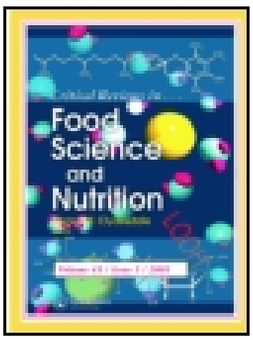

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Application of Enzymes in Food Processing

Jennylynd James and Benjamin K. Simpson

Department of Food Science and Agricultural Chemistry, Macdonald Campus of McGill University, 21111 Lakeshore Rd., Ste-Anne de Bellevue, Quebec, Canada, H9X 3V9

Referee: Dr. Maurice R. Marshall, Dept. of Food Science and Human Nutrition, Pesticide Research Lab., Univ. of Florida, Gainesville

ABSTRACT: Enzymes offer potential for many exciting applications for the improvement of foods. There is still, however, a long way to go in realizing this potential. Economic factors such as achievement of optimum yields and efficient recovery of desired protein are the main deterrents in the use of enzymes. Changing values in society with respect to recombinant DNA and protein engineering technologies and the growing need to explore all alternative food sources may in time make enzyme applications more attractive to the food industry. Research is continuing on the commercially viable enzymes in use today to improve various properties such as thermostabilities, specificities, and catalytic efficiencies. New and unique enzymes continue to be developed for use in enzymatic reactions to produce food ingredients by hydrolysis, synthesis, or biocatalysis. An aggressive approach is needed to open new opportunities for enzyme applications that can benefit the food industry.

KEY WORDS: *enzymes, applications, food industry.*

I. INTRODUCTION

Enzymes are biological catalysts that act to speed up chemical reactions. They are specific, effective in small amounts, and react under mild conditions of temperature and pH. Enzymes are derived from natural sources and may be readily inactivated after a desired transformation has taken place.[1]

Unlike inorganic catalysts, enzymes are highly specific, catalyzing the transformation of only a single substrate or the splitting of a small group of closely related compounds or a specific bond. This minimizes byproduct formation in large-volume reactions. The capacity of enzymes to react under mild conditions of temperature and pH (up to 100°C and pH 3 to 10) achieves a reduction in energy costs. Low usage levels make enzymes economical and practical for commercial application.[1] Because they are derived from plants, animals, or microbial sources, enzymes are perceived as natural, nontoxic food components and are preferred over chemical aids as food-processing aids by consumers.[2] Based on these properties, enzymes find numerous applications in industry. Reaction times for industrial enzymes vary from a few minutes to up to several days. During catalysis, the enzyme forms a transient complex with the substrate that may decay to form free enzyme and product (or substrate).[1] Thousands of enzymes, each capable of catalyzing a unique reaction, have been identified, and many are important to the food industry today.

II. TRADITIONAL USES OF ENZYMES IN THE FOOD INDUSTRY

A. Early Enzyme Use

Enzymes have been used since ancient times in an empirical manner, and special knowledge has been handed down from generation to generation of craftsmen, housewives, and chefs. One example is the use of the mucous membrane of weaning calves to preserve and improve the quality of milk for human consumption.[3] Other examples of early uses of enzymes include the addition of saliva to starchy products in preparation of fermented liquors, preparation of malted barley and moldy bran for starch saccharification, fermentation of grape juice to wine, conversion of

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milk to curds and whey in containers made of animal stomachs, meat tenderization with papain in unripe papaya fruit, baking of bread with yeast by ancient Egyptians, and use of molds to make various oriental fermented foods.[4-6]

The discovery of enzymes of the digestive tract was yet another milestone in the impact of enzymes on food products and the food industry. A few historical moments in food-related *enzymology* are illustrated in Table 1. Technical enzyme preparations have been in use since the beginning of this century. Beer had been stabilized with papain preparations since about 1911. Pectinases were used to improve the pressing quality of Concord grapes and for the clarification of fruit juices in the 1930s.[7] The first generation of enzyme preparations was unspecific, because no accurate knowledge of substrate nor enzyme activity was available. Manufacturers, however, have been constantly trying to improve the specificity of technical enzymes. Table 2 lists some of the early enzyme-related patents in the food industry. Takamine (1914) of Japan was the first to patent an enzyme from the microorganism *Aspergillus oryzae*.[4] Prior to 1960, the function and structure of enzymes was not too clear, but many applications had been found in the food industry (Table 3).

B. Current Enzyme Applications

Common use of enzymes in the food industry includes ingredient production and texture modification with industrial applications such as the production of high-fructose corn syrup, beverage clarification, brewing, baking, production of low-lactose milk, and meat tenderization. However, it is rare that the consumer would use enzymes directly.[7] Many food enzymes are used to degrade various biopolymers. Their specificity and high reaction rates under mild reaction conditions are favored over competing chemical treatments. Industrial food enzymes fall into three main groups: hydrolases, oxidoreductases, and isomerases. Bulk enzymes such as proteases, amylase, glucoamylase, pectinases, and cellulases (all hydrolases) have been produced using mainly two microbial genera: *Bacillus* and *Aspergillus*. Extracellular enzymes released by these microorganisms can simply be concentrated from the spent culture broth after removal of cells at the end of the production phase.[3]

Newer uses of microbial enzymes are emerging within the meat, fish, plant protein, and vegetable oil sectors. Their share of the total market for food enzymes is still quite small. Unfamiliarity with enzymes among potential users and lack of well-defined application areas of great economic significance are the main reasons for the slow advancement of enzymes in food production.[8]

1. Enzymes and Biotechnology

The application of microbial enzymes has its roots in Asia, while in North America and Western Europe it was more usual to use enzymes from plants and animals.[9] Microbial enzymes have

TABLE 1
Early Discovery of Enzymes

Year	Enzyme	Scientist
1831	Ptyalin (amylase)	Leuchs
1833	Diastase (amylase)	Payen and Persoz
1836	Pepsin activity	Schwann
1837	Emulsin activity	Leibig and Wholer
1846	Invertase activity	Dubonfant
1856	Trypsin activity	Corvisart
1897	Enzymes converting glucose to ethanol	Buchner

Adapted from Neidleman, S. L., *Food Technol.*, 45(1), 88, 1991.

TABLE 2
Some Early Enzyme Patents in the Food Industry

Year	Enzyme	U.S. patent no.	Inventor	Title
1894	Amylases	525,823	J. Takamine	Process of Making Diastatic Enzyme
1906	Amylases	836,699	J. Takamine	Diastatic Substance and Method of Making Same
1911		991,560	J. Takamine	Enzyme
	Malt protease	995,820	L. Wallerstein	Beer and Method of Preparing Beer
	Proteases	995,823		Preparation of Use in Brewing
	Pepsin	995,824		Method of Treating Beer or Ale
	Papain	995,825		Method of Treating Beer or Ale
	Bromelin	997,826		Method of Treating Beer or Ale
	Yeast protease	997,873		Method of Treating Beer or Ale
1915	Amylases	1,129,387	S. Franknel	Manufacture of Diastase
	Amylases	1,513,640	I. Pollak	Diastase Preparation and Method of Making Same
1917	Amylases	1,513,641	A. Boidin	Malt Extract and Method of Making Same
1922	Invertase	1,437,816	H. S. Paine and J. Hamilton	Process for Preparing Fondant or Chocolate Soft Cream Center
1923	Amylases, protease, lipase	1,460,736	J. Takamine	Enzymatic Substance and Process of Making Same
1932	Amylases, papain	1,854,353-5	M. Wallerstein	Method of Making Chocolate Syrups
	Amylases	1,858,820	R.Douglas	Process of Preparing Pectin
1933	Invertase	1,919,675	L. Wallerstein	Invertase Preparation and Method of Making Same
1937	Proteases	2,077,477-9	L. Wallerstein	Process of Chillproofing and Stabilizing Beers and Ales

Adapted from Neidleman, S. L., *Food Technol.*, 45(1), 88, 1991.

largely been considered by the North American food industries as merely substitutes for the traditional enzyme sources and not as processing aids.

Progress made in sterile technology as well as development of antibiotic fermentations and mass cultivation of single organisms have laid the foundation for technical production of microbial enzymes.[3] Introduction of pectinases for the clarification of fruit juices in the 1930s was probably the first example of industry's acceptance of microbial enzymes for an application in which enzyme technology had not been used previously.[8] The commercial use of isolated microbial enzymes, on a large scale, was enhanced in the 1960s following the inclusion of alkaline proteases in washing powders. Many of the bulk of enzymes used industrially today are of microbial origin, because, unlike plants and animals, microorganisms multiply rapidly and are not subject to the same political and agricultural policies that regulate the production of livestock for slaughter.[10,11]

Enzyme technology involves a great deal of overlap with the fields of fermentation and tissue culture technology, because these methods are also used for enzyme production. In fermentation technology and plant tissue culture, whole organisms are used. The multistep chemical conversions catalyzed by these processes require enzymes acting in sequence. Complex cofactors may be required. Traditional enzyme technology only requires one or two chemical reactions. Thus, instead of the inconvenience of using whole organisms, the enzyme is extracted from the plant,

TABLE 3
Pre-1960 Applications of Enzymes in the Food Industry

Enzyme	Source[a]	Food product	Application
Amylase and protease	2,3 2	Baked food	Bread and cracker baking
Amylase	2,3	Beer	Mashing
Protease	1,2,3		Chillproofing!
Glucose oxidase	2	Carbonated drinks	Oxygen removal
Amylase	2,3	Cereals	Precooked baby food
Amylase	2,3		Breakfast food
Amylase	2	Chocolate	Syrup
Pectinase	2		Bean fermentation
Pectinase and hemicellulase	2		concentrate
Protease	1,2,3	Condiments	Flavor ingredients
Invertase	2	Confectionary	Soft center, fondants
Rennin	1	Dairy	Cheese production
Lipase	1		Cheese favoring
Catalase	1,2		Milk sterilization with hydrogen peroxide
Protease	1		Off-flavor prevention in milk
Protease	1,2,3		Protein hydrolysates in milk
Lactase	2		Whey concentrates, ice cream, frozen desserts, milk concentrates
Glucose oxidase	2		Oxygen removal from dried milk
Amylase	2,3	Distilled beverages	Mashing
Glucose oxidase	2	Egg	Glucose removal
Pectinase	2	Fruit juice	Clarification, filtration
Protease	2,3	Meat	Meat tenderizing, casing tenderizing condensed fish solubles
Invertase	2	Molasses	High-test molasses
Amylase and amyloglucosidase	2	Starch	Corn syrup
Pectinase	2	Wine	Pressing, clarification, filtration

[a] 1 = animal, 2 = microbiological, 3 = plant.

Adapted from Neidleman, S. L., *Food Technol.*, 45(1), 88, 1991.

animal, or microbial source, purified, and the isolated form used to catalyze a reaction.[7]

Recombinant DNA methods are having a significant impact on enzyme technology. Some of the benefits include increased enzyme production and improvement of enzyme properties such as thermostability and ability to operate outside the normal pH range. Even though plants and animals may serve as sources of several useful enzymes, their use as sources of industrial enzymes may be limited by availability, cost, and various political, economic, and environmental factors. However, this limitation may be obviated by isolating the enzyme from a plant or animal source and amplifying it in a microorganism. Methods that have been employed include screening from nature, mutagenesis, genetic engineering and cloning techniques, polymerase chain reaction (PCR), protein engineering, and chemical derivatization.

The three main cloning techniques employed include "shotgun" cloning, cDNA cloning, and synthetic DNA cloning. "Shotgun" cloning is rela-

tively simple to carry out because the gene sequence coding for a particular protein does not have to be known. The disadvantages of this method, however, are that a very specific selection method is required to identify recombinant strains, genes that contain introns would not be accurately expressed, and where prokaryotes are used to produce enzymes, these microorganisms would have to recognize foreign DNA and express it. There is no problem of introns being translated in cDNA cloning. Selection methods tend to be simpler than those of "shotgun" cloning and less laborious. However, mRNA required for production of cDNA may not be available in sufficient quantities. The cloning of cDNA is technically more difficult to carry out and codon choice may not be optimal for the host cell. Synthetic DNA cloning has the advantage that the sequence of gene promoters and ribosomal binding sites can be optimized and changed. Thus, highly specific mutations and enzyme production are possible. The disadvantage here, however, is that the gene sequence must be elucidated first and there is usually a problem with degeneracy.[12]

PCR has been applied in all cloning techniques. This process has been used to generate specific sequences of cloned double-stranded DNA for use as probes, to generate probes specific for uncloned genes by selective amplification of particular segments of cDNA, to generate libraries of cDNA from small amounts of mRNA, to generate large amounts of DNA for sequencing, and for the analysis of mutations. The main limitation of this method, however, is the high rate of misincorporation because Taq DNA polymerase lacks the editing function of other polymerases like the Klenow fragment in *Escherichia coli* (Pol I). PCR is still a very useful tool that can be applied in protein engineering and the production of beneficial food enzymes.[13]

DNA technology and its means of improving enzyme production using cloning and expression, with protein engineering techniques to form unique enzymes, have led to the making of new enzymes based on specific or desired functional needs. If the three-dimensional structure or primary amino acid sequence is known, the process becomes even more feasible.[14] The development of successful commercial products for food-processing enzymes and additives by genetic engineering technology requires a concerted approach of genetic manipulation, protein engineering, and process development contributed by scientists and engineers from diversified disciplines, as seen in Figure 1.

Genetic engineering has made it possible to isolate particular genes coding for enzymes, from organisms whose genetics are unknown. Using *in vitro* recombination these genes can be introduced into microorganisms that have been used in food preparation for centuries, with subsequent synthesis of the novel gene products (enzymes). Genetic engineering methods provide the opportunity to increase gene dose and so affect product yield. Plant and animal enzymes may be produced more efficiently when cloned into a microbial cell and should be produced under highly regulated conditions.[15]

Protein engineering involves protein crystallography, interactive computer graphics, databases, and other forms of commercial modeling to generate knowledge of the relationship among sequence, three-dimensional structure, and function. The necessary sequence changes for novel properties are suggested and carried out by site-directed mutagenesis to alter one or a few specific amino acids in a molecule. The progress made in the genetic engineering and protein engineering stages are only useful when a large-scale process has been developed to cultivate the "new" organisms in a fermenter and to purify a large enough quantity of active product for product formulation and toxicology testing. Optimization of conditions for the cloned gene products is an important step in translating labscale experiments into commercial success.[16]

Enzymes of biotechnological interest that have been cloned fall into two main categories: those that have applications *in vitro* in some commercial processes and enzymes involved in biosynthetic pathways leading to commercially useful products. Polysaccharases and proteases are the main groups of genetically engineered enzymes applied commercially. The α-amylases of several species of bacilli have been cloned and in some cases partially or totally sequenced. Fungal glucoamylase genes from *Aspergillus awamori* and *A. niger* have been cloned. In both cases cDNA clones were prepared as probes and the genes identified using purified mRNAs that en-

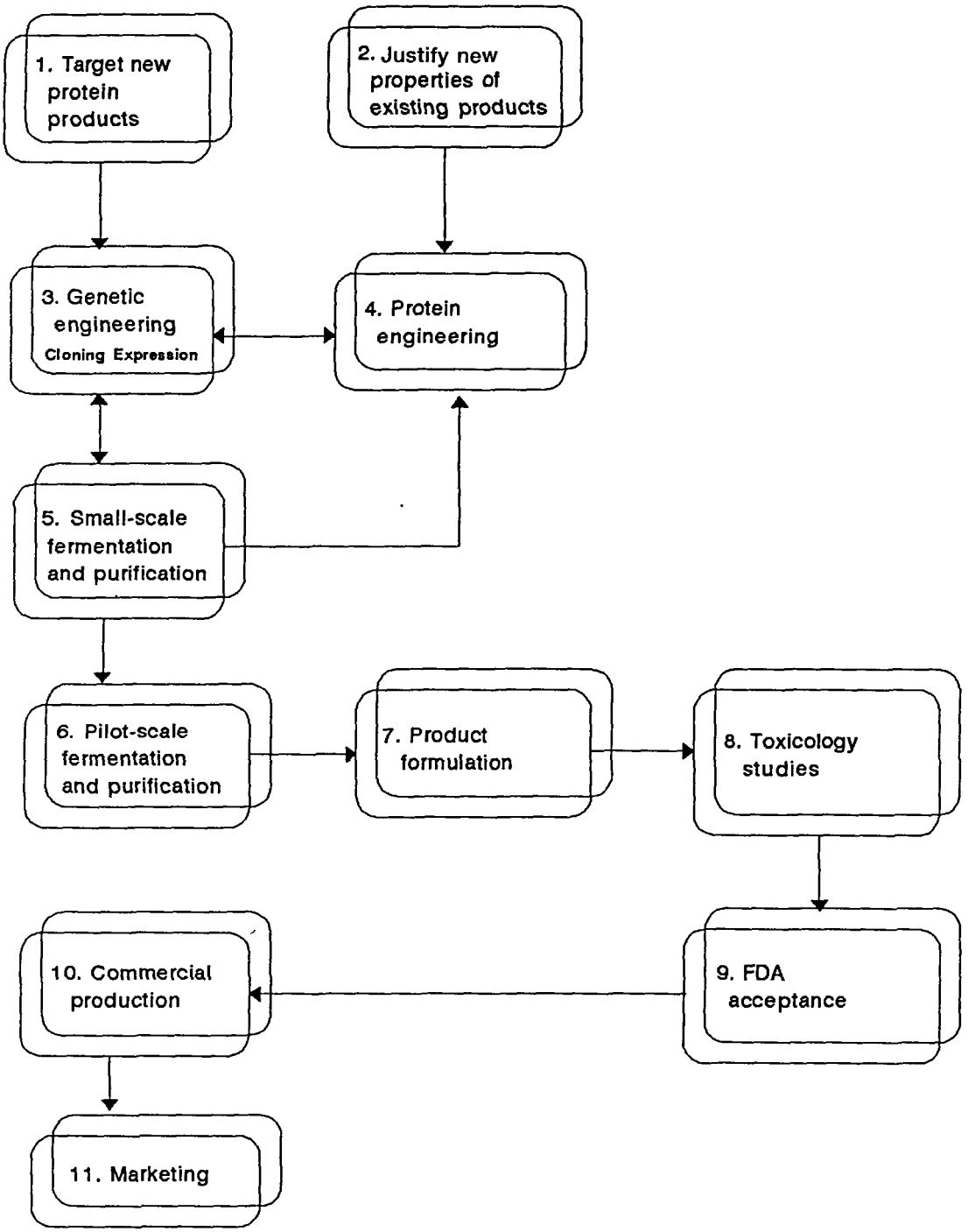

FIGURE 1. Flowchart for commercial development of recombinant food-processing enzymes and food additives. (Adapted from Lin, Y. L., *Food Technol.*, 41(10), 104, 1987.)

coded the glucoamylases. Cellulases from both bacterial and fungal sources have been cloned. There are reports of cloning of genes from organisms that code for protease production. One example is the cloning of a thermostable neutral protease gene from *Bacillus stearothermophilus* and its expression in *B. subtilis*. The gene coding for calf chymotrypsin (rennin), a proteolytic en-

zyme of great commercial importance, has been cloned and sequenced in *Escherichia coli* using a tryptophan promoter from the host bacterium. The reader is referred to the review by Peberdy[15] for further information on genetic engineering in relation to enzymes.

2. Bulk Enzymes and Immobilization

Traditionally, enzymes were used in bulk form, for example, specific proteases for hydrolysis of casein in cheese production. Through mass production of secreting cells, extracellular enzymes can be concentrated from the spent culture broth after removal of the cells at the end of the production phase. Enzymes thus became available at low cost and were used only once and then discarded or left in the product in a denatured form. This is a simple approach to enzyme use not requiring complex equipment or technical staff. Bulk enzymes are distributed for use either as concentrated liquids or as dry powders.

Alternatively, the enzyme may be immobilized, that is, constrained one way or another within the confines of a solid support to permit it to be reused from one operation to another.[17] Other advantages of enzyme immobilization include: (1) a reduction in the need for huge quantities of enzymes thus reducing costs, (2) the ability to reuse the enzyme many times more than when in the soluble form, (3) no residual enzyme left in the product, (4) increased stability of the enzyme (5) improved enzyme behavior (pH optima shifted to more advantageous pH on immobilization to certain supports), (6) the possibility of a continuous process, which permits better quality control of the product, (7) less additional processing, (8) lower labor costs, and (9) advantageous use of multiple enzyme systems. The procedure has been explored since the mid-1960s. Substrate can now be continuously added to a support as product is removed.

Solid supports used for enzyme immobilization include polysaccharides, inorganic supports, fibrous proteins, synthetic polymers, hydrogels, and hollow fibers. Enzymes can be constrained or held to these supports by adsorption, covalent attachment, crosslinking, entrapment, microencapsulation, or by other methods.[17] Examples of polysaccharide supports include diethylaminoethyl (DEAE)-Sephadex, which provides a polycationic, weakly basic anion exchange group. Enzymes having a relatively high content of acidic amino acids remain firmly bound to DEAE-cellulose or DEAE-Sephadex even at high substrate concentration as long as certain physical conditions such as ionic strength and pH are maintained.

Some of the disadvantages of enzyme immobilization include: (1) the cost of immobilization, (2) requirement for highly skilled personnel, (3) unique sanitation and toxicology problems, and (4) loss of enzyme activity; for example, aminoacylase from *Aspergillus oryzae* adsorbed on DEAE-Sephadex lost 40% of its activity over a 32-d period when used at 50°C for continuous hydrolysis of acetylated L-methionine.[17]

Chitin is another carbohydrate support material used in the immobilization of enzymes, as described with glucose isomerase and glucoamylase attached by simple adsorption. The deacetylated form of chitin is chitosan and this polymer has a large percentage of free amino groups. Chitosan has been used along with gluteraldehyde to prepare immobilized acid-tolerant lactose.[17] Porous glass, alumina, nickle oxide on nickle screen, silica alumina impregnated with nickel oxide, porous ceramics, and sand are some of the inorganic materials to which enzymes have been attached. Inorganic supports have the following advantages: (1) structural stability over varying conditions, (2) excellent flow properties in reactors, (3) inertness to microbial attack or attack by enzymes, (4) ease of adaptability to various particle shapes and sizes, and (5) good regeneration capability.

General methods used to immobilize enzymes include adsorption, covalent binding, and entrapment. For a very informative discourse on immobilized enzymes in the food industry, the reader is referred to the review article by Pitcher.[18] The influence of the use of enzyme immobilization on the food industry is illustrated by the corn starch industry, where glucose isomerase is immobilized for the production of high-fructose corn syrup. This syrup has become a very competitive sweetener in the food industry.[19]

It is also possible to immobilize whole cells instead of isolated enzymes. This approach has several advantages: (1) it avoids the lengthy and

expensive operations of enzyme purification, (2) there is preservation of the enzyme environment and reduction of its degradation, (3) sophisticated transformations are performed more easily, especially those requiring several enzymes and cofactor regeneration, and (4) a multipurpose catalyst may be provided.[19] However, there are limitations to the approach, namely, (1) rate limitation due to diffusion of substrate and products through the cell wall, and (2) undesirable reactions of the substrate or product, causing a loss of specificity. Continuous processes are favored by cell immobilization.

C. Dairy Industry

For quite some time, rennet obtained from the stomach of calves was the only technical enzyme used in cheese making, apart from the specific use of esterases from sheep and goat gland extracts. Demand for cheese and dairy products is on the increase worldwide. The dairy industry has responded to these demands through mechanization and consolidation and by taking advantage of improvements and new technology. Microbiological proteases from *Mucor* or *Endothia parasitica* have been used as substitutes for animal rennet, which is becoming scarce. However, microbiological rennin has been found to be less specific than pure chymosin.[7]

Pepsin and chymosin-like enzymes from harp seal and cold-temperature-adapted fish species have been shown to be able to curdle milk, but similarly to the microbial enzymes, are relatively less specific. The introduction of microbial chymosin produced by a genetically engineered organism is considered the most significant future breakthrough in microbial rennet production.[8] Improvements are needed in the area of shortened cheese ripening time and use of the waste product whey.

1. Microbial Proteinases in the Dairy Industry

Microbial rennet substitutes derived from *Mucor miehei, M. pusillus* and *Escherichia parasitica* are utilized in countries such as Holland and the U.S. Commercial products derived from these rennet substitutes are listed in Table 4. Mucor rennets are marketed as liquids stabilized with salts and permitted preservatives. The enzyme from *E. parasitica* is less stable and is usually marketed in a solid form. Religious and ethnic regulations against the use of animal-derived enzymes have encouraged the search for alternatives to calf rennet in the United States. European countries have a larger supply of calf rennet and are in the practice of using various blends of calf rennet and bovine or porcine pepsin.[21]

In Cheddar cheese manufacture, the rennet is added to milk at a temperature of 31°C. The milk-clotting enzyme is added at a rate of 150 to 200 ml/100 kg of milk, and clotting occurs within 10 to 15 min. A temperature of 35°C is used in Swiss cheese manufacture. Chymosin is used as the standard of evaluation for other milk clotting en-

TABLE 4
Commercial Microbial Rennets

Microbial source	Supplier	Product name
Mucor pusillus	Dairyland Food Laboratories	Emporase
Mucor miehei	G.B. Fermentation Industries	Fromase
Mucor miehei	Chr. Hansen's Laboratories	Hannilase
Mucor miehei	Miles Laboratories	Marzyme
Aspergillus niger	Miles Laboratories	Milenzyme
Mucor miehei	Pfizer, Inc.	Morcurd
Mucor miehei	Novo Laboratories	Rennilase

Adapted from Ward, O. P., *Microbial Enzymes and Biotechnology,* Fogarty, W. M., Ed., Applied Science Publishers, New York, 1983, 251.

zymes. Comparative trials indicate that microbial enzymes from *M. miehei* compare favorably with rennet in cheese making with respect to curd yield and firmness. Various types of cheeses prepared by microbial rennet were judged satisfactory by organoleptic tests. However, it has been reported that microbial rennets produce more bitter products, particularly in young cheeses.[21] Most other proteolytic enzymes cause extensive digestion of milk proteins that leads to unacceptable flavor, poor texture, and reduced yield.[21]

Studies of acceleration of the complex biochemical reactions in cheese ripening have focused on proteinases, with less emphasis on lipases and peptidases, in hard/semihard internally bacterially ripened cheeses. Accelerated ripening did not occur with increased rennet levels. Use of an optimal level of Neutrase reduced the ripening by approximately 50%, but the product thus obtained had a softer body and was more brittle than cheese of the same age.[22] A combination of Neutrase and streptococcal cell-free extract (CFE) gave better results with increased proteolysis, but no increased flavor intensity. This combined enzyme preparation is now being produced commercially by Imperial Biotechnology of London, U.K., as a product called Accelase and is to be used in large-scale commercial cheese-making trials.[22]

2. Lactases

Lactose is a disaccharide composed of glucose and galactose residues linked together by an α-(1→4) glycosidic bond. It is a major component of milk and appears in whey during cheese manufacture. Lactose is less sweet and less soluble than its component sugars and is thus of little use commercially, because it needs to be broken down into its component sugars.

It is estimated that 70% of the world population is deficient in intestinal lactase (β-galactosidase), the enzyme necessary to digest lactose. Certain individuals from various ethnic groups in the U.S., namely, African-Americans (45 to 81%), Mexican-Americans (47 to 74%), Asian-Americans (65 to 100%), Native Americans (50 to 75%), and people of northwestern European ancestry (6 to 25%), have been found to be lactose intolerant. Thus, one-third of the U.S. population may be presumed to have some form of lactose intolerance.[23]

The enzyme lactase, which coverts lactose into its subunits (glucose and galactose), can thus find useful commercial applications in the food industry. The development of processes involving soluble or immobilized lactase has been very slow, possibly due to the high cost of the enzyme in correlation to the value of the substrates.[9] The potential for enzymatic modification of lactose has been recognized since the 1950s, but production of lactose-modified dairy products became possible only with the commercial development of processes for the isolation of the enzyme from microbial sources. Lactases generally used for lactose hydrolysis are extracted from yeast such as *Kluveromyces lactis (K. marxianus)* and *K. fragilis* or from fungi such as *Aspergillus niger* and *A. oryzae.* These lactases differ widely in their properties, especially in their pH optima.[23,24]

A dairy-modified lactose-reduced milk is available on the market. It is well accepted despite the slightly sweeter taste brought about by hydrolysis. One promising process is the Tetra Lacta System, developed by Tetra Pak International AB. In this process milk is sterilized by an ultrahigh-temperature (UHT) treatment, after which it is mixed with sterile enzyme solution in low concentrations. Hydrolysis of lactose then takes place during storage of the packed milk (1 to 2 weeks — over 80% conversion of lactose).[8]

Lactase capsules were introduced by the Kremers Urban Co., Millwaukee, WI and Lactaid, Inc. began commercial marketing of tablets to health professionals in 1985. Lactase-containing tablets are now available for both consumers and health professionals. Each tablet contains β-D-galactosidase from *A. oryzae* and is taken with a meal. For example, in 1990 Winthrop Consumer Products, Division of Sterling Drugs, New York, NY introduced a chewable tablet known as Dairy Ease.[23]

Lactase has been employed in the manufacture of ice cream. Partial replacement of the skim milk powder and sugar with hydrolyzed sweet whey in ice cream formulae gives a smoother

texture and an acceptable flavor. The possibility of producing low-fat (3 to 5%) ice cream mixes that are comparable in flavor and texture with ordinary ice cream (10% fat) is being investigated.[8] Complete replacement of sucrose in ice cream mix with hydrolyzed-lactose syrup depressed the freezing point slightly.

In general, when cheeses are prepared from hydrolyzed-lactose milk, a faster rate of acid development is observed during the initial stages of cheese making, thereby reducing manufacturing time. Faster ripening time for flavor and texture development has been observed, as well as higher cheese yield. Reported disadvantages, however, include off-flavor development, sweet taste, failure of curd to knit properly during cheddaring, and crumbly body.[23]

3. Future of Enzymes in the Dairy Industry

Genetic engineering offers the advantage of directed manipulation of microorganisms to achieve significant improvements on existing processes. Research progress has been slow in the area of diary products for the following reasons: (1) higher intrinsic value of pharmaceutical targets, (2) difficulty of culturing in the laboratory — dairy starter organisms have complex nutritional growth requirements, (3) few selection markers, and (4) lack of secretion of proteins and other substances.

In the dairy industry, genetic engineering technology has targeted the cloning of starter culture organisms with genes coding for long-term resistance to bacteriophage infection and the elimination of culture rotations. Improved rates of acid production and substrate use have also been important areas of research. Formation of cultures that give new or improved textures and flavors, with accelerated flavor development, have been targeted. Production of antimicrobial compounds that reduce spoilage and inhibit harmful microorgansisms is an area of major concern. Above all, fermentation-based production of microbial calf chymosin will prove to have enormous economic savings.

The ability to use the full potential of genetic engineering in lactic streptococci was initiated when Kondo and McKay (1982) reported successful transformation of plasmid DNA in *Streptococcus lactis* protoplasts; however, the frequency of transformation was low. Using a polyethylene glycol-induced uptake system, an increase in efficiency of about 300-fold was achieved.[25]

Also important was polyethylene glycol-induced transfection of protoplasts of *Streptococcus lactis* subsp. *diacetylactis.* The efficiency of genetic exchange reported was 50,000 transfectants per microgram of phage DNA. By shuttling plasmid vectors between organisms, such as *Escherichia coli* or *Bacillus subtilis*, and the streptococci, the vector permits chromosomal fragments to be cloned and manipulated in the well-developed *E. coli* or *B. subtilis* system and then transferred to the streptococci for further study.[25] Several research groups have developed recombinant DNA techniques for the introduction of the prochymosin gene from calf stomach cells into microorganisms such as *E. coli* or *Saccharomyces cerevisiae.* Table 5 gives a partial list of companies with patent applications filed on microbial calf chymosin. One problem experienced with recombinant proteins produced in *E. coli* is the formation of soluble granules that are catalytically inactive, packed inside the cell. These inclusion bodies of protein present major problems in industrial scale-up and enzyme recovery.

D. Protein Modification

Food protein modification using microbial enzymes has played an important role in bread manufacture. Strong gluten of low extensibility reduce the volume of the baked product. This situation is remedied by using fungal proteinases to achieve limited, specific gluten degradation.[7] Enzymatic hydrolysis of proteins has been applied to improve their whipping capacity or emulsifying power. In the confectionary industry, protein modification using microbial enzymes has been used in the production of foaming agents and specialty protein hydrolysates for dietetic purposes. The development of industrial protein

TABLE 5
Companies and Investigators Who Have Published Research, Have Patent Applications Filed, or Have Patents on Microbial Calf Chymosin

Company or investigator	Microorganism
T. Beppu, U. of Tokyo	*E. coli*
Celltech, U.K.	*E. coli* and *S. cerevisiae*
Codon, CA	*E. coli*
Collaborative Research	*E. coli* and *S. cerevisiae*
Genencor	*E. coli* and *B. subtilis*
Genex	*E. coli*
Gist Brocades	*Kluveromyces*
Unilever	*E. coli*

From Barach, J. T., *Food Technol.*, 39(10), 73, 1985. With permission.

hydrolysis processes was hampered by the formation of bitter-tasting peptides. Examples of enzyme-modified proteins are discussed below.

1. Soy Protein Hydrolysates/Health Foods

The treatment of soya flour or isolated soy protein with proteases produces proteins with modified functional properties, such as increased isoelectrical solubility and improved whipping capacity, foam stability, and emulsifying capacity.[7] Soy protein hydrolysates have great potential as ingredients in low-pH foods such as beverages where applications depend on a soluble product that does not cause problems of gelation, turbidity, and off-flavor to the food produced. The major problem encountered with soy protein hydrolysates is the problem of bitter peptide formation. The degree of bitterness is related to the degree of hydrolysis. A compromise thus has to be established.[20]

The production of isoelectric, soluble soya protein hydrolysate (ISSPH) and the decolorization of the blood cell fraction of slaughterhouse blood are both processes that use *Bacillus licheniformis* protease (Alcalase®), a product used in the detergent industry. The neutral protease from *B. amyloliquefaciens* (Neutrase) is used in the production of soya milk for increasing its protein content.[8] Traditionally, soy milk is produced by initial soaking of the beans for at least 3 h, depending on water temperature. The beans are then ground in water to bean ratio of 10 to 1. The slurry is filtered and the resulting milk heated to boiling for about 30 min to improve its nutritional value and flavor. The product has a typical bean flavor caused by enzymatic lipid oxidation during the wet grinding process.[26,27]

It was found that enzymes improved the yield of soy milk compared with traditional procedures. The neutral protease Neutrase gave superior results at neutral pH. Olsen and Alder-Nissen[27] devised a method whereby hydrolysates of soya protein could be produced, removing compounds responsible for bitter off-flavors. Figure 2 gives the structures of precursors and off-flavor compounds in soybean. "Cooked soybean" off-flavor results from the presence of phenolic acids such as syringic, vanillic, ferulic, gentistic, salicyclic, *p*-coumaric, and chlorogenic acids. Ferulic and *p*-coumaric acids have been found to be precursors for the soybean off-flavor compounds, which were identified as 4-vinyl-guaiacol and 4-vinyl-phenol.

Soya white flakes are washed at pH 4.5 with water in a four-step extraction process to produce a concentrate with a low level of beany off-flavors. The concentrate is immediately hydrolyzed

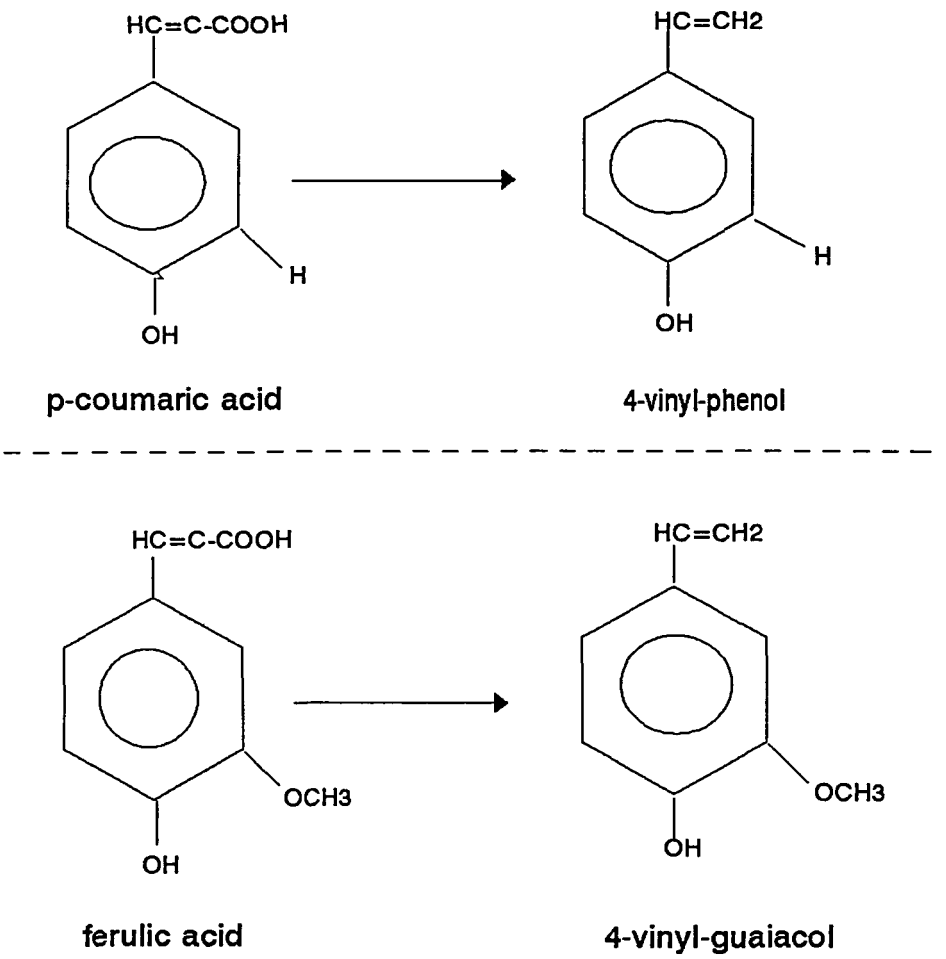

FIGURE 2. Precursors and off-flavors from soybean. (From Olsen, H. S. and Adler-Nissen.[27] With permission.)

with the alkaline protease Alcalase to a specified degree of hydrolysis. Hydrolysis is terminated by lowering the pH and the supernatant is then recovered by centrifugation.[28]

E. The Starch Industry

1. High-Fructose Corn Syrup Production

The method of acid hydrolysis of starch forming glucose was developed by Russian chemist Kirchoff in 1811, and de Saussure in 1815 established the basis for the commercial production of syrups and crude sugars. Although acid-converted corn syrup was useful in many applications, the lack of sweetness relative to that of sucrose and the formation of reversion products limited the use of glucose syrups. The introduction of enzymatic processes with liquefying bacterial amylase and saccharifying amyloglucosidase today allows yields of up to approximately 90% glucose. The use of immobilized glucose isomerase has made it possible to produce isomerase syrup with high fructose content.[7] A large percent of microbial enzymes produced are utilized in the starch industry. The large-scale process of high-fructose syrup production was developed in the mid-1970s using Novo's commercial preparations of thermostable α-amylase (for the initial degradation of starch to dextrins) and immobilized glucose isomerase (for converting glucose to fructose).

The second process step, the saccharification of dextrins to glucose, is carried out by soluble glucoamylase from *Aspergillus niger*. Immobilized glucoamylase has proved inefficient because the maximum relative glucose content obtained is lower than that obtained with soluble glucoamylase.[8,29]

High-fructose corn syrup (HFCS) has become an established product for the corn wet milling industry, and almost every major corn processor in the U.S. has an HFCS plant in operation. HFCS is used as a less expensive direct replacement of sucrose.[30] A simplified flow diagram for the production of HFCS starting from starch was described by Bucke.[32] The need for multiple treatment during HFCS production arises from the nature of starch itself. Starch consists of two fractions. The amylose fraction consists of long, unbranched chains of D-glucose units linked by α-(1→4) bonds. In contrast, the amylopectin fraction is highly branched and has both α-(1→4) and α-(1→6) bonds.[31] The main challenge in the early saccharification steps is that α-amylases act mainly on 1→4 bonds so that addition of a debranching enzyme like pullulanase or isoamylase, which specifically attack α-(1→6) glycosidic bonds, is now widely practiced.[31] Commercial preparation of debranching enzymes exhibiting the right combination of pH and temperature, activity, and stability has begun.

The heart of the process is the enzymatic isomerization step using immobilized glucose isomerase. Two enzymes, α-amylase and glucoamylase, are used in a soluble form to hydrolyze starch. Syrup containing typically 90 to 95% dextrose is fed to the isomerization reactor system. A series of pretreatment steps such as separation of residual protein, carbon treatment, and ion exchange are needed to condition the feed syrup for isomerization. A series of posttreatment steps, including evaporation, are needed to obtain the end product.[30,32]

The amylase from *B. amyloliquefaciens*, which has a temperature optimum of approximately 70°C, was initially used to liquefy starch to form dextrin. This enzyme is also used today in brewing and for the starch breakdown in brandy manufacture. Maize starch production requires temperatures in excess of 100°C for complete starch digestion. There was thus the need for bacterial amylases with high-temperature stability, such as that from *B. licheniformis*, which has optimum working temperature 90°C.

2. Maltose Production

Amyloglucosidase from *A. niger* and *Rhizopus* is used for saccharification of dextrins to glucose, which is then isomerized to fructose in high-fructose syrup production. The reaction is slow: 45 to 60 h at pH < 5.0 and temperature 60°C.[7] Exoacting maltogenic α-amylase has been described. It is also the first near commercial example of an industrial enzyme produced by genetically engineered organisms. In trials, more glucose was obtained than with plant β-amylases.

The demand for high-maltose syrups in Japan is growing. Most Japanese prefer the much milder sweet flavor of maltose to the very sweet flavor of sucrose. Therefore, a lot of high-maltose syrup instead of sucrose has been recently used for making cakes and soft drinks.[33] By treating starch paste with β-amylase, maltose may be obtained in a yield of about 80%, with 20% of dextrins being produced simultaneously. Maltose syrups produced from corn starch are of two types. One type is 30 to 50% maltose and 6 to 10% glucose (high-maltose syrup) corresponding to 43 to 49 dextrose equivalent (DE) corn syrups (Table 6). The other is 30 to 40% maltose and 30 to 50% glucose (fermentable syrup) representing 65 to 70 DE (Table 6).

Maltose production can be divided into the production procedure for high-maltose syrup and the fermentable syrup. For high-maltose syrup production, two common procedures used are (1) the acid-enzyme conversion process, and (2) the multiple-enzyme process. For the first process, a maltose-producing saccharifying enzyme such as barley β-amylase is added and the residual enzyme is deactivated by heating. In the multiple-enzyme precursor, starch is gelatinized and the preliminary starch depolymerization is brought about by enzymes such as fungal α-amylase or bacterial α-amylase instead of acid. Barley

TABLE 6
Compositional Data of Syrups

Sample	Dextrose equivalent	% Saccharide, carbohydrate basis						
		DP_1	DP_2	DP_3	DP_4	DP_5	DP_6	DP_7
Corn syrup, AC	27	9	9	8	7	7	6	54
Corn syrup, AC	36	14	12	10	9	8	7	40
Corn syrup, AC	42	20	14	12	9	8	7	30
Corn syrup, AC	55	31	18	12	10	7	5	17
Corn syrup, HM, DC	43	8	40	15	7	2	2	26
Corn syrup, HM, CD	49	9	52	15	1	2	2	19
Corn syrup, DC	65	39	31	7	5	4	3	11
Corn syrup, DC	70	47	27	5	5	4	3	9
Corn syrup, DC, E	95	92	4	1	1			2[b]

[a] Data supplied by cooperating member companies of the CRA.
[b] Sum of $DP_{5,6,7}$.

Note: DP = degree of polymerization; AC = acid conversion, DC = dual conversion (acid-enzyme), HM = maltose, E = enzyme conversion.

From Maeda, H. and Tsao, G. T., *Process Biochem.*, 14(7), 2, 1979. With permission.

β-amylase is then added to continue the conversion. Microbial β-amylase has been known to be very stable in supply and economical to use.

A. oryzae α-amylase is reported to be the most effective of many amylases tested. Staley Corn Processing, Inc. has applied a debranching enzyme for the production of high-maltose syrup of 80% maltose content as a commercial product. Barley β-amylase and microbial pullulanase have been used as debranching enzymes. It was found that *B. cereus* var. mycoides accumulated large amounts of β-amylase and pullulanase in the broth at the same time when cultivated with meat extract and mild casein as the nitrogen sources. These enzymes find great industrial applications.[33]

F. Brewing Industry

Traditionally, manufacture of beer is divided into stages: a main fermentation stage and a maturation stage. During the main fermentation, the bulk of fermentable sugars in the wort (an aqueous extract of malted barley) is metabolized into ethanol, while during maturation, flavor, carbon dioxide content, haze stability, attenuation, and other properties of the beer are adjusted carefully.[34,35] Proteinases and exopeptidases present in the malt cause the increase in soluble nitrogen compounds required for the brewing process. If unmalted grain is used, the missing proteolytic activity must be supplemented by exogenous protease preparations. In selecting proteases, the following conditions must be observed: (1) high-temperature stability at 55 to 60°C, (2) optimum activity between pH 6 to 7, (3) insensitivity to natural proteinase inhibitors, and (4) activity at very low protein concentrations. Traditionally, this function was carried out using papain.[7]

In beer manufacture, proteinases act to increase the actual yield of wort and the level of α-amino nitrogen in the wort, and to remove chill

haze from beer during finishing. It is agreed that a level of 140 to 180 mg of α-amino nitrogen per liter of wort is required for normal fermentation.[20]

Beer contains complexes of proteins with carbohydrates and tannin, which are soluble in the newly pasteurized product, but tend to become less soluble with time as the beer is cooled. The complexes are insoluble, but do not precipitate. They instead form unacceptable turbidity in the beer. This turbidity is called chill haze. The nature of chill haze and its formation vary depending on the type and source of barley malt hop adjuncts and processing variables used in the particular brewing (5 to 70% proteins, 1 to 55% tannins, and 3 to 80% carbohydrates).[35] In North America, where beer is chilled before serving, the problem is unavoidable; thus, chill proofing or prevention of chill haze formation is required.

Examples of haze-removing material are adsorbents such as bentonite, wood chips, silica, nylon powder, and activated carbon. These solid particles would have to be removed at the end of the process, thus requiring additional costs for filtering. There would also be a reduction in yield because some final product remains trapped in the filters. Around 1914, the Wallerstein Company used proteolytic enzymes to hydrolyze proteins involved in haze formation. For example, papain has been immobilized on chitin and beer passed over columns of the enzyme for chill proofing.[34,35]

The brewing industry is quite conservative in the use of microbial enzymes; however, the enzyme acetolactate decarboxylase isolated from several microorganisms has been applied to catalyze the degradation of α-acetolactic acid to acetoin. During beer maturation processes, the oxidative transformation of α-acetolactic acid into diacetyl and the subsequent reduction of diacetyl to acetoin by reductases from the yeast are usually rate limiting for the overall maturation process (Figure 3). Acetoin is a major flavor component in beer and thus its formation is critical in beer production.

Godtfredsen et al.[34] determined the occurrence of α-acetolactate decarboxylases among lactic acid bacteria. *Streptococcus diacetylactis* strain FD-64-D was found to generate a decar-

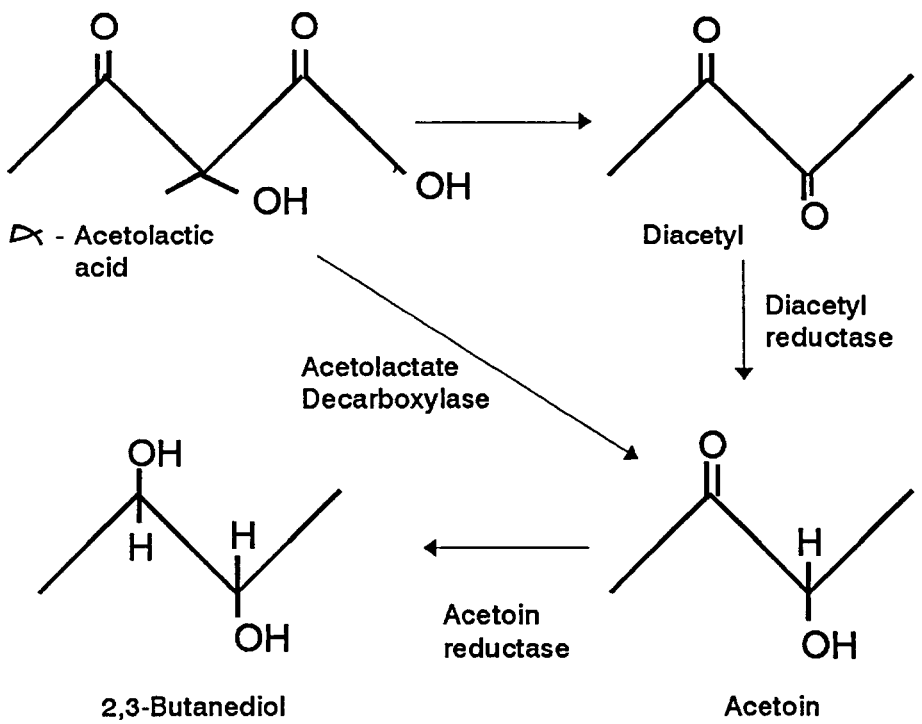

FIGURE 3. Key reactions in diacetyl generation in and removal from beer. (Adapted from Godtfredsen, S. E., Rasmussen, A. M., Ottesen, M., et al., *Appl. Microb. Biotechnol.*, 20, 23, 1984.)

boxylase exhibiting a satisfactory activity and an excellent stability at the pH prevailing in beer and wort. *Lactobacillus casei* DSM 2546 produced a decarboxylase with improved solubility characteristics and also exhibited satisfactory activity and stability at low beer pH. Based on these superior qualities, the enzyme from *L. casei* was evaluated for solubility for pilot-scale production. Thus, acetolactate decarboxylase production from lactobacilli was not considered to be profitable even though it has GRAS status, because it requires too much zinc for stabilization. Therefore, more suitable bacterial sources are required for the production of acetolactate decarboxylase.[8]

Microbial enzymes are being used in the production of low-calorie beer. The main difference of the "lite" beers compared with "normal" beers is that "lite" beers are relatively lower in calories (i.e., ~ 15 to 50% lower). Brewing techniques in "lite" beer formation include a low original gravity and a complete fermentation. One simple production method is to dilute a regular beer. However, this may upset flavor balance. A more complex technique includes the use of dextrose as an adjunct to reduce the residual carbohydrate content of the beer or addition of amyloglucosidase to wort to achieve the same results. However, with the enzyme technique, it is difficult to remove all residual enzyme activity in the final beer, which leads to a distinctly sweeter product on the market. Glucoamylases and fungal β-amylase are used commercially for this purpose.[8]

G. Lipid Modification

Lipases are produced by plants, animals, and microorganisms. Plant lipases have not found commercial applications, but microbial and animal lipases have been used successfully in producing novel ingredients.[36] Bacteria of the genera *Pseudomonas, Achromobacter,* and *Staphylococcus,* as well as yeasts (i.e., *Candida* and *Torulopis*), and molds (i.e., *Rhizopus, Aspergillus,* and *Geotricum*) have been shown to produce lipases.

Glycerol ester hydrolases or lipases are enzymes that hydrolyze tri-, di-, and monoglycerides present at an oil-water interface. Although the reaction seldom goes to completion, hydrolysis of a triglyceride by lipase can yield monoglycerides and free fatty acids. Lipases have been used for the interesterification of triglycerides, but these reactions are commonly carried out using inorganic catalysts in the oil and fat industry. Compared with inorganic catalysts, lipases are more substrate specific, that is, they will catalyze the interesterification of only certain types of fatty acids in particular positions in the triglycerides.[8]

Vegetable and animal fats are commonly used as substrates for lipolysis. The ease with which fat or oil is hydrolyzed depends on the lipase itself. The substrate, however, has to be emulsified because a true lipase by definition acts at the oil-water interface. Glycerol esters are the preferred substrates.

Microbial lipases may be divided into two groups, depending on their positional specificity. There is a nonspecific group that releases fatty acids from all three positions of the glycerol moiety. The nonspecific lipases also cause complete breakdown of triglycerides to free fatty acids and glycerol. The second group is specific in that hydrolysis takes place at the Sn1 and Sn3 positions preferentially to give free fatty acids and the di- and monoglycerides as reaction products.[36] One good example of this is the system pioneered by Unilever Research in which one of the major lipids of palm oil, 1,3-dipalmitoyl-2-oleyl glycerol (POP), is transformed into more valuable compounds. One or both of the palmitic acid residues are substituted with stearic acid to form palmitoyl-2-oleyl-3-steroyl glycerol and steroyl-2-oleyl-3-steroyl glycerol, that is, POS and SOS, respectively.[32]

These lipids (POS and POP) are the main components of cocoa butter.[8] In this process the enzyme is immobilized and hydrated before use, and the substrate is a particular grade of palm oil, mixed with tristearin or stearic acid in an organic solvent. These reactions may be termed transesterification: a process used in the oil industry to modify the composition and hence the physical properties (crystallinity and melting characteristics) of triglyceride mixtures. In this process a chemical catalyst is used to promote acyl migration between glyceride molecules so that products consist of glyceride mixtures in which the fatty acyl groups are randomly distributed among the

glyceride molecules. The process can also be applied to mixtures of triglycerides and free fatty acid, resulting in the formation/production of interesterified triglycerides randomly enriched with the added fatty acid.[37]

Controlled lipolysis has been applied in the generation of short-chain fatty acids, which in turn are oxidized into carbonyl compounds such as methyl ketones, which are responsible for milk aroma. Numerous procedures describing the applications of various lipolytic enzymes have been patented, and a few examples of these patented procedures are summarized in Table 7. Different lipolytic enzymes yield differing amounts of products with the same substrate; for example, the addition of lamb gastric extracts combined with lamb pregastric esterase in the manufacture of Italian cheese leads to the development of the picante flavor of Romano and Provolone.[36] Inclusion of pregastric calf esterase and fungal esterase resulted in typical Fontina cheese flavor. In the U.S., effective August 13, 1974 enzyme-modified cheese became an acceptable optional ingredient in processed cheese, processed cheese food, or processed cheese spread.[36]

1. Production of β-Monoglycerides

Monoglycerides are used widely in the food industry as emulsifiers. They can be produced by the base-catalyzed hydrolysis of triglycerides at a high temperature. This chemical process, however, is not specific and a statistical distribution of various glycerides (mono-, di-, and triglycerides) and glycerol are obtained. Enzyme-derived monoglycerides are formed using a method of lipase-catalyzed alcoholysis of triglycerides. These

TABLE 7
Patents Pertaining to use of Lipolytic Enzymes in Food Ingredient Manufacture or Processing

Application	Country	Patent no.	Year of issue
Modification of fat in milk	U.S.	1,966,460	1930
	U.S.	3,469,933	1969
Use in Italian cheese	U.S.	2,531,329	1950
	U.S.	2,794,743	1957
Use in American cheese	U.K.	1,326,516	1971
	Canada	911,248	1971
	Canada	911,250	1971
	U.S.	3,650,768	1972
Enzyme-modified milk powder	U.S.	2,531,329	1950
	U.S.	2,794,743	1957
Lipolyzed milk	U.S.	2,638,418	1953
Lipolyzed milk fat "buttery" flavor	Japan	3,187/70	1970
	U.K.	1,251,272	1971
	Japan	72–45108	1972
	Canada	912,905	1971
	U.S.	3,469,993	1972
Lipolyzed milk fat "cultured cream" flavors	U.S.	3,469,993	1969
Lipolyzed milk fat "blue cheese" flavors	U.S.	2,965,492	1960
	U.S.	3,100,153	1963
	U.S.	3,072,488	1963
Lipolyzed milk fat "cheese-like" flavors	U.K.	1,326,516	1971
	U.S.	3,780,182	1973
Use in yogurt	Japan	3107/71	1971

From Kilara, A., *Process Biochem.*, 20(4), 35, 1985. With permission.

monoglycerides possess good emulsification characteristics due to the β position of the fatty acid, as opposed to the α position in the chemically derived compound.[38]

2. Fatty Acids and Health

Polyunsaturated fatty acids have been the focus of research activities for a number of years because of their potential health benefits. Vegetable oils such as coconut oil are made up mostly of medium-chain fatty acids. Corn and soybean oil contain mostly long-chain polyunsaturated fatty acids, while palm oil has about equal amounts of long-chain saturated and polyunsaturated fatty acids. Polyunsaturates in large amounts may increase cancer risk. Hydrogenation converts polyunsaturated fats to saturated fatty acids or isomeric trans forms. Both of these are believed to raise serum cholesterol levels and are thus undesirable with respect to adverse effect of high cholesterol levels to human health.[39]

Omega-3 fatty acids are esterified mainly at the C_2 position of glycerol in the triglycerides of fish oils. The health benefits of omega-3 fatty acids, eicosapentaenoic acid and docosahexaenoic acid in fish oil, have been the subject of active research in recent years. Enrichment for these acids can be carried out using a method of lipase-catalyzed alcoholysis.[38] For example, Menhaden oil (which contains about 25% omega-3 fatty acids), when hydrolyzed by lipase and followed by separation of the esters, cholesterol, and fractional crystallization, formed product with 60 to 70% omega-3 fatty acids.

H. Fruit and Juice Processing

Fruit and juice processors use enzymes for a variety of benefits, which include: increased juice yields, improved efficiency of juice filtration, improved juice stability for concentration, enhanced juice clarity, reduced juice bitterness, and increased rate of fruit dehydration.[1] Enzymes used most often in juice processing are pectinase, cellulase, hemicellulase, amylase, and arabinase. Naringinase and limonase have been used to hydrolyze naringin and limonin, the bitter compounds found in grapefruit juice. Biomacerase may be used in the production of stable purees from a wide variety of fruits and vegetables, such as carrots, tomatoes potatoes, celery, and pears. Biopectinase ML is specifically formulated to hydrolyze pectin, cellulose, and hemicellulose more extensively than traditional pectinase products.[40]

Aspergillus, Rhizopus, and *Trichoderma* are the chief fungi used as sources of enzymes to increase juice yield and reduce juice viscosity. Structural polysaccharides that interfere with juice extraction, filtration, clarification, and concentration are broken down by pectinase, cellulase, and hemicellulase. Macerating enzymes are also used to extract juice from citrus fruits and some tropical fruits (such as pineapple) that have a great deal of juice trapped in the pulp. One example is the treatment of papaya with two macerating enzymes. This achieves a 12 to 30% increase in juice yield.[1] It is now customary to treat apple mash with enzymes prior to pressing. This serves to get rid of a clouding agent called araban, which comes from the cell walls. Araban consists of a linear chain of α-1,5-arabinofuranosyl residues with α-1-3 branches. Debranching arabinosidases and endoarabinase are needed to break down araban and prevent precipitation.[41]

III. POTENTIALLY NEW OR INCREASED USES OF ENZYMES IN THE FOOD INDUSTRY

A. Inulinase

The importance of fructose because of its comparative sweetness vs. sucrose has stimulated a search for alternative sources of fructose. Various plants of the compositae family, especially the Jerusalem artichoke or topinambour, contain up to 16% inulin, the fructose polymer in which fructofuranose units are joined by a β-1,2 linkage as opposed to furosans, which have an α-2,6 glycosidic bond. Jerusalem artichokes can be grown as a high-yield crop. Inulinase, an enzyme produced industrially from *Aspergillus* species, can almost completely hydrolyze inulin (up to 99%)

at pH 4.5 and 60°C.[5] The enzyme has not been put into use because of political decisions that have prevented the financing for European farmers to grow these crops.[8]

B. Marine Food Quality Assessment

The application of enzyme methods to marine food analysis provides an objective, inexpensive, and rapid approach to seafood quality. If these methods are adapted to a visual basis, enzyme analysis could be carried out anywhere, dockside or aboard ships. Enzyme analysis of marine foods can be used for quality assessment at critical points where this type of determination has an impact for grading and purchasing.[42]

The compound hypoxanthine, a metabolic byproduct of ATP breakdown, is used as a measure of fish quality. Using the enzyme xanthine oxidase, which converts hypoxanthine to uric acid, followed by differential spectrophotometry in the UV region, fish freshness can be determined. The K value is used in the determination of fish freshness, where K is defined as:

$$K = \frac{[H_xR]+[H_x]}{[IMP]+[H_xR]+[H_x]}$$

where H_xR = inosine, H_x = hypoxanthine, IMP = inosine monophosphate, all degradation products of ATP. If K = 1, then [IMP] = 0, thus denoting poor-quality fish. A K value of 0.2 or lower denotes high-quality fish.

Rapid visual enzyme methods were designed for convenience aboard ship, dockside, or in a food-processing plant. A strip of absorbent paper is soaked in resazurin, xanthine oxidase, and a buffer. When the enzyme test strip is moistened with flounder extracts and allowed to react for 5 min at room temperature, the color changes from blue to pink, and the intensity is correlated with a laboratory colorimeter. Diamines developed after the hypoxanthine peak can also be used to determine the onset of spoilage (Figure 4).[42]

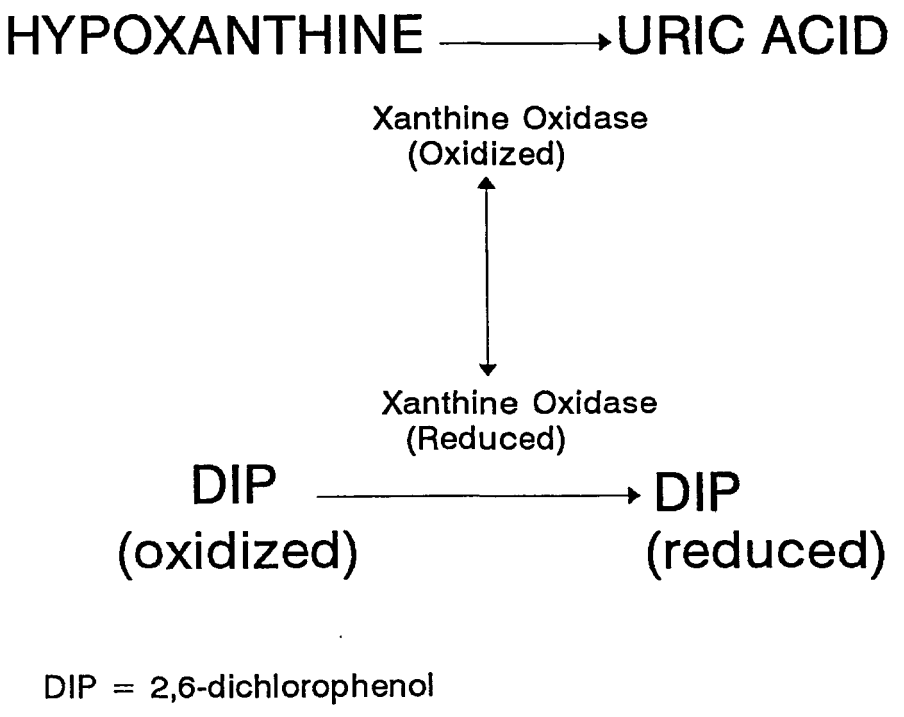

FIGURE 4. The reduction of DIP by xanthine oxidase in the presence of hypoxanthine. (Adapted from Jahns, F. D. and Rand, A. G., in Ory, R. L. and St. Angelo, A. J., Eds., *Enzymes in Food and Beverage Processing,* American Chemical Society, Washington, D.C., 1978, 266.)

C. Baking Industry

Some of the functions of enzymes in baked products include: flour quality improvement, retardation of staling, dough improvement, and more efficiently machinability. Enzymes are also used to enhance bread crust color, bleach flour, improve the quality of high fiber baked products, and reduce the phytate content in whole-grain formulations. Fungal and bacterial enzymes available for use in bakery processing include α- and β-amylases, proteases, amyloglucosidases, pentosanase, glucanase, and phytase. The most important of these are amylases and proteases.[1]

Bakers have added a surfactant to bread to retard staling. These compounds reduce the tendency for retrogradation by complexing with both the amylose and amylopectin fractions of starch. A novel approach is the addition of α-amylase directly to flour for the conversion of starch to maltose for yeast fermentation and for retarding staling. Addition of α-amylase is believed to cleave a few bonds in the soluble region of the starch, leaving the insoluble regions separated by regions that can bend. Another theory in the retardation of staling is that the enzyme is thought to shorten the chain length of amylopectin from 19 to 21 units to 12 to 15 units, which reduces its tendency to retrograde.[1]

Enzymes for the baking and milling industries may be used to control dough properties and improve the quality of the finished product. The enzymes Milenzyme® fungal protease, Clarase®, HT-proteolytic, bromelain, and Tenase® act on the raw material to enhance leavening action and modify the protein structure, thus contributing to flavor development. Milenzyme fungal protease, derived from *Aspergillus oryzae,* is used in making yeast-raised breads. It hydrolyzes the interior bonds of gluten and modifies the extensibility properties of dough.[40]

Doughs containing high levels of the wheat protein gluten show improved machinability and better baked quality when formulated with proteases. Sodium bisulfite, a reducing agent, had been used to weaken gluten by hydrolyzing the disulfide linkages in the protein. However, this compound was found to destroy vitamin B (thiamine). Instead of chemical agents, bacterial protease, an endoenzyme that weakens the gluten without affecting the nutritional quality of the flour, is now used. The enzyme acts on the inner peptide bonds of gluten and results in reduced elasticity and improved extensibility of the dough.[40] HT-proteolytic is a protease derived from *Bacillus subtilis* that is useful for conditioning high-protein flours in crackers, cookies, pizza crust, and other specialty baked products. It softens the dough for improved extensibility with reduced elasticity. The enzyme is effective at pH range of 5.0 to 9.5 and up to 60°C.

Fungal proteases are added to bread formulations to improve loaf symmetry and uniformity, to improve grain and texture, and to provide a softer crumb. Fungal proteases contain both exo- and endoenzyme activities. Amino acids liberated by the exoenzyme activity during hydrolysis of gluten react with sugars in the Maillard reaction. This improves flavor and crust color, contributing to yeast growth and ability to form gas.[1] Pentosanase incorporated into bread formulations breaks down the pentosans, yielding easier-to-handle doughs and finished products with a larger loaf volume.

D. Fat Degradation

Fat concentrations in excess of 0.025% have a detrimental effect on the whipping properties of egg white, which normally contains about 0.04% fat. Fat in egg white may be eliminated using lipase MAP 10, which is derived from *Mucor* and recommended for use at the rate of 1 kg/45,000 lb. Compared with pancreatic lipase, which is currently being used by many egg processors, MAP 10 has Kosher status and does not contain protease, which affects the egg flavor.[40]

E. Bacterial Detection and Elimination

Enzymes can now be used for the detection of bacterial contamination in dairy, meats, fish, poultry, and other food products. This is accomplished by an instrument called the Catalasemeter. It measures, within minutes, the amount of catalase enzyme in a sample. This relative catalase value

can be correlated with a specific level of bacteria or abnormality in a culture.[40]

1. Antimicrobial Enzymes

Enzymes have been demonstrated to play an important role in extending the shelf life of foods by preventing the growth of spoilage organisms. This can be achieved by several methods:[43] (1) functioning to deprive the problem organism(s) of a necessary nutrient; (2) production of reaction products harmless to humans or animals, but bacteriostatic or bactericidal in nature; (3) destruction of an outer membrane, cell wall, or cell membrane component, causing a change in permeability or a physical disruption of the cell wall, resulting in death; or (4) inactivation of an essential enzyme by a "killer enzyme".

An example of an enzyme that deprives an organism of a necessary nutrient is glucose oxidase-catalase, which, in the presence of excess glucose, depletes oxygen, preventing the growth of obligate aerobes. Oxidases that generate H_2O_2 in a system that does not contain catalase or peroxidases are lethal, because of the residual H_2O_2 present and the depletion of oxygen. Xanthine oxidase, found in milk, may also generate toxic H_2O_2 in the absence of catalase. Xylitol is being used in toothpaste as an anticariogenic ingredient. Xylitol is converted to xylitol-5-phosphate by the enzyme xylitol phosphorylase. This phosphate cannot be further metabolized by *Streptococcus mutans,* a cariogenic organism, thus killing the organism.[43]

Enzymes that attack cell walls, causing opening up and wall permeability, can cause cell death. Examples include proteases, lysozyme, chitinases, peroxidase, various hydrolases, phenolases, and phosphatases. Crude trypsin, combined with lysozyme and lysolecithin, is reported to lyse *E. coli* and *Shigella flexner*.[39]

Cell wall degradation using bacteriolytic enzymes is a novel method of coping with bacterial contamination in food. Bacterial cell wall has a sugar backbone consisting of *N*-acetyl-muramic acid and *N*-acetyl glucosamine linked together through β-1→4 bonds. The sugar backbone is cross-linked by peptides to form a peptidoglycan layer. Some bacteriolytic enzymes hydrolyze the β-1→4 bond, while some hydrolyze the peptide bonds. A few new bacteriolytic enzymes have been developed as replacements for lysozyme found in egg white (*N*-acetyl-muramidase). The effectiveness of three bacteriolytic enzymes compared to that of lysozyme is presented in Table 8. Bacteriolytic enzyme SP417 produced by *Nocardiopsis dassonvillei* lyses Gram-positive as well as Gram-negative bacteria, including *Staphylococcus aureus* and *Pseudomonas aeruginosa.* Enzyme SP417 produced by *Bacillus pabuli* differs from SP416 in that it lyses Gram-negative organisms, including *Escherichia coli, Vibrio parahaemolyticus,* and *Salmonella arizona.*[44]

Bacteriolytic enzyme SP455 produced by strain *N. dassonvillei* is active on only a few bacteria, in particular, *P. aeruginosa.* Bacteriolytic enzymes may be used in the food industry to extend shelf life of unprocessed or little-processed foods. They may be used to control the process of fermented food production, such as cheese or salami, and act as a processing aid in releasing intracellular products from bacteria. Disinfection of equipment, packaging material, and process water can all implement the action of bacteriolytic enzymes.[44]

"Killer enzymes" or "antienzyme enzymes" fall into two main groups: proteases and sulfhydryl oxidases for -SH enzymes. Other important enzymes include those able to inactivate cytosolic aspartate aminotransferase. Mutastein, a protein produced by *Aspergillus terreus,* has been shown to be an effective inhibitor of cyclodextrin glucosyltransferase and other α-glucosyl-transferases and to prevent dextran by *Streptococcus mutans.*[43]

F. Proteases

Acid proteases are available commercially to perform many, varied functions, such as degradation of haze-forming proteins in fruit juices, hydrolysis of gelatin, preparation of casein hydrolysates, and the digestion of soya protein.

There has been an upsurge of interest in proteases from aquatic species because of their special properties. Proteases used in industry have

TABLE 8
Comparison of Bacteriolytic Enzymes

Microorganism	Activity[a]			
	SP416	SP417	SP455	Lysozyme
Gram-positive				
Bacillus cereus	0	0	0	0
Bacillus subtilis	++	+	+	+
Corynebacterium liquefaciens	++	+	+	+
Lactobacillus plantarum	++	0	0	0
Listeria inocua	0	0	0	0
Micrococcus kristinae	+	0	0	0
Micrococcus luteus	++	++	++	+++
Micrococcus sedentarius	++	0	+	0
Staphylococcus aureus	++	0	+	0
Streptococcus lactis	++	0	nd	nd
Streptococcus faecalis	0	0	0	0
Streptococcus faecium	++	0	0	0
Gram-negative				
Campylobacter fetus	++	0	nd	nd
Enterobacter aerogenes	0	0	0	0
Escherichia coli	++	+++	+	0
Pseudomonas aeruginosa	+++	+++	+++	0
Salmonella arizona	++	++	0	0
Serratia marcescens	0	+	0	0
Vibrio parahaemolyticus	++	++	++	0

[a] SP416 was dosed at 160 mg/l, SP417 at 8 mg/l, SP455 at 1600 mg/l, and pure lysozyme at 1000 mg/l. The reactions were run at pH 7 for 20 min at 30°C. Enzyme activity (lysis) was measured as decrease in turbidity of the bacterial suspension at 660 nm. 0 indicates less than 10% lysis; +, 11 to 25% lysis; ++, 26 to 50% lysis; +++, more than 50% lysis; and nd, not determined.

From Neilsen, H. K., *Food Technol.*, 45(1), 102, 1991. With permission.

been mainly derived from plant, animal, and microbial sources. Marine proteases have been underutilized for the following reasons:[45] (1) relatively few studies have been carried out on marine proteases; there is thus a lack of information about their potential; (2) fluctuations exist in supply due to seasonal nature of source material; and (3) personnel are reluctant to work with fish offal because of the odor.

There is a large pool of diversified species adapted to a variety of habitat conditions in the marine environment. Genetic variations within species as well as adaptation to different environmental conditions have resulted in differences in the properties of proteases. These unique properties can be applied in the food industry in many areas. For example, trypsin has been isolated from many species, including Greenland cod, Atlantic cod, and cunner fish. Compared with bovine trypsin, the turnover number of fish trypsins is one order of magnitude higher for both amidase and esterase reactions. Fish chymotrypsins were also found to have a higher rate of reaction and to hydrolyze more peptide bonds in various protein substrates than mammalian trypsin from beef pancreas.[45]

Pepsins from cold-water fish exhibit a low Arrhenius activation energy, low-temperature optimum, and low thermal stability compared with warm-habitat and warm-blooded animals. Fish pepsins are also resistant to autolysis at low pH. These pepsins are inactivated at relatively mild heat treatment, a useful property in cheese manufacture after the milk clotting reaction is complete.[45]

Fish proteases have traditionally been applied in the seafood industry to reduce viscosity of stickwater in the fish meal industry. They are

used for preparation of fish protein hydrolysates for food or animal feed and fermentation processes, depending on endogenous proteases. Recent applications include the removal of skin and scales, the hydrolysis of membranes and other supportive tissue that surrounds roe, roe sacks, and other tissues, and recovery of pigment and flavor extracts.[45]

G. Artificial Sweetener: Aspartame

Aspartame is a dipeptide of the methyl ester L-aspartic acid linked to L-phenylalanine. Aspartame is marketed under the brandname Nutrasweet (Nutrasweet, Inc., Mount Prospect, IL). Aspartame may be used in the body similarly to any other protein, but its caloric value is insignificant. Individuals suffering from the condition phenylketonuria (absence of the enzyme phenylalanine 4-monooxygenase) should avoid aspartame and products that include it as an ingredient.[46]

The key step in aspartame production is the formation of the peptide bond between the α-carboxyl group of L-aspartic acid and the amino group of L-PheOMe. Aspartame can be chemically produced by the reaction of carbobenzoxy-L-aspartyl anhydride with L-PheOMe, with the reaction taking place at the α-carbonyl (Figure 5). There is a 7% byproduct from the reaction with the β-carbonyl. Phenylserine is used as the building block in the enzymatic phenylalanine-sparing route to aspartame production. Enzymatic synthesis of aspartame starting with phenylserine methyl ester is outlined in Gross.[38] The metalloprotease used is thermolysin, which catalyzes the dipeptide bond formation in organic solvent such as ethyl acetate with a 93% dipeptide yield.[38] Then α-aspartame is obtained in a single step by catalytic hydrogenolysis with a yield of 85%.

Enzymatic synthesis of aspartame has the advantage of regiospecificity where no protection of the β-carbonyl of α-L-aspartic acid is necessary. An inexpensive racemic mixture of isomers of phenylserine methyl ester can be used as starting material because the reaction is highly stereospecific. Only the α-erythro isomer of phenylserine methyl ester reacts with aspartic acid (Figure 5).[38] In the chemical synthesis of aspartame the reaction is first complicated by blocking of the amino group of carbobenzoxy-L-aspartyl anhydride. Reaction of this anhydride with L-PheOMe is not specific because the amino group of L-PheOMe can react with either the α- or β-carbonyl group of the anhydride to form two stereoisomers. It is thus necessary to have a final purification step to remove the β-aspartame (~7%) present, thus giving low yields and increasing production costs.

IV. OTHER POTENTIAL ENZYME APPLICATIONS

A. Food Quality

Enzymes are useful in determining several of the quality indices of foods. Some of these are listed in Table 9. Substrate kits and simple assay techniques need to be developed for routine application of these indices in the quality control of foods. In order to change the solubility and functional properties of proteins, enzymes can be used specifically to modify the side-chain amino acid residues in proteins or to hydrolyze peptide bonds. Proteolytic enzymes can form bitter peptides, which is one problem experienced in the cheese industry. Enzymes may be used in the removal of toxic and antinutritive components. Plants such as cassava, lima beans, sorghum, linseed, and kernels of almonds have high levels of cyanogenic glycosides. HCN is liberated from the cyanogenic glycosides by the action of the thioglycosidase, this compound being able to react with cytochrome oxidase, a key respiratory enzyme. Proper cooking would distill the HCN; however, more research is needed on the use of thioglycosidases in reducing toxicity.

Oligosaccharides (e.g., raffinose, stachyose, and verbascose) found in beans cannot be hydrolyzed in the human small intestines because of the absence of α-galactosidase. Thus, the use of beans in human diet is limited by the development of flatulence caused by the presence of these undigested oligosaccharides. There is a need to develop α-galactosidase from microbial sources to aid in digestion of bean oligosaccharides, perhaps by pretreatment of the beans.[47]

Nucleic acid content in single-cell protein continues to be a major deterrent in its use for

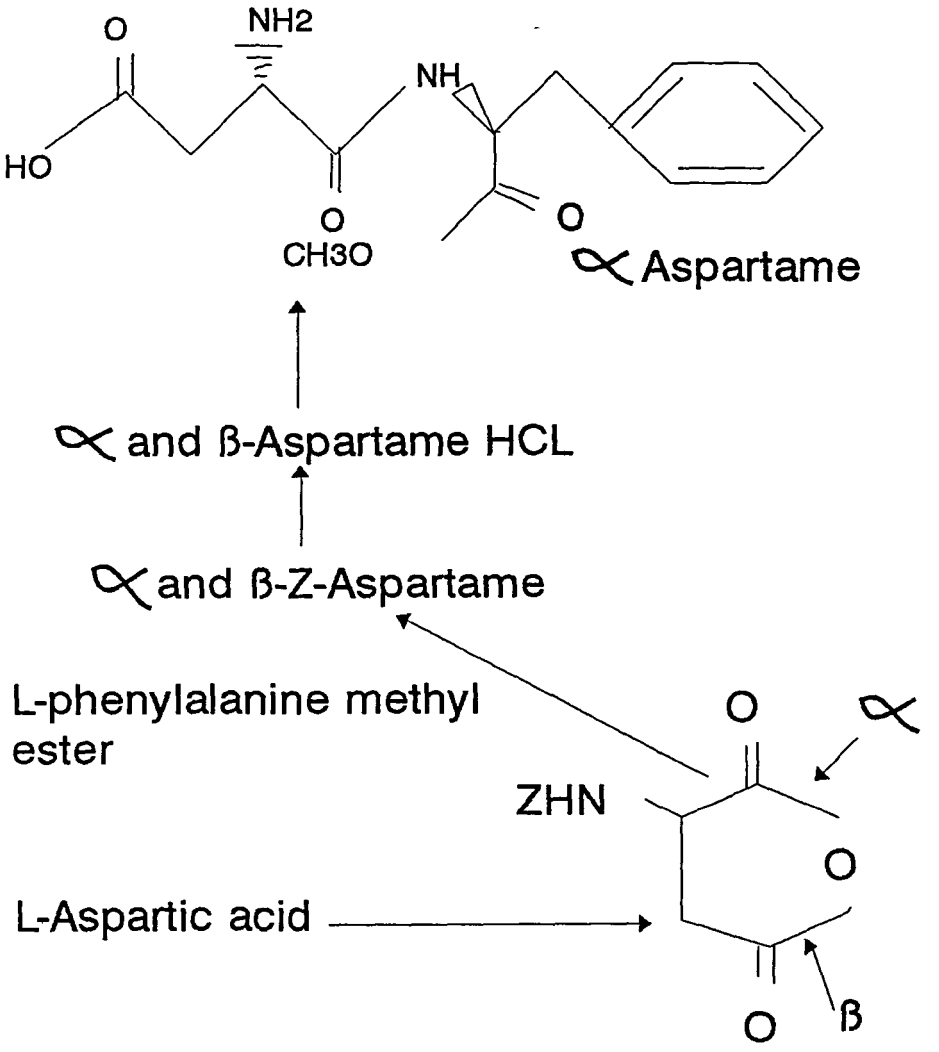

FIGURE 5. Chemical synthesis of aspartame. Z = CO_2CH_2Ph. (Adapted from Gross, A., *Food Technol.*, 45(1), 96, 1991.)

human consumption. The nucleic acid is known to crystallize out as uric acid in the joints causing the condition gout. Nucleases need to be developed to find a solution to this problem.

B. Protein Supplementation

Apart from removal of toxic substances and improvement of functional properties, nutritional quality can be improved by supplementation with limiting amino acids. Japan has been one of the world leaders in amino acid production since 1969. Systems have been developed using aminoacylase immobilized to DEAE-cellulose on a commercial scale to resolve D- and L-mixtures of methionine, valine, tryptophan, and phenylalanine.[46,47]

The protein in the human body consists of L-amino acids that are continuously taken up with the food. Pure L-amino acids infusion solutions are used in artificial feeding to supply the necessary protein structural elements. These are obtained in special bioreactors with the aid of enzymes. By a method of enzyme immobilization, the catalyst can be retained in a reaction vessel with continuous flow through. The adult human

TABLE 9
Indices of Food Quality

Index	Significant enzyme	Food product
Proper heat treatment	Peroxidase	Fruits and vegetables
	Alkaline phosphatase	Milk, dairy products, ham
	β-Acetylglucosaminidase	Eggs
Freezing and thawing	Malic enzyme	Oysters
	Glutamate oxaloacetate transaminase	Meat
Bacterial contamination	Acid phosphatase	Meat, eggs
	Catalase	Beans
Maturity	Sucrose synthetase	Potatoes
	Pectinase	Pears
Freshness	Lysolecithinase	Fish
	Xanthine oxidase	Hypoxanthine content of fish
Sprouting	Amylase	Flour
	Peroxidase	Wheat
Color	Polyphenol oxidase	Coffee, wheat
	Succinic dehydrogenase	Meat

Adapted from Whitaker, J. R., *Enzymes. The Interface between Technology and Economics,* Danehy, J. P. and Wolnak, B., Eds., Marcel Dekker, New York, 1980, 53.

requires about 60 g of protein per day. L-Amino acids are used as food supplements and in infusion solutions on a large scale.[48] Stereoselectivity is one of the important characteristics of enzymes. They catalyze reactions with only one enantiomeric and diastereomeric pair of chiral compounds, although some enzymes such as racemases are exceptional in that respect. Enzymatic processes have been developed for optically active amino acids that are important as nutrients and starting materials in the pharmaceutical industry.

C. Oxidoreductases

Oxidoreductases are being employed as catalysts in food systems to convert cholesterol to the nontoxic coprostanol. If this process is fully developed, it will serve to replace the current approach of cholesterol extraction by steam distillation and supercritical fluid extraction.

D. Invertase

Several enzymes have found application in the food industry, although on a small scale. One such example is invertase. Invertase is used for making candies with a soft center. The enzyme is added to sucrose candy that is covered with chocolate. With time, the sucrose is partially hydrolyzed to glucose and fructose. This makes the candy soft.[8]

V. CONCLUSIONS

Change in availability of energy and protection of the environment may make some enzymatic processes more attractive. Active research is being carried out to make improvements in dairy products. The following are being developed: *Streptococcus lacti* with significantly improved resistance to bacteriophage; lactic acid

bacteria with improved ability to produce lactic acid, especially under adverse conditions of temperature and salt level; single-strain starters for yogurt, Italian cheese, and buttermilk, unique yogurt cultures that produce biopolymers for texture and as sweeteners; fermentations based on source of microbial calf rennet; and industrially important recombinant DNA products from the lactic acid bacteria.[40]

Recombinant DNA techniques have augmented the potential for new developments in the food enzymology area. Some specific examples cited point out these developments:[40] (1) thermostable bacterial α-amylase suited for maltose syrup production is being produced by Novo; (2) recombinant chymosin is being investigated, among others, by Genencor, Genex, Dairyland Food Laboratories, and companies in Japan; (3) accelerated cheese ripening systems are being perfected by Miles under the commercial name Natur Age; (4) heat lability of microbial rennets is being improved to approach the heat lability of calf chymosin; (5) glucoamylase expressed by recombinant DNA is being studied by CPC International; (6) Cetus Corporation is trying to clone glucoamylo-cellulase and oxidase to better breakdown cellulose and glucose.

The aim now is to produce enzymes that offer technological advantages over currently available enzymes, but do not appreciably alter the sensory attributes of the products. Processes using the new generation of enzymes will realize cost savings due to the engineered technologically desirable properties. At this stage there is a general understanding of how enzymes work at the molecular level. It is hoped that DNA technology will lead to the development of enzymes that can perform what is now considered "impossible", for example, manufacture of Cheddar cheese from soyprotein and production of beer from any carbohydrate source within 1 or 2 days.

REFERENCES

1. **Dziezak, J. D., Ed.,** Enzymes: catalysts for food processes, *Food Technol.,* 46(1), 78, 1991.
2. **Simpson, B. K. and Haard, N. F.,** Cold-adapted enzymes from fish, in *Food Biotechnology,* Knorr, D., Ed., Marcell Dekker, New York, 1987, 495.
3. **Kula, M. R.,** Trends in enzyme technology, in *Biotec 1. Microbial Genetic Engineering and Enzyme Technology,* Hollenberg, C. P. and Sahm, H., Eds., Gustav Fisher, New York, 1987, 77.
4. **Neidleman, S. L.,** Enzymes in the food industry, *Food Technol.,* 45(1), 88, 1991.
5. **Demain, A. L.,** Industrial microbiology, *Science,* 214, 987, 1981.
6. **Litchfield, J. H.,** Single cell proteins, *Science,* 219, 740, 1983.
7. **Wasserman, B. P., Montville, T. J., Korwek, E. L., and Hogan, J. D.,** Food Biotechnology, *Food Technol.,* 42(1), 133, 1988.
8. **Alder-Nissen, J.,** Newer uses of microbial enzymes in food processing, *Tib-tech,* 5(6), 170, 1987.
9. **Zubay, G.,** *Biochemistry,* Addison-Wesley, Reading, MA, 1983.
10. **Fogarty, W. M.,** Microbial amylases, in *Microbial Enzymes and Biotechnology,* Fogarty, W. M., Ed., Applied Science Publishers, New York, 1983, 1.
11. **Godfrey, T. and Reichelt, J.,** *Industrial Enzymology — The Application of Enzymes in Industry,* The Nature Press, London, 1983, 1.
12. **Lee, B. H.,** *Fundamentals of Food Biotechnology,* VCH Publishers, New York, 1996.
13. **Sambrook, J., Fritsh, E. F., and Maniatis, T.,** *Molecular Cloning. A Laboratory Manual,* 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring, NY, 1989, 14.5.
14. **Penet, C. S.,** New applications of industrial food enzymology; economics and processes, *Food Technol.,* 45(10), 98, 1991.
15. **Peberdy, J. F.,** Genetic engineering in relation to enzymes, in *Biotechnology,* Rehm, H.-J., Reed, G., and Kennedy, J. F., Eds., VCH Publishers, Weinheim, Germany, 1987, 326.
16. **Lin, Y. L.,** Genetic engineering and process development for production of food processing enzymes and additives, *Food Technol.,* 41(10), 104, 1987.
17. **Olson, A. C. and Korus, R. A.,** Immobilized enzymes, in *Enzymes in Food and Beverage Processing,* Ory, R. L. and St. Angelo, A. J., Eds., American Chemical Society, Washington, D.C., 1976, 100.
18. **Pitcher, W. H.,** *Immobilized Enzymes for Food Processing,* CRC Press, Boca Raton, FL, 1980.
19. **Rouxhet, P. G.,** New ways of enzyme utilization — immobilization of biocatalysts, *Brewers Digest,* 9, 40, 1986.
20. **Ward, O. P.,** Proteinases, in *Microbial Enzymes and Biotechnology,* Fogarty, W. M., Ed., Applied Science Publishers, New York, 1983, 251.
21. **Shaker, K. A. and Brown, R. J.,** Effects of enzyme choice and fractionation of commercial enzyme preparations and protein recovery in curd, *J. Dairy Sci.,* 68, 1074, 1985.
22. **Fox, P. F.,** Acceleration of cheese ripening, in *Food Biotechnology,* Marcel Dekker, New York, 1988, 133.

23. **Holsinger, V. H. and Kligerman, A. E.,** Applications of lactase in dairy foods and other foods containing lactose, *Food Technol.*, 45(1), 92, 1991.
24. **Woychik, J. H. and Holsinge, V. H.,** Use of lactase in the manufacture of dairy products, in *Enzymes in Food and Beverage Processing,* Ory, R. L. and St. Angelo, A. J., Eds., American Chemical Society, Washington, D.C., 1976, 67.
25. **Barach, J. T.,** What's new in genetic engineering of dairy starter cultures and dairy enzymes, *Food Technol.*, 39(10), 73, 1985.
26. **Eriksen, S.,** Application of enzymes in soy milk production to improve yield, *J. Food Sci.*, 48, 445, 1983.
27. **Olsen, H. S. and Alder-Nissen, J.,** Industrial production and applications of a soluble enzymatic hydrolysate of soya protein, *Process Biochem.*, 14(7), 6, 1979.
28. **Rackis, J. J.,** Enzymes in soybean processing and quality control, in *Enzymes in Food and Beverage Processing,* Ory, R. L. and St. Angelo, A. J., Eds., American Chemical Society, Washington, D.C., 1978, 244.
29. **MacAllister, R. V.,** Manufacture of high fructose corn syrup, using immobilized glucose isomerase, in *Immobilized Enzymes for Food Processing,* Pitcher, W. H., Ed., CRC Press, Boca Raton, FL, 1980, 81.
30. **Venkatasubramanian, K.,** High fructose corn syrup. Plant design and process economics, in *Enzymes. The Interface between Technology and Economics,* Danehy, J. P. and Wolnak, B., Eds., Marcel Dekker, New York, 1980, 35.
31. **Finn, R. K.,** Conversion of starch to liquid sugar and ethanol, in *Biotec 1. Microbial Genetic Engineering and Enzyme Technology,* Hollenberg, C. P. and Sahm, H., Eds., Gustav Fisher, New York, 1987, 101.
32. **Bucke, C.,** Industrial glucose isomerase, in *Topics in Enzyme and Fermentation Biotechnoogy,* Wiseman, A., Ed., John Wiley & Sons, New York, 1977, 147.
33. **Maeda, H. and Tsao, G. T.,** Maltose production, *Process Biochem.*, 14(7), 2, 1979.
34. **Godtfredsen, S.E., Rasmussen, A. M., Ottesen, M., Rafn, P., and Peitersen, N.,** Occurrence of α-acetolactate decarboxylases among lactic acid bacteria and their utilization for maturation of beer, *Appl. Microb. Biotechnol.*, 20, 23, 1984.
35. **Finley, J. W., Stanley, W. L., and Walters, G. G.,** Chill proofing beer with papain immobilized on chitin, *Process Biochem.*, 14(7), 12, 1979.
36. **Kilara, A.,** Enzyme-modified lipid food ingredients, *Process Biochem.*, 20(4), 35, 1985.
37. **Macrae, A. R.,** Extracellular microbial lipases, in *Microbial Enzymes and Biotechnology,* Fogarty, W. M., Ed., Applied Science Publishers, New York, 1983, 225.
38. **Gross, A.,** Enzymatic catalysis in the production of novel food ingredients, *Food Technol.*, 45(1), 96, 1991.
39. **U.S. Council for Coconut Research/Information (U.S.C.C.R.I.)** Now the truth about coconut oil, N.W., Washington, D.C., 1992.
40. **Anon.,** Enzymes—fermentation, *Food Technol.*, 39(10), 80, 1985.
41. **Uhlig, H.,** Food enzymes, in *Biotec 1, Microbial Genetic Engineering and Enzyme Technology,* Hollenberg, C. P. and Sahm, H., Eds., Gustav Fisher, New York, 1987, 109.
42. **Jahns, F. D. and Rand, A. G.,** Enzyme methods to assess marine food quality, in *Enzymes in Food and Beverage Processing,* Ory, R. L. and St. Angelo, A. J., Eds., American Chemical Society, Washington, D.C., 1978, 266.
43. **Scott, D.,** Antimicrobial enzymes, in *Food Biotechnology,* Knorr, D., Ed., Marcel Dekker, New York, 1988, 199.
44. **Neilsen, H. K.,** Novel bacteriolytic enzymes and cyclodextrin glycosyl transferase for the food industry, *Food Technol.*, 45(1), 102, 1991.
45. **Haard, N. F. and Simpson, B. K.,** Proteases from aquatic organisms and their uses in the seafood industry, in *Fish Processing — Biotechnological Applications,* Martin, A., Ed., Elsevier, New York, 1993.
46. **Giese, J. H.,** Alternative sweetness and bulking agents, *Food Technology,* 47(1), 114, 1993.
47. **Whitaker, J. R.,** Some present and future uses of enzymes in the food industry, in *Enzymes. The Interface Between Technology and Economics,* Danehy, J. P. and Wolnak, B., Eds., Marcel Dekker, New York, 1980, 53.
48. **Soda, K. and Tanaka, H.,** Enzymatic synthesis of optically active amino acids from racemic starting materials, in *Biotec 1, Microbial Genetic Engineering and Enzyme Technology,* Hollenberg, C. P. and Sahm, H., Eds., Gustav Fisher, New York, 1987, 93.