteolysis to tenderize whole muscle meat.

Extraction Processes. 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 or fruits. An important development

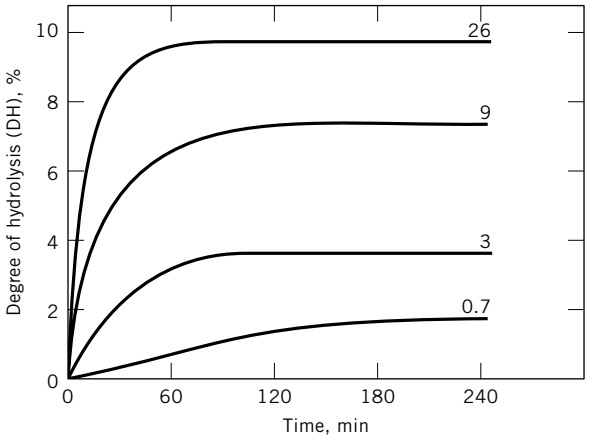

Fig. 13. Removal of α-acetolactate during fermentation.

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. Viscozyme (Novozymes A/S) from a selected strain of *A. niger* (143) contains >10 different enzyme activities that offer the possibility of utilization in various commercial 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. 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). Arabinan 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 arabinanase side activity. The use of cell-wall degrading enzymes is also found in winemaking.

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 (144).

Proteases are widely applied in extraction processed of heparin and chondroitin sulfates (cf. the section Extraction of Complex Polysaccharides) and for making yeast extracts.

7.4. Animal Feed Application. Enzymes are today widely applied in the feed industry for enhancing digestibility and thus nutritional value of feed, especially in monogastrics like pigs and chickens, which unlike ruminants, are unable to utilize fully components in plant-based feed stock. Enzymes like β-glucanases and xylanases or mixtures of various carbohydrases are well known in the industry for increasing the digestibility of feed with a high content of cellulose and hemicellulose. Benefits recorded in feeding trials include increased daily weight gain, increased feed conversion ratio, reduced mortality, and a reduced amount of sticky droppings in chicken farming (145). In short, the enzymes provide an increased output at a given feed cost.

During the last 10 years, a significant enzyme application within the feed industry has developed to allow monogastrics to utilize the phosphorus bound in phytic acid in cereal-based feed. Phytase is applied to liberate inorganic phosphorus from phytic acid. Phytic acid contains ~85–90% of the total phosphorus in the plant and is largely inaccessible to monogastrics. Two particular factors have contributed to this development; the ban of bone meal as a source for

cheap inorganic phosphorus due to the bovine spongiform encephalopathy (BSE) and the increasing focus on the phosphorus and nitrogen outlet from intensive animal production in many countries. The addition of phytase results in a significant reduction in the phosphorus outlet from monogastrics. Furthermore, the use of recombinant DNA technology allows for highly cost-efficient production of the phytase, which to some degree closes the gap between utilizing phytase and adding inorganic phosphorus (1).

Enzymes can be added to the feed together either with the premix, or the granulated enzyme products may be mixed with feed components and subjected to pelleting. Alternatively, liquid enzyme can be applied directly on the final feed.

A smaller enzyme application within the feed industry includes hydrolysates of vegetable protein sources for calf milk replacement. 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 enzymatic modification, it is possible to make wheat gluten, 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.

7.5. Enzymes for the Textile Industry. The textile industry was one of the first industries to use enzymes, cf. section 1.1.1. Thus, crude amylases were introduced as early as at the turn of the twentieth century to "desize" (remove) starch from woven fabric, thereby overcoming the fiber-damaging effects of conventional acid-based processes. Since then, enzymes have been introduced in a number of steps in the manufacturing of textiles to provide a variety of benefits (77). The mild enzymatic treatments have lead to improved process quality, efficiency, and effectiveness and reduced environmental impact. In the manufacturing of cotton-based textiles, enzymes have now, as indicated in Figure 14, been introduced in almost all the wet processing steps (1).

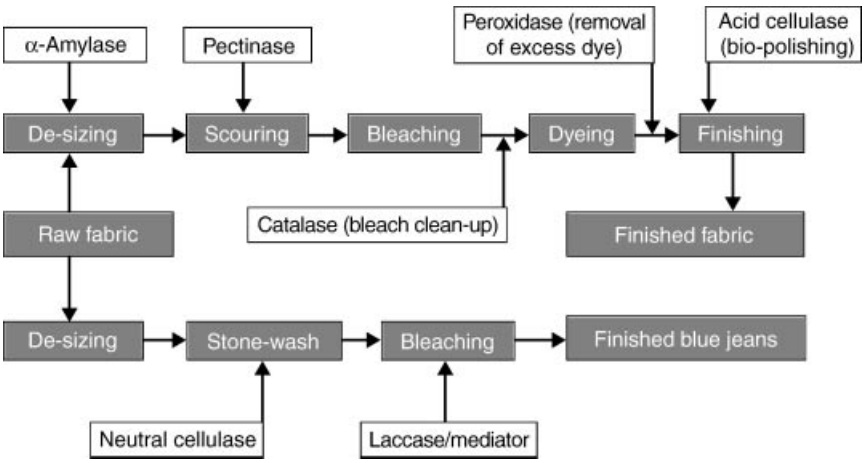

Fig. 14. Enzymes used in various unit operations in textile wet processing and the manufacturing of Denim. Reproduced with permission from (1).

Desizing. 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. Enzymatic desizing is one of the oldest nonfood applications of commercial amylases.

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 below.

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 s.

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 (cf. Table 4) 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 was originally carried out by lightweight pumice stones that were 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.

Microbial cellulases have developed within the textile industry as a tool for fabric finishing, in particular for denim garment finishing (77). Cellulases can generate the stone-washed look with far less damage to the fabric compared to the pumice stone based process. Today, cellulases for denim blue jeans processing represent the largest market for textile enzymes.

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 (146) is as follows: Load garments into an 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 obtained. 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 bio-stoning, a process with great future potential (146).

Oxidoreductases in the Textile Industry. Hydrolytic enzymes have a very dominant position among the enzymes used on an industrial scale. These enzymes (amylases, cellulases, lipases, proteases, etc) catalyze reactions in which a chemical bond is broken by addition of a water molecule. Oxidoreductases (cf. section 6.1.4.6) have a completely different mode of action as they catalyze the transfer of electrons from one substrate to another. This may lead to degradation of some substrates and polymerization of others. Within the textile industry several large-scale industrial applications have, rather uniquely, been established for oxidoreductases.

During cotton bleaching, fabric whiteness is increased and "motes" (dark seed coat fragments) are decolorized or degraded. Immediately after bleaching using hydrogen peroxide, the first oxidoreductase for textile applications, catalase, can be used to efficiently decompose residual hydrogen peroxide to (*1*) stop the bleaching reaction before fiber damage can occur, (*2*) prevent hydrogen peroxide interference with subsequent dyeing, and (*3*) save water and time by reducing the number of rinses needed after bleaching, before dyeing (77,147).

The latest "revolution" in denim finishing has been the introduction of laccase for indigo decolorization. Compared to other enzymes, laccase has relatively low substrate specificity, acting on a range of substituted aromatic compounds. Laccase-catalyzed oxidation of these compounds using atmospheric dioxygen as the primary oxidant produces transient intermediates that can degrade upon further oxidation, condense to form oligomers or polymers, or can transfer the oxidation effect to other molecules. This transfer of oxidizing power via a so-called "mediator" compound makes it possible for laccase to catalyze oxidation reactions outside its direct substrate specificity or steric accessibility. A commercial product based on a laccase-mediator system selectively decolorizes indigo to give fabric-safe enhanced abrasion (77,148).

Peroxidases, like laccases, catalyze electron-transfer reactions. Instead of dioxygen, peroxidases use hydrogen peroxide as the primary oxidant. As with laccase, peroxidase catalytic activity can be enhanced or adjusted to target specific substrates by using a mediator compound. This feature has been used in

developing a peroxidase system that can selectively remove unfixed dyestuff and reduce the number of rinse cycles needed after reactive dyeing (149).

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 (150).

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.

7.6. 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.

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 related to these resinous substances can be avoided by using lipases (151). Triglycerides are important constituents of wood 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 using 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 $\sim$1 h 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 ground-wood pulp for the production of newsprint (152).

Bleach Boosting. Bleaching reduces the highly colored residual lignin component and improves the brightness and brightness stability of chemical pulp. Traditionally, chemical pulp bleaching is accomplished by several bleaching stages including chlorine, chlorine dioxide, alkaline extraction, and hypochlorite. Because of the environmental issues associated with the chlorinated organic substances generated during chlorine bleaching, the paper industry is already moving toward elemental chlorine-free bleaching (ECF) and totally chlorine free bleaching (TCF) technologies, which include oxygen, ozone hydro-

gen peroxide, and peracetic acid. Xylanase aided prebleaching gained popularity in the late 1980s (153). It was found that xylanase treatment of pulp could improve the accessibility of bleach chemicals and the release of chromophores by the degradation of reprecipitated xylan on the fiber surfaces. For mills that are still using chlorine bleaching, xylanase treatment provides a simple way to decrease the use of active chlorine, therefore reducing the negative environmental impact. For mills that are using ECF and TCF bleaching sequences, xylanase treatment can either help the mill reduce the use of bleach chemicals or increase the brightness ceilings. It is generally accepted that xylanase treatment can lead to ~10–15% savings for softwoods and 20–25% savings for hardwoods (154). Xylanase treatment can also eliminate the need for extra capital investment, which is of great interest for the mills that have limited chlorine dioxide generation capacity (155).

Anionic Trash Reduction. Pectin is a minor component of wood. It is distributed in the middle lamella between fiber walls and in cell junctions. It is also present in ray cell walls and pit membranes. In general, pectin content of wood is ~1–2%. Pectin is α-(1-4) linked polygalacturonan with both galacturonic acid and its methyl ester moieties. Both hardwood and softwood pectin are highly methylated (156). During the alkaline bleaching process, pectin is demethylated and dissolved in the liquor becoming a significant part of the dissolved and colloidal substance (DCS). Since polygalacturonic acid has a very high negative charge density, it will interfere with cationic retention/drainage aids, lower wet strength, increase deposit formation, and reduce sheet brightness (157). Such negatively charged polymers are often referred to as "anionic trash", which will increase the "cationic demand" of the pulp stock. Recently, it is reported that pectinase treatment of a thermomechanical pulp (TMP) could lower the cationic demand by >40% (158). It seems that the ability of polygalacturonic acids to complex cationic polymers depends strongly on their degree of polymerization (DP). When the pectin polymers are degraded to small oligomers ($DP < 6$), they lose the ability to bind to cationic polymers used in the wet end of the papermaking processes. A recent mill trial in Canada using a commercial pectinase demonstrated ~28–60% reduction in the cationic demand of peroxide bleached TMP pulps (159). The reduction in cationic demand provides significant savings in wet-end chemicals.

7.7. Other Industrial Enzyme Applications. The applications discussed in the previous sections account for the bulk of industrial enzymes used today. Enzymes have, however, also found use in a number of other industries, and new applications are constantly being developed. Some of the more important further areas of applications, either in terms of volume of enzyme used or in terms of development potential, are discussed below.

Leather Processing. Processing of skin and hides into leather has been based on enzymes ever since 1908 when Otto Röhm patented the first standardized bate containing pancreatic enzymes (160). 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 (77) are reduced process time, increased opening up of fibrous structure, cleaner surface, increased softness, improved area yield, and reduced need for chemicals.

Before the skins and hides can be tanned, protein and fat between the collagen fibers must be partially or totally removed. The protein can be removed by proteases and the fat removed by lipases, as well as by surfactants and organic solvents. Proteases are used mainly in the soaking, bating, and enzyme-assisted unhairing steps. Lipases are used for degreasing by hydrolyzing fat on the flesh side and inside the skin structure. The use of lipases greatly reduces the need for surfactants or organic solvents thereby providing obvious environmental benefits.

Modification of Fats and Oils. Several enzyme-based processes have been introduced in the fats and oils industry. Immobilized lipases are used as catalysts in interesterification or transesterification to produce triglycerides with changed melting characteristics or altered nutritional properties. Even though this use of immobilized lipases was first described in the 1980s, the process has not until recently been sufficiently cost-effective to be introduced in true large-scale applications such as, eg, the production of margarine. Even though enzyme production had become considerably more efficient, the cost of immobilization remained an obstacle. Recent developments have, however, changed this picture. A new process for immobilizing lipases based on granulation of silica has dramatically lowered process cost, and processes based on this new material are now used for the production of commodity fats and oils with no content of trans-fatty acids (161). Another recently introduced process is the removal of phospholipids in vegetable oils (degumming), using a highly selective microbial phospholipase (162). This enzymatic application has enabled savings of energy as well as water for the benefit of both the industry and the environment.

Personal Care. The potential allergenic effect of enzymes (cf. Chapter 8) excludes their use in traditional personal care products such as leave-on skin creams and shampoo. Safe applications of enzymes have, however, been developed in several areas. Proteases are used to clean dentures and contact lenses effectively under very mild conditions. Also, lipases have found some use in contact lens cleaning. Furthermore, the residual hydrogen peroxide used for disinfections can be neutralized using a catalase. Finally, enzymes such as glucoamylase and glucose oxidase are used in certain toothpastes to provide more effective cleaning and an antimicrobial effect.

Oil Drilling. In underground oil and gas drilling, different types of drilling mud are used to cool the drilling head, to transport stone and grit up to the surface, and for controlling the pressure underground. The drilling mud builds up a filter cake to ensure low fluid loss. Biopolymers in the mud glue particles together during the drilling process to make a plastic-like coating, which acts as a filter. These biopolymers may be starch, starch derivatives, cellulose (carboxymethylcellulose), or polyacrylates. During drilling, a clean-up process is carried out to create a porous filter cake or to completely remove it. The buildup of cake can cause serious drilling problems including the sticking of the drill pipe. Conventional ways of degrading the filter cake glue involve treatment with strong acids or highly oxidative compounds. Such harsh chemicals give rise to a number of problems including safety, environmental impact, equipment corrosion, and formation damage. Enzyme-based clean-up processes have emerged as an attractive alternative based on enzymes such as cellulases, mannanases, and amylases that effectively degrade the biopolymers used. The extreme down-hole temperatures often experienced do, however, pose a problem

to the application of enzymes limiting their use to wells operating at more moderate temperatures.

Organic Synthesis. Enzymes offer many potential advantages when used as catalysts for chemical synthesis. Their unique properties are, first of all, an often outstanding chemo-, regio-, and, in particular, stereoselectivity. Furthermore, enzymes are highly efficient catalysts working under very mild conditions. Enzymes do, however, also have some drawbacks that may limit their potential use, such as the ability to accept only a limited number of substrates and a moderate operational stability. Ways of overcoming most of these potential limitations exist and they pose in most cases more of a perceived than a real problem. Well over 100 different enzyme-based processes have been implemented on an industrial scale (163). A few processes are performed on a multithousand tonnes per year scale such as the manufacturing of acrylamide using a nitrile hydratase, the thermolysin-catalyzed synthesis of the low calorie sweetener aspartame and the synthesis of β-lactam antibiotics using a penicillin acylase (164). The majority of the established processes are, however, performed on a much smaller scale (100 kg–100 t/year). In most of these processes the unique enantioselectivity of the enzymes is utilized for the production of single-enantiomer intermediates used in the manufacturing of drugs and agrochemicals. As a consequence of their selectivity, few enzymes have a broad applicability. Exceptions do, however, exist, best exemplified by the *Candida antarctica* lipase B. This lipase has in numerous publications been shown to be a particularly efficient enzyme catalyzing a great number of different reactions including both regio- and enantioselective syntheses (165).

Extraction of Complex Polysaccharides. Polysaccharides with important medical use such as heparin and chondroitin sulfate are produced by extraction of various animal materials such as porcine lung or intestinal mucosa and bovine trachea. To obtain products with the desired purity and properties, very mild process conditions must be used. To boost extraction yields, proteases are added during the extraction to break down the tissue material and to liberate the polysaccharides without degrading the products.

Cork Industry. In the processing of corks for wine bottles, enzymatic steps have recently been introduced. To provide clean corks with the desired light look, a hydrogen peroxide bleaching step is usually employed. To remove excess hydrogen peroxide after the bleaching, a catalase is used.

Although cork is still the preferred choice for sealing a bottle of wine, the use of cork is not without problems. Cork can impart unpleasant flavors to the wine giving rise to the distinct mouldy “corked” taste that makes wine undrinkable. Recently, a phenol oxidase has been introduced to solve this problem (Suberase, Novozymes A/S). The enzyme polymerizes phenolic compounds in cork, thereby immobilizing the compound(s) responsible for the off-flavor or precursors of the latter.

8. Environmental and Safety Aspects

The industrial use of microbial enzymes is an important contribution to the development of Industrial or White Biotechnology. These terms describe the

industrial use of biological processes to produce products and services. 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 at 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 that are complementary. The first is physical containment in a fermentor 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 avoid dust generation, enzymes are supplied as liquids, encapsulates, or immobilized preparations along with safe handling guidance.

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 safety 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.

8.1. Regulatory Aspects. The safety assessments of microorganisms and enzyme products described above are subject to approval by authorities in many cases. For the use of genetically modified microorganisms in containment, harmonized EU (European Union) legislation applies, and similar regulation is in place in other parts of the world. For the enzyme products, specific legislation is quite limited, most national authorities have preferred to use or adapt existing legislation on chemicals, food, and feed additives. In the EU, however, specific enzyme guidelines have been prepared for the evaluation of food and feed

enzymes and product approval applies in Denmark and France. 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 Manufacturers and Formulators of Enzyme Products (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.

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:

- Those considered to be food stuffs, such as *A. oryzae*, the most prominent example of this category.
- Those considered harmless contaminants of food, such as *Aspergillus niger, Bacillus s., B. licheniformis*, and *Saccharomyces cerevisiae*; and
- 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.

Toxicological studies on food enzyme preparations focus on components derived from fermentation rather than the enzyme protein itself. Studies and uses of many types of enzymes have shown that enzymes *per* se are not toxic when used in food. 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 lead, coliforms, *E. coli*, and *Salmonella*. Furthermore, they prescribe the absence of antibacterial activity and, for fungal enzymes only, mycotoxins.

Feed Enzymes. In contrast to food applications, enzymes are used in feed primarily as active additives, eg, functioning as digestibility enhancers in chicken and pigs. The JECFA/FCC purity specifications for food-grade enzyme have been adopted. Harmonized EU legislation and guidelines apply for the assessment and approval of enzymes used as feed additives.

Technical Enzymes. Chemical control legislation sets the requirements for classification, warning labeling, and safety data sheets for all enzyme preparations. All enzymes are classified as potential respiratory sensitizers due to their protein nature. When an enzyme is used for a technical application, ie, industrial but nonfood and nonfeed, its regulatory status is generally determined by its compliance with chemical substance inventories, eg, EINECS in the EU or TSCA in the United States. In some cases, they are exempted as naturally occurring substances.

Enzyme manufacturers have developed nondusting product formulations and safe handling guidelines that effectively prevent human exposure, sensitization, and allergies.

9. Economic Aspects

Worldwide consumption of industrial enzymes amounted to ~$2.15 billion in 2003 (Fig. 15); about one-third was accounted for by the U.S. market. Estimation of worldwide consumption is difficult because official production figures are scarce. Furthermore, a relatively large portion of the production of starch-processing enzymes and the production of detergent enzymes to a lesser extent is for internal consumption. Also, the currency used for the estimation influences the result considerably.

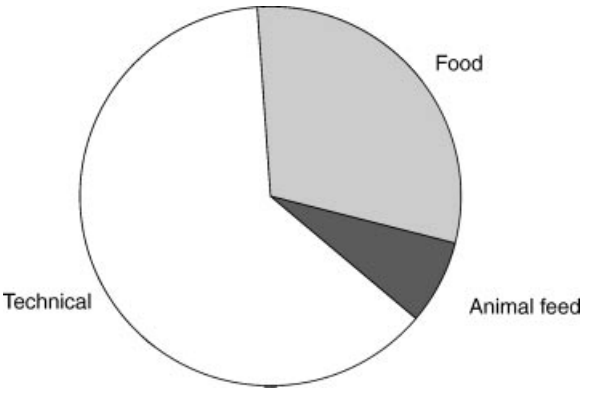

Fig. 15. Segmentation of the industrial enzyme market. In the year 2003, the market totaled $ 2.15 billion.

The growth in volume of the enzyme business from 1990 to 2003 is estimated to be 5–10%/year. The technical industry segment, covering a broad range of industries, with the detergent, starch, textile, fuel alcohol leather, and pulp and paper industries as the most important, accounts for ~60% of total enzyme sales. The detergent industry is still the most important segment accounting for ~35% of total enzymes sales. The technical enzymes segment is, however, the most mature segment and the long-term annual growth is only projected to be at the level of 5%. Many technical enzyme segments, including the important detergent segment, are rather mature and this growth is very limited. A few industries, such as the fuel alcohol industry, are, however, growing at double-digit growth rates. The food and feed segments are both expected to grow at a rate of 10–15%. This segment is driven by the introduction of enzymes into new applications, leading to a further expansion and diversification of the market.

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