Enzymes, Plant and Fungal

Processing

Identification

Chemical Names:
There are many different plant and fungal enzymes used in processing. Among the chemical names for pectinase are poly(1,4-α-D-galacturonide)glycanohydrolase, poly(1,4-α-D-galacturonide)lyase, and pectin pectylhydrolase.

Other Names:
The model enzyme is pectinase. Among the other names for pectinase are pectin lyase, pectin methylesterase, pectinesterase,and polygalacturonase. See the attached table of other enzymes commonly used in food processing.

CAS Numbers:
Pectinase: 9032-75-1

Other Codes:
Enzyme Commission numbers for the major components of pectinase: Pectin methylesterase--3.1.1.11; Pectin lyase: 4.2.2.10; polygalacturonase: 3.2.1.15

Recommendation

Synthetic / Non-Synthetic:	National List:	Suggested Annotation:
Non-synthetic (consensus)	*Allowed 95%+ Allowed 50%+ (consensus)*	*Enzymes derived from edible, non-toxic plants or non-pathogenic fungi that are not genetically engineered as defined by the NOSB may be used in processed foods labeled as "Organic." Incidental ingredients used in the production of enzyme preparations must be non-synthetic as defined by OFPA and the NOSB, or be substances that appear on the National List of ingredients allowed for use in foods labeled as "Organic." This includes water and substances that are insoluble in food but removed from the foods after processing. (2-1-1; see reviewer 1 for discussion)*

Characterization

Composition:
Enzymes are proteins composed of up to 20 amino acids (Nielsen et al., 1991). The active components of enzymes consist of the biologically active proteins. These proteins have highly complex structures and may be conjugated with metals, carbohydrates and / or lipids. The model enzyme for this review, pectinase, actually refers to a combination of at least six different enzymes (Wingard, Katchalski-Katzin, and Goldstein, 1979). The principle enzymes in pectin are pectin methylesterase, pectin lyase, and polygalacturonase (Food Chemicals Codex, 1981). Pectinase is marketed in powder or liquid form (White and White, 1997).

Properties:
Enzyme preparations may consist of whole cells, parts of cells, or cell-free extracts from the source used. Active components have known molecular weights that range from 12,000 to several hundred thousand (Food Chemicals Codex, 1981). Enzymes may be in liquid, semi-liquid, or dry form. Enzymes in general and pectinase in particular is readily soluble in water. Enzymes are practically insoluble in alcohol, in chloroform, and in ether. The liquids are generally in aqueous solution, having many of the same properties of water, with the liquid form boiling point slightly above 100° C (212° F). Dry preparations are off-white to tan amorphous, finely divided powders. Liquids usually range in color from tan to dark-brown.

Individual preparations are generally characterized by functionality and activity rather than the properties of the product. The color of preparations may vary from virtually colorless to dark brown (National Academy of Sciences, 1981). For example, pectinase hydrolyzes the pectin molecule (Reed, 1975).

How Made:

cessing and scale-up appears to be comparatively easy. Commercially available machinery can be employed to accomplish separation of the phases, and much knowledge in chemical engineering can be applied to the description and development of such processes. It is hoped that in this way intracellular enzymes can be made available in the future in large quantities and at a lower cost.

ACKNOWLEDGMENTS

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Enzymes are produced by cellular anabolism, the naturally occurring biological process of making more complex molecules from simpler ones. Source organisms for food processing include bacteria, fungi, higher plants, and animals (White and White, 1997). Enzymes may be extracted from a given source organism by a number of different methods (Nielsen, et al., 1991). Most of the organisms that produce commercial enzymes are considered fungi of some sort. These organisms include the molds *Aspergillus Niger, Rhizopus oryzae, Rhizomucor meihei*, blights such as *Endothia parasitica* and yeasts such as *Candida* spp and *Saccharomyces* spp. A considerable amount of research has been conducted on genetically modifying fungi and other organisms to increase the yields and consistencies of enzymes. Many of the prospective donor organisms are pathogenic and are being screened for genetic sequences to be inserted into non-pathogenic hosts (see, for example, Surgey, Robert-Budouy, and Condemine, 1996). Continuous improvement of production methods is possible without the use of recombinant DNA techniques. For example, classical methods of hybridization can also be used to improve enzyme-producing organisms (see Solis, Flores, and Huitron, 1997).

The model enzyme used for this TAP review, pectinase, is generally produced by a fungal source organism. Enzymes derived from higher plants are discussed more fully in the review of enzymes used for livestock production. Animal derived enzymes are not considered for the purpose of this review. The NOSB has previously considered bacterial enzymes for processing of food for human consumption (NOSB, 1995).

Until recently, all enzymes produced and used for food were from these naturally-sourced biological products. Pectinase and other enzymes can be produced by a wide number of methods. One source of commercial pectinase is the mold *Aspergillus niger* grown by controlled fermentation (Aunstrup, 1979). The substrate often contains various grains and synthetic nutrients.

Isolation of the enzymes from their intracellular sources generally begins with separation from the media, usually by physical means such as centrifuging and sorting by specific gravity. The cell walls of the organisms are then burst through a mechanical process of homogenization, similar to that used on milk. Extracellular production--where the fermentation organism excretes the enzymes in a form that can be safely isolated--does not necessarily involve breaking the cell walls of an organism to recover the enzyme. However, techniques such as ion-exchange may be used to remove impurities in extracellular production (Lilly, 1979).

Further extraction, purification, and standardization from this point generally involves use of synthetic substances. Because extraction is pH dependent, the pH may be adjusted through the use of various strong acids, such as sulfuric acid, and bases such as sodium hydroxide. Other chemical extractants may be organic solvents, such as acetone; polymers, such as methylcellulose or polyvinyl alcohol; glycol ethers such as polyethylene glycol (PEG); or salts, such as sodium phosphate (White and White, 1997). Organic solvent extraction has been declining for a number of years (Pariza and Foster, 1983; a continuing trend confirmed by reviewers). Specific enzymes are then precipitated or absorbed by the use of a variety of chemical constituents and / or ion exchange columns. Final purification removes the extractants by further centrifugation, adsorption to a suitable adsorbent, and subsequent elution (Albertsson, 1971; Kula, 1979).

The isolated material is molecularly and functionally the same as produced by the functioning cell, thus non-synthetic. Enzymes that are molecularly the same, but not functionally the same are called 'denatured.' Recent technological advances in genetic engineering have made it possible to alter cellular genetic content, resulting in new production capabilities of the cell. The NOSB has considered other such alterations to be synthetic.

Specific Uses:
Enzymes have a wide variety of uses (ETA, 1999). Specific applications of pectin lyase is in juice clarification, extraction, wine clarification and production, cloud stabilization of citrus juices, extraction of citrus juices, and use in production of vegetable and fruit purees. In particular, pectinase is used primarily to depolymerize and esterify plant pectins in fruits such as apples, lemons, cranberries, oranges, cherries, grapes, and tomatoes, to name a few. The application of pectinase enables the entire fruit to be liquified. This has the effect of improving saccharification and thus sweetness, reducing waste and energy use per unit of juice produced, improving aroma and color; enhancing clarity, removing haze, preventing gel formation, and increasing fruit juice yield (Neilsen et al., 1991; White and White, 1995).

dye Cibacron Blue F3GA is known to interact with the nucleotide-binding site of dehydrogenases and kinases. Recent experiments show that this property can be exploited as well for affinity partition (Kula *et al.*, 1979). From the results presented in Fig. 15 it can be concluded that a spacer is needed between the water soluble polymer (monomethoxy-polyethylene glycol) and the affinity ligand to allow a specific binding of the ligand to the enzyme. This result is somehow surprising in view of the comparatively low molecular weight ($\overline{MW}$ 5000) of the polymer. It may be attributed to the rather rigid secondary structure of polyethylene glycol in solution (Rosch, 1971), which interferes with the need of proper orientation and immersion of the ligand toward the enzyme. Such considerations are known from affinity chromatography. In affinity chromatography, elution can be performed by increasing the concentration of the ligand in solution. Similarly formaldehyde dehydrogenase could be displaced by addition of NAD^+ to a system containing Cibacron Blue F3GA bound to polyethylene glycol as an affinity ligand in a two-phase system (Fig. 15).

As yet there are only a few examples for improvements in the selectivity of partition which lead to a greater separation power by liquid–

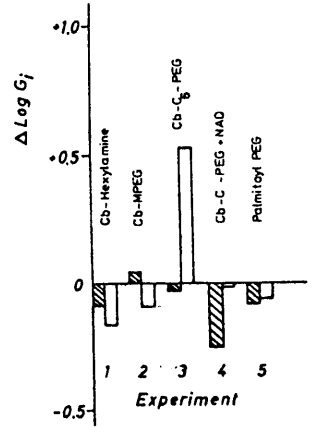

Fig. 15. Effect of affinity partition expressed as $\Delta \log G_i$, ▨ formate dehydrogenase ⊓ formaldehyde dehydrogenase $G = K(V_t/V_b)$ (partition ratio): (7% w/w dextran T 500, 5% w/w polyethylene glycol, 50 mmol/kg sodium acetate, pH 7.5, 5 mmol/kg potassium phosphate, 25% crude extract of *C. boidinii*, top phase 2.0 ml, bottom phase 1.5 ml, 10% of polyethylene glycol in the system replaced by modified polyethylene glycol as indicated). cb = Cibacron Blue F3GA, MPEG = monomethoxy-polyethylene glycol, cb-c_6-PEG = Cibacron Blue-hexamethylenediamine-polyethylene glycol. (From Kula *et al.*, 1979.)

liquid techniques. But the results promise that the concept developed here will eventually become true. Greater selectivity has to be paid for. Since it is necessary to synthesize modified polymers covalently linked to ion-exchange groups, hydrophobic group or biospecific ligand recovery processes have to be developed for the repeated usage of these expensive modified polymers to keep the proportional cost as low as possible. Much work still needs to be done to understand the different linked equilibria in such selective systems, as well as to meet technological and economic necessaries.

XII. REMOVAL OF POLYMERS

From earlier reports in the literature (Albertsson, 1971, 1977), one gains the impression that the removal of the polymers introduced into the enzyme solutions to form a phase system is an obstacle for the application of aqueous two-phase systems on a large scale. However, we found that diafiltration through ultrafiltration of membranes of the appropriate cutoff range is a fast and efficient way to remove the water-soluble polymers, especially polyethylene glycol (Hustedt *et al.*, 1978). As the molecular weight of the polyethylene glycol in a phase system is much smaller and the molecular weight of the dextran larger than the molecular weight of most enzymes, this method appears generally applicable. Another simple way to separate an enzyme from most of the polymers of a polyethylene glycol rich upper phase is by the addition of salt, thereby establishing a new phase system and extracting the desired enzyme into the salt phase (Hustedt *et al.*, 1978b; Albertsson, 1971). Other possibilities include centrifugation for very large molecules or particles (Albertsson, 1971), adsorption of the enzyme on a suitable adsorbent, and subsequent elution (Albertsson, 1971), or precipitation with salt (Albertsson, 1971). Only the last two appear also suitable for large-scale work. Some of the methods listed offer opportunities for not only removing the polymers but also for increasing the specific activity of the desired enzyme at the same time or removing contaminating activities by proper selection of the parameters of the process (Hustedt *et al.*, 1978b).

XIII. CONCLUSIONS

Liquid–liquid partition of enzymes in aqueous two-phase systems is a very versatile and efficient method for the extraction of enzymes especially suited for the demands of the large-scale isolation of intracellular enzymes. The method can be adopted for continuous pro-

Action:
Enzymes increase the rate of biochemical reactions and decrease the time for those reactions to reach equilibrium. They are not consumed in the chemical reactions and, as such, their action is catalytic. For example, two constituents of pectinase are pectin methylesterase and polygalacturonase. Pectin methylesterase demethylates pectin; polygalacturonase hydrolyzes the a-1,4-galacturonide bonds in pectin (Food Chemicals Codex, 1981). A large variety of pectic enzymes are available both in liquid or solid forms and in various strengths, as measured by the level of activity. In the case of pectin, this is measured by the ability of the enzyme to hydrolyze the glycosidic bond between the biopolymer pectin of repeating chains of the sugar galactose or galacturonic acid. The amount of pectin in fruits depends on the maturity, degree of ripeness, variety, and subsequent storage conditions of harvested fruit (Reed, 1975).

Combinations:
Enzymes often are included in whole cells or parts of the cells of the source (National Academy of Sciences, 1981). They are often packaged with various carriers that do not have catalytic activity that may or may not be synthetically derived (White and White, 1997). Synthetic preservatives are almost always added during processing, and may be present in the final preparation to prevent microbial growth, stabilize the preparation, and maintain the desired enzymatic activity (Pariza and Foster, 1983). Other incidental ingredients in enzyme preparations function as carriers, stabilizers, humectants, and diluents.

Enzymes are usually used in combination with other enzymes. For example, pectinase is often used with cellulases, hemicellulases, and proteases. Several of these are also produced by *A. niger* (White and White, 1997). Some of these materials are on the recommended National List.

Status

OFPA
The substance is used in handling and is non-synthetic but is not organically produced (7 USC 6517(b)(1)(C)(iii)).

Regulatory
Enzymes are considered food additives under the Food, Drug, and Cosmetic Act. See 21CFR 184 for various specific GRAS listings. Pectinase has been self-declared GRAS by the Enzyme Technical Association (ETA, 1999).

Status among Certifiers
Most US certifiers have allowed the use of fungally derived enzymes documented to not be from genetically engineered sources. Specific conditions for extractions and incidental additives does not appear to be uniform among US certifiers at this point.

Historic Use
Enzymes contained in various ingredients have been used to prepare foods since before recorded history. However, production and application of pure enzymes has become increasingly sophisticated over the past century. The first use of pectinase in fruit juice processing dates back to the 1930s (Nielsen et al., 1991). Steady supplies of purified, standardized pectic enzymes have been commercially available for about fifty years. Enzymes have been used in a broad number of applications by organic food processors for as long as organic processed food has been on the market.

International
In general, enzyme standards for international trade are set by the Joint FAO/WHO Expert Committee on Food Additives (1990). The Codex Alimentarius Commission organic food guidelines allow "[a]ny preparations of microorganisms and enzymes normally used in food processing, with the exception of microorganisms genetically engineered/ modified or enzymes derived from genetic engineering" (Joint FAO/WHO Food Standards Programme, 1999). The most recent edition of the IFOAM *Basic Standards* considers enzymes acceptable for use in organic food processing provided they are based on the established Procedure to Evaluate Additives and Processing Aids for Organic Food Products (IFOAM, 1998). These standards are parallel to, but not exhaustively covered by the OFPA criteria.

$$\ln K = \ln K_{el} + \ln K_{h\,phob} + \ln K_{h\,phil} + \ln K_{conf} + \ln K_{lig} \qquad (8)$$

where K_{el}, $K_{h\,phob}$, $K_{h\,phil}$, K_{conf}, and K_{lig} stand for partition coefficients depending on electrical, hydrophobic, hydrophilic, conformational, and ligand effects. Each of these terms can be used in principle to manipulate and enhance the selectivity of partition for a given protein.

The influence of the interfacial electrical potential has been discussed before. In addition to the potential generated by unequal distribution of small ions, liquid ion-exchangers can be employed to shift the protein across the interface. In Fig. 14 the partition coefficient of pullulanase is plotted as a function of the DEAE-dextran concentration in a polyethylene glycol–dextran system as an example. This approach can be compared to batch adsorption on solid ion-exchangers, and in a multistep procedure it may be related to ion-exchange chromatography. Substituted dextrans are already commercially available, and the synthesis of ion-exchangers derived from polyethylene glycol has been described by Johansson (1970b).

The hydrophobicity of the phases is not identical in an aqueous multiphase system. Hydrophobic interactions can be further modulated by including different nonionic detergents into the phase system. This way a membrane-bound protein phospholipase A has been purified (Albertsson, 1973). Special attention should be paid to derivatives of the polymers constituting the phase system. It is noteworthy that a number of commercially available detergents are derived from polyethylene glycol so that such specialities for liquid–liquid extraction can be supplied at reasonable cost. The potential of hydrophobic interactions for the purification of proteins is well documented for hydrophobic chromatography on solid supports. One can assume that these principles can be also exploited for the partition in aqueous phase systems.

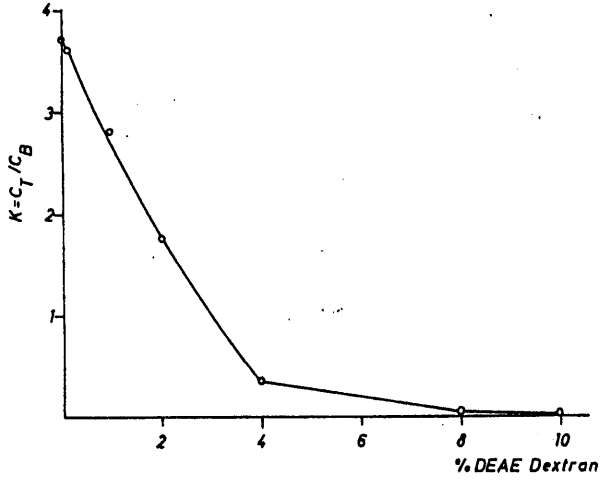

Fig. 14. Influence of the concentration of DEAE-dextran on the partition coefficient of pullulanase: (9% w/w polyethylene glycol 4000, 1.25% w/w dextran T 500; 100 m*M* sodium phosphate pH 7.8). The abscissa indicates the amount of dextran replaced by DEAE-dextran.

For example, Shanbhag and Johansson (1974) demonstrated that serum albumin can be selectively extracted from plasma by addition of small amounts of palmitoyl–polyethylene glycol to a polyethylene glycol–dextran system. It is known that serum albumin interacts with fatty acids. The last example may, therefore, also be considered as a special case of ligand interaction. The specific interaction between enzymes and their substrates, products, or inhibitors forms the basis of many separation procedures and is commonly called affinity chromatography (Jacoby and Wilchex, 1974). Similiar principles can be applied to partition linking the ligand in question covalently to one of the polymers forming the phase system. Flanagan and Barondes (1975) derived, from theoretical arguments, an equation describing affinity partition in polyethylene glycol–dextran systems:

$$\Delta \log K_{enz} = n(\log K_{PEG-L}) \qquad (9)$$

where n denotes the number of ligand binding sites of the enzyme and K_{PEG-L} is the partition coefficient of the modified ligand in the absence of the enzyme. From the theory it follows that oligomeric enzymes with $n > 1$ should preferentially be extracted by affinity partition. Attempts to verify Eq. (9) have not been successful so far. The deviation from the expected behavior may be explained by other equilibria in the system that lower the apparent ligand concentration, e.g., ligand–ligand interaction or formation of micelles.

Affinity partition has been employed for purification of the cholinergic proteins from the electric organ of *Torpedo californica* (Flanagan *et al.*, 1975), Δ_{5-4}-3-oxosteroid Isomerase (Hubert *et al.*, 1976), trypsin (Takerkart *et al.*, 1974), and S-23 myeloma protein (Flanagan and Barondes, 1975) using a specific ligand, an inhibitor, or the determinant group of an antigen, respectively, bound to polyethylene glycol for biospecific interaction and extraction. We could show that the specificity of extraction for dehydrogenases could be enhanced by addition of NADH bound to polyethylene glycol to the phase system (Kula *et al.*, 1979). Coenzymes like NADH serve in this regard as general ligands and can be employed for the extraction of classes of enzymes. Also the

OFPA 2119(m) Criteria

(1) The potential of such substances for detrimental chemical interactions with other materials used in organic farming systems.
As this is a processing material, the substance is not used in organic farming systems.

(2) The toxicity and mode of action of the substance and of its breakdown products or any contaminants, and their persistence and areas of concentration in the environment.
See processor criteria (3) below.

(3) The probability of environmental contamination during manufacture, use, misuse or disposal of such substance.
This is considered below under processor criteria (2).

(4) The effect of the substance on human health.
This is considered in the context of the effect on nutrition (3) below as well as consideration of GRAS and residues (5) below.

(5) The effects of the substance on biological and chemical interactions in the agroecosystem, including the physiological effects of the substance on soil organisms (including the salt index and solubility of the soil), crops and livestock.
As this is not released into the agroecosystem, there is no direct effect.

(6) The alternatives to using the substance in terms of practices or other available materials.
See discussion of alternatives in (1) below.

(7) Its compatibility with a system of sustainable agriculture.
This is considered more specifically in the context of organic handling in (6) below.

NOSB Processing Criteria

A SYNTHETIC PROCESSING AID OR ADJUVANT may be used if;

1. An equivalent substance cannot be produced from a natural source and has no substitutes that are organic ingredients.

   Enzymes frequently offer the only way to achieve a desired technical effect. Nearly all commercially prepared foods contain at least one ingredient that has been made with enzymes. In a number of cases, the alternatives would be prohibited for use in organic production (e.g. sulfuric acid); in other cases, the alternative would be chemical modification (e.g. sodium hydroxide used to produce starch). Some enzymes are essential for the production of certain foods, for example α-amylase to produce barley malt or rice syrup; or various coagulents used to produce cheeses. In the case of pectinase, different fruits can be processed into juice, wine, oil, or preserves with lower yields (Faigh, 1995), longer processing times (Gist-Brocades, 1993), and subjectively lower quality (Chang, et al., 1994).

2. Its manufacture, use and disposal does not contaminate the environment.

   Production of enzymes is generally conducted in controlled, closed environments. Materials necessary for their manufacture generally do not in and of themselves constitute an environmental hazard. Good manufacturing and handling practices are sufficient to protect workers from any negative effects of exposure, although inhalation or other ingestion of enzymes can have irritating or allergenic effects on some people.

   The fermentation process is relatively efficient and closed. Because of their catalytic nature, enzymes theoretically can react indefinitely, and relatively small amounts are effective in performing their functions.

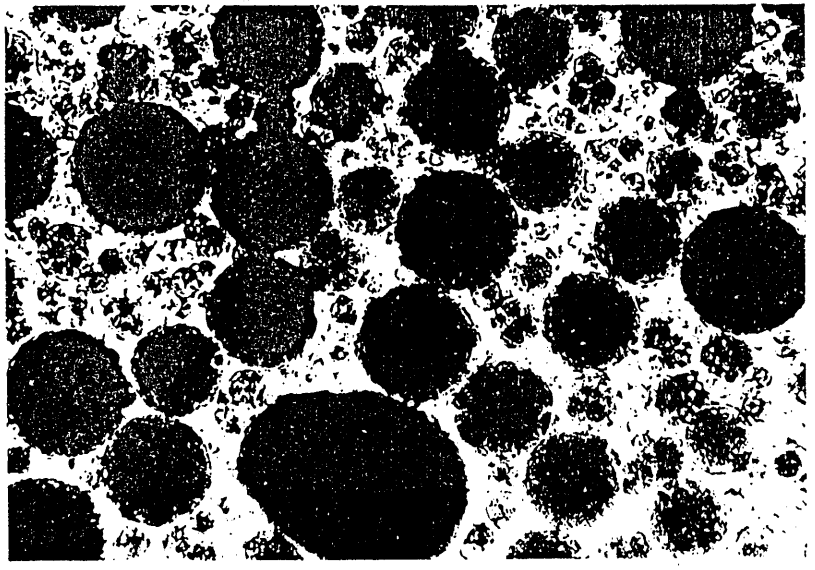

Fig. 11. Microscopic picture of a two-phase system including cell homogenate of *Lactobacillus cellobiosus* (19.2% w/w polyethylene glycol, 13.5% potassium phosphate, pH 5.0). Diameter of the droplets ≤200 μm.

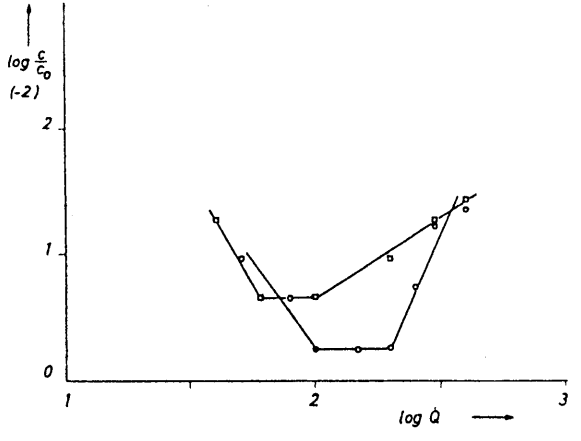

Fig. 12. Throughput characteristic of a polyethylene glycol–dextran system in the α-Laval separator "Gyrotester B" under two different conditions of flow resistance set up by the number of regulating screws (c is the concentration of disperse phase (dextran) in the effluent, c_0 means the concentration of disperse phase in the feed and $\dot{Q}$ the throughput): O—O = 4 regulating screws, 13.5 mm; □—□ = 1 regulating screw, 13.5 mm (system: 10% w/w polyethylene glycol 4000, 2% w/w dextran T 500). (From Kroner *et al.*, 1978.)

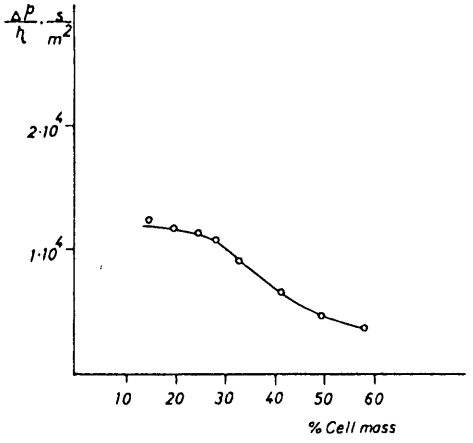

Fig. 13. Quotient of the density difference ($\Delta\rho$) and the viscosity (η) as a function of concentration of *K. pneumoniae* cells (conditions as in Fig. 7). (From Kroner *et al.*, 19

from laboratory data enzyme isolations with a scale-up factor of 50 (Kroner *et al.*, 1978). Further scale-up in industrial dimensions a pears also possible. The high accuracy of calculated yields and con dence in scale-up data arise from the fact that in contrast to many otl separation methods partition does not depend on the absolute conce tration but on the ratio of the concentration, which is constant fo given set of parameters. There are a number of other devices a methods developed in the chemical industry such as extraction c umns of different design, mixer–settler, Graesser contactor, counte current distribution, and so forth that could possibly be used for co tinuous processes. Multistep procedures are of great importance if consider purification and separation of enzymes by partition followi the removal of cells and cell debris. We described one example (H tedt *et al.*, 1978b) the separation of 1,4-α-glucan-phosphorylase fr glycosyl transferase for such a concept. In this case we were success in a single-step partition. In general, multistep procedures will necessary to achieve the desired separation. Obviously a wealth knowledge in chemical engineering can be used for such develo ments. We are currently investigating how different devices me tioned above can be adopted for work with aqueous two-pha systems.

XI. SELECTIVITY OF PARTITION

The partition coefficient can be described as the sum of several m or less independent terms (Albertsson, 1977):

Enzymes need to be replaced when they are degraded by physical conditions (e.g. heat) or removed with the processed food.

Release of enzymes into the environment is generally not a concern. They are active in very low concentrations, and each enzyme's action is specific to a very narrow range of substrate(s). They can be relatively stable molecules, but are generally degradable by heat or other environmental factors. Enzymes in the environment may accelerate the rate that pollutants are metabolized (Tinsley, 1979). This may be detrimental, beneficial, or have no net effect, depending on the substrate and metabolite.

Escape of enzyme-producing organism into the environment is not considered an environmental concern (Nielsen, et al., 1991). Genetically-engineered organisms, particularly microorganisms--may change the nature of this concern. Wild-type producing strains have shown a fair ability to be controlled in open ecosystems by natural competition. Genetically-engineered strains, on the other hand, may have far-reaching consequences if released into the environment. At present, there is insufficient data and experience with such strains to regard their potential interactions as safe, in anything but a very controlled environment, and even then this may not be a certainty.

3. If the nutritional quality of the food is maintained and the material itself or its breakdown products do not have any adverse effect on human health.

Enzymatic activity on foods is specific and transformational, usually resulting in a significant change in the characteristics of the substrate. The new food product may have a significantly different effect on the human system when ingested. For example, consider the difference between corn meal and corn syrup, or milk and cheese. That a transformation occurs is not by itself enough to say whether the ultimate effect on human health is positive or negative. Most studies show that nutritional quality as measured by vitamin and mineral content, as well as other parameters, is maintained (Braddock, 1981). In some cases, because of the enzyme's role in the removal of the non-nutritional part of the food and making the nutrients of the food more digestable, enzymes can measurably improve the nutritional quality of food. Other indicators of quality are arguably improved (Chang et al., 1994).

There is an on-going debate in human nutrition as to the advantages of whole over processed foods. This is discussed further in the reviewer comment section. A recent study based on an analysis of 46 supplements for the quality of their antioxidants composition demonstrated that natural intact food sources were better (Tufts, 1999). By implication therefore, the pectin in an apple, and the overall nutritional value of an apple, is much greater to the consumer than is depectinized, filtered apple juice.

There is the potential for enzymes to pose a threat to human health and safety. As proteins, enzymes can cause allergic reactions in sensitive individuals (Tucker and Woods, 1995). Enzymes can remain active after digested and there is concern that novel enzymes--particularly some of the more potent ones being developed by genetic engineering--will attack human tissues in some instances (Tucker and Woods, 1995). Perhaps the greatest concern with fungal enzymes is the presence of mycotoxins from either the source organism or a competing organism that invades the fermentation media. Many of these organisms are capable of producing antibiotics. While Good Manufacturing Practices require that non-pathogenic strains be used, quality control and Hazard Analysis Critical Control Point (HACCP) need to be sufficient to ensure that both the strains and the media avoid contamination with pathogens and toxins. The organism used as a case study for this review, *A. niger*, provides a good example. *A. niger* is capable of producing low levels of toxins, but most strains are considered non-toxigenic because the levels of toxins are so low (Pariza and Foster, 1983).

Enzymes are widely used for therapeutic purposes (Jayaram, Ahluwalia, and Cooney, 1991; Cichoke, 1999). While there are a number of contraindications that need to be considered in a number of cases, and a recognition that not all uses of enzymes are beneficial or desirable, they are generally not a threat to human health when properly handled and used.

Finally, after processing and packaging, the enzyme may be prone to spoilage by a microbial contaminant. For this reason, preservatives are almost always added during processing and after final preparation (Pariza and Foster, 1983).

Experiments show that the conventional equations derived in chemical engineering (Hemfort, 1970) can be used to estimate the adequate selection of machine settings for the regulation of the back pressure of the lower phase:

$$\rho_u(r_s^2 - r_u^2) = \rho_l(r_s^2 - r_l^2) \tag{6}$$

$$r_l = \frac{r_s^2(\rho_l - \rho_u) + \rho_u r_u^2}{\rho_l} \frac{1}{2} \tag{7}$$

where ρ_u is the density of the upper phase, ρ_l the density of the lower phase, r_l the outlet diameter for the lower phase, r_u the outlet diameter for the upper phase, and r_s the radius of interphase line in the disk stack. The density difference of aqueous two-phase systems, however, is smaller than usually encountered with organic phases. Therefore, a finer adjustment of the regulating devices, e.g., gravity disks sometimes becomes necessary in order to obtain optimal results.

As the manufacturer will usually supply only a standard set of gravity disks, appropriate intermediate sizes have to be ordered separately or they can be easily machined in a local mechanics shop. As discussed above, a much higher throughput is expected for liquid–liquid separation as compared to solid–liquid separation. That cell debris is indeed included in the dispersed lower phase is demonstrated in Figs. 10 and 11, which also show that the particle droplet size is much larger than cell debris size. For a more complete description of the separator, see Kroner *et al.* (1978).

Dispersed aqueous two-phase systems show a rather fast coalescence under most conditions. The limit particle size calculated from a plot of log c/c_0 over log $\dot{Q}$ (Fig. 12) is smaller than expected from estimates of the average droplet size in the feed, which we interpret to be a result of the additional dispersion by the high radian acceleration in the open disk stack separator (Kroner *et al.*, 1978).

Equation (1) shows that the throughput is inversely proportional to the viscosity. Therefore, the viscosity of the dispersion is the most important parameter besides the limit particle diameter which will limit the throughput. For a given aqueous two-phase system the viscosity changes with increasing concentration of cells and cell constituents added, as does the density of the suspension. The increase in the density difference counteracts the increase in the viscosity to some extent; but at higher cell concentrations the influence of the viscosity is dominating, and the throughput will drop. In Fig. 13 the quotient of the density difference and the viscosity is plotted as a function of the cell concentration. It can be seen that up to 30%, moist *K. pneumoniae*

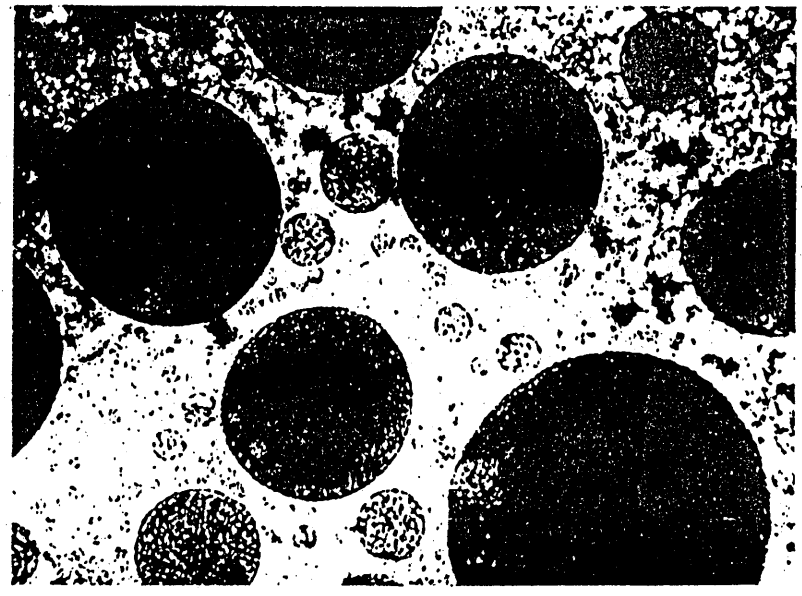

Fig. 10. Microscopic picture of a two-phase system with *E. coli* cell homogenate (10% w/w polyethylene glycol 6000, 10% w/w potassium phosphate, pH 7.0). Droplet diameter ≤350 μm.

cells can be included in the system without adverse effect on the throughput. This corresponds to 7–7.5% of dry weight material in the dispersion—a much higher value than can be realized in conventional solid–liquid separation of comparable probes. In fact, the maximal concentration of cell constituents is determined more often by parameters such as the partition coefficient and the phase ratio, than by the throughput of the separator (Kroner *et al.*, 1978).

In most of the cases investigated, we observed the continuous removal of cells and cell debris with the lower phase. If the build-up of a sediment in the periphery of the separator bowl becomes a problem, the use of a solid ejecting type separator is indicated. For polyethylene glycol–salt systems with a high solid content, a decanter centrifuge may be the best choice. We are currently investigating the performance of various separator designs commercially available for the separation of aqueous two-phase systems containing cells and cell debris. For all these machines industrial experience is available for scale-up of processes that can be used for the development of technology for large-scale enzyme isolation procedures. We have been able to predict

4. Is not a preservative or used only to recreate/improve flavors, colors, textures, or nutritive value lost during processing except in the latter case as required by law.

   Enzymes in and of themselves generally would not be considered preservative materials. The products of enzyme activity could conceivably act as preservatives, but these would be from the breakdown of the food material itself, not from an outside source. Food qualities are changed by enzymatic activity, but this change should not necessarily be construed as a means of re-creating qualities of the original product lost in processing. The product is substantially different from the raw ingredient(s). While enzymes can be used to transform food into a more stable product, these processed foods are generally identified as different from their raw ingredients. For example, raspberry jelly is considered to be different from raspberries. The use of pectinase neither increases nor decreases the shelf life of a raw product. In a natural situation, various enzymes are produced by either the plant itself (Kays, 1991) or various organisms to accelerate decay, decompose cell walls, increase sugar content, and release the nutrients contained in the fruit and other plant organs in the senescence process.

5. Is Generally Recognized as Safe (GRAS) by FDA when used in accordance with Good Manufacturing Practices (GMP), and contains no residues of heavy metals or other contaminants in excess of the tolerances established by FDA.

   Enzymes are unchanged by their action on their substrates; they remain as they are, and active, until denatured by heat or other factors, or until the substrate is exhausted. Depending on the process, enzymes may be removed from the final product, or denatured and left in, or may even be potentially active. How they are labeled in final product formulations should be dependent on the specific outcome for the product in question. As was mentioned above, carriers, preservatives, or other commercial enzyme formulation components are also potential residues in finished foods.

   Many enzymes are classified as GRAS, although such determination has not been universally made. GMPs, quality control measures, and analytical protocols can reduce the risk of mycotoxins being included in fungal enzyme formulations as by-products of the manufacturing process (Pariza and Foster, 1983). Implementation of HACCP plans can take further steps to reduce risk to food safety posed by enzymes.

   A number of fungal enzymes are generally and specifically considered GRAS. The Enzyme Technical Association has made a self-declaration of GRAS for a number of enzymes (ETA, 1999).

   The Food Chemicals Codex places the following limits on residues:

   Arsenic (as As) not more than 3 ppm.
   Coliforms: not more than 30 per g.
   Heavy metals as lead: not more than 0.004%.
   Lead (Pb): not more than 10 ppm.
   Salmonella spp: Negative by test.

   The Food Chemicals Codex also states that "[a]lthough tolerances have not been established for mycotoxins, appropriate measures should be taken to ensure that the products do not contain such contaminants."

6. Is compatible with the principles of organic handling.

   Enzymes have been used in organic processing for as long as organic processed food has been marketed, and are currently being used by certified organic processors. An industry survey of organic food processors regarding the compatibility of various processes found that enzymes were rated between 2.5 and 2.7 on a scale of 1 to 5, or approximately mid-range, as compatible with organic processing (Raj, 1991).

   In certain food products, enzymes are the only way to produce the desired product, such as barley malt or rice syrup, or for certain cheeses. In others, such as production of certain invert starches, the alternatives--sulfuric or phosphoric acid--would not be compatible with an organic handling system, and may result in

The physical parameters of the dispersions are also influenced by the presence of microorganisms and cell debris. The density changes are not very pronounced in the two phases of a polyethylene glycol–dextran system. The density difference increases slightly but steadily and reaches values around 0.13 gm/cm³. The viscosity of the dispersion increases with cell concentration, slowly at first, up to approximately 30%. Above this value, the viscosity rises abruptly. The pronounced rise in viscosity occurs at around the same concentration when the partition coefficient for the enzyme investigated begins to fall. Detailed data on the change of physical parameters in polyethylene glycol–salt systems with increasing concentrations of cells and cell debris are not yet available. The analysis is hampered in such cases by the precipitates present, as discussed earlier. The physical parameters are very important for the separation and will be discussed later.

X. TECHNICAL ASPECTS OF EXTRACTION AND SEPARATION

The extraction of a desired enzyme and the separation of phases can be accomplished in a variety of ways. The highly advanced technology developed in chemical engineering over the past decades has to be adopted for the special case of aqueous two-phase systems. Single-step operations have been successfully performed with different devices (Kroner *et al.*, 1978; Hustedt *et al.*, 1978a; Kula *et al.*, 1977). The rather low interfacial tension (Albertsson, 1971) aids the extraction, as very large surface areas are easily generated, and equilibrium of partition is reached rapidly even for compounds of high molecular weight. In fact, it is difficult to measure mass transfer rates and the approach to equilibrium under operational conditions (Shanbhag, 1973). Adequate mixing was found sufficient for equilibrium partition. Mixing can be accomplished by propeller-type impellers as well as by vibro mixers or other means.

The use of a settling tank is the cheapest way to achieve a phase separation. In Fig. 9, separation of a polyethylene glycol–salt system is plotted as a function of time. For such systems the incorporation of a settling tank in the process is an alternative to centrifugal phase separation, especially if one considers that most enzymes are stable under these conditions for extended periods of time and unattended operation is possible in off-hours.

For the separation of polyethylene glycol–dextran systems a centrifugal separator has to be employed as the time needed for settling

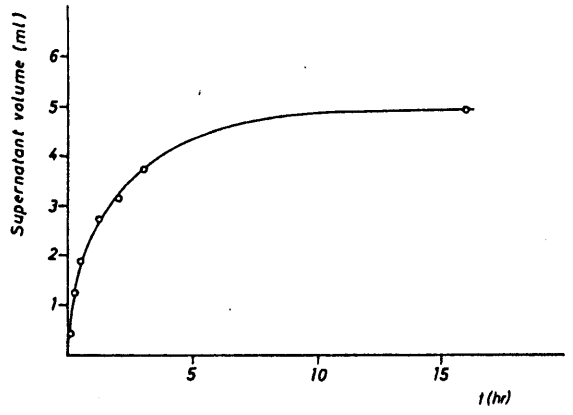

Fig. 9. Course of the separation of a polyethylene glycol–salt system including cell debris in a glass tube (height ~8 cm, diameter ~1.2 cm) (system: 14% w/w polyethylene glycol 1550, 7.5% w/w potassium phosphate, pH 7.7, 49% cell homogenate of *Streptomyces* species).

becomes too large. The optimal operational conditions for a separator depend on the physical characteristics of the dispersion to be separated. The density difference of the phases is the dominating factor of the correct positioning of the interface, which will determine the purity of the phases. Aqueous two-phase systems have been separated in an open disk stack separator to yield the upper phase in a purity better than 99.8% and with only 2% loss (Table II) (Kroner *et al.*, 1978).

TABLE II
DETERMINATION OF THE OPTIMAL LENGTH OF THE REGULATING SCREW

Length of screw (mm)	Phase ratio top/bottom in operation	Purity of phases (%)	
		Top	Bottom
15.5	Liquid leaves at the inner port only	ND	ND
14.5	29	≈90	≈100
13.5	9	≥99.8	≈82
12.5	8	≥99.5	≈75
10.5	4	ND	ND
8.5	Most liquid leaves at the outer port	ND	ND

System: 9% w/w PEG 4000, 2% w/w dextran T 500; $\Delta\rho = 0.050$ gm/cm³, $\rho_u/\rho_l = 0.95$, phase ratio $V_u/V_l = 8.8$; ND = not determined (From Kroner *et al.*, 1978.)

products of lower quality. There are some cases in which microbial fermentation can offer a more holistic approach to processing than the use of isolated enzymes. One such example is the use of *Mucor mehei* to produce certain kinds of cheeses.

7. There is no other way to produce a similar product without its use and it is used in the minimum quantity required to achieve the process.

   For a number of foods, enzymes are essential to the identity of the food. Even where they are not required to be used to make an identifiable food, they are needed to produce a food of the quality consumers expect. In determining the standard of identity of natural juice, juice extracted using pectinase is usually considered minimally processed (Haight and Gump, 1995). Because they are effective in small amounts, very little of any enzyme is needed to process a given food. An industry survey found the amount of enzymes used in processing is in all cases less than 0.06%. The maximum amount of pectinase used in baked goods was found to be 0.000002% (Pariza and Foster, 1983).

Discussion

Condensed Reviewer Comments

None of the reviewers have a commercial or financial interest in pectinase in particular or enzymes in general.

Reviewer 1
NOTE: The following review covers enzymes as a general class of materials, using pectin [enzymes] as an example in parts of the discussion.

Research included by OMRI for this review suggests that processing of foods with enzymes can enhance the nutritive value of foods by breaking down "indigestible" food components, thereby making certain nutrients more available in the final product. One example given is that pectinase activity on plums in juice manufacture can result in greater availability in the final juice product of antioxidant components, which otherwise might not have been yielded during processing. How such laboratory trials correlate to human nutrition is not clear from the information presented, and not completely known - that is, whether such enzymatic treatments would be necessary to make said antioxidants as available in the human gut, or whether the altered food overall is definitely better than the original whole food. (This question/example could be extended to other fruits and nutritional components thereof, which are processed similarly with pectinases.) Generally speaking, processing yields of total juice from various fruits is increased when pectinases are used, as more of the fruit can be liquefied and separated from the seed and fiber; this is the primary reason enzymes are used in fruit processing.

There are some potential drawbacks to considering the advantages in exclusion to other effects of use of these enzymes, both from a nutritional standpoint and from an organic foods perspective. The assumption given in the first paragraph in this section of the NOSB database for this part of the criteria, i.e. that so-called "non-nutritive" or "indigestible" food components serve no positive function as part of a human diet and can therefore be removed, is based on incomplete knowledge at best. For example, the pectin in an apple, and the overall nutritional value of an apple, is much greater to the consumer than is pectinized, filtered apple juice. In which instances an isolated food component is desirable or valuable and which cases it is not is subject to variance from one commodity to another. We do not fully understand the complex balance of nutrients and how they interact on human nutrition for any agricultural product, and we should therefore be careful in choosing which components we deem appropriate to keep in the product and which to discard. Research continually shows how previously unidentified or poorly-understood food components can play significant roles in human health and nutrition. The value of food fiber is a good case in point.

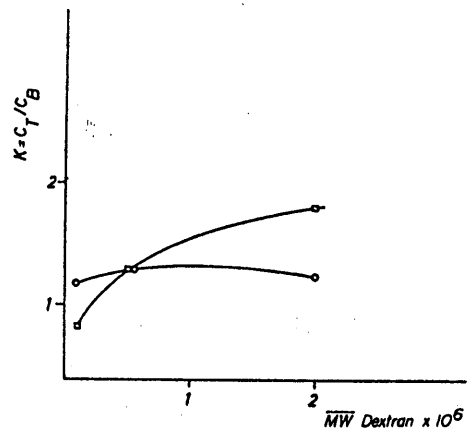

Fig. 3. Dependence of the partition of two enzymes on the average molecular weight of dextran: O—O α-glucosidase from *Saccharomyces carlsbergensis* (10% w/w polyethylene glycol 4000, 5% w/w dextran, 100 m*M* potassium phosphate, pH 6.3); □—□ isoleucyl-tRNA synthetase from *Escherichia coli* (9.2% w/v polyethylene glycol 6000, 6.2% w/v dextran, 73 m*M* potassium phosphate, pH 7.0).

weights $>10^5$. While the dextran concentration also is not a very critical variable, the polyethylene glycol concentration influences the partition coefficient and has to be considered for process development. Looking at the phase diagram, it becomes evident that high concentration ratios of polyethylene glycol and dextran are normally required to achieve a high volume of the top phase and a high phase ratio. On the other side, increasing the concentration of polyethylene glycol will lower the partition coefficient, and an optimal compromise between these variables has to be found. The lower limit of the concentration of polyethylene glycol is given by the phase diagram. In addition, the cost of polymers constituting the system has to be considered when the optimal concentration of the polymers is evaluated. Therefore, the tendency is to lower the dextran concentration, which is much more expensive than polyethylene glycol at present.

In this respect it is fortunate that the average molecular weight of dextran at values $>10^5$ has little influence on the partition coefficient, and expensive dextran fractions of narrow molecular-weight distribution can be replaced by crude dextran with a rather wide molecular-weight distribution for enzyme extractions. However, crude dextran shows a rather high viscosity in solution. The viscosity is not so much apparent in the disperson of both phases, but the resulting lower phase will be very viscous, and the large difference in the viscosity of the two

phases will lead to separation problems. We could reduce the viscosity of crude dextran to acceptable levels by hydrolysis under very mild conditions so that a more economic carrier for the phase system would be available (Kroner *et al.*, 1979).

VII. INFLUENCE OF IONS INCLUDED IN THE PHASE SYSTEM

Small ions have partition coefficients in aqueous two-phase systems around 1, but most of the time not exactly 1. This will lead to a differential concentration increase in one of the phases. If the counterion does not have the same partition coefficient, an interfacial potential is generated, as discussed in detail by Albertsson (1971) and Johansson (1970a). For a manipulation of partition coefficients in aqueous two-phase systems, the ratio of ions, rather than their concentration, is the dominant factor. For multivalent anions the dissociation is pH-dependent, and therefore so is the partition coefficient. This is very pronounced in the case of phosphate, where the monovalent ion $H_2PO_4^-$ has a partition coefficient of 0.96 and the divalent ion HPO_4^{2-} one of 0.74 in a system made up of 7% (w/w) dextran T 500 and 7% (w/w) polyethylene glycol 4000 (Johansson, 1970a). As a consequence, in phosphate buffers a high interfacial potential is generated at pH values above 7 between the phases in a polyethylene glycol–dextran system. The lower phase becomes negatively charged, and proteins with isoelectric points below 7 are preferentially exported to the upper phase. Therefore, many proteins show a remarkable pH-dependent increase in the partition coefficient in phosphate buffers (Fig. 4), which can be exploited for the extraction (Kroner *et al.*, 1978). The ionic strength should have no further influence on the partition of proteins above a certain threshold level to minimize donnan potentials. But we frequently observed increasing partition coefficients of enzymes by increasing the phosphate concentration (Fig. 5) (Hustedt *et al.*, 1978b; Kroner and Kula, 1978). The reason for this is poorly understood at present. Further work is needed to differentiate salt effects in such systems and to distinguish between partition, exclusion, solubility changes, and other possible mechanisms.

VIII. INFLUENCE OF TEMPERATURE

The temperature will effect the phase diagram as well as the partition and the stability of the proteins. In our experience the hydrophilic polymers used enhanced the stability of enzymes so that separation

A purely materials-based approach to processing of organic foods is flawed, in that the effects of individual processing steps and their associated materials can be cumulative. Analysis of each component step in the process does not necessarily reveal the total effect of all processes combined to make the final product. For this reason, although use of enzymes may not in and of themselves be seen as negative, use may be an integral part of a negative outcome as regards one of the goals of organic foods production, namely wholesome foods of high nutritional value. Fruit and juice processing is sometimes a good example of this; although initial yield of juice from the fruit may be higher than without use of the enzyme(s), the final products often are only a shadow of the original material. The nutritive values of corn meal and corn syrup are starkly different from each other; this is partly due to the action of enzyme, and partly due to the subsequent isolation of the product of the enzyme's activity. In such cases, it could be said that the nutritional value of the organic agricultural commodity has largely been lost.

Allowance of use of enzymes on organic foods therefore poses a potential danger as regards the nutritional value of the finished product. Non-specific allowance of all enzymes (or allowance of a specific class of enzymes used non-specifically, i.e. on any commodity), can lead ultimately to production of organic food products which lack much of the nutritive value of the original agricultural component(s).

What is needed, therefore, is a broader principle on which to base decisions as to whether or not materials such as this are appropriate for particular foods. In this discussion, nutritive value is the determining criterion. The annotation as proposed in the NOSB database file should therefore be amended with a statement similar to the following: "Use of enzymes in any given process is subject to overall evaluation of the final nutritional value of the finished product compared to its initial ingredient(s). Such evaluation shall take into account all processing steps involved, not just those involved with the use of the enzyme(s). In cases where the nutritional profile of the raw ingredient(s) is deemed to have been substantially weakened, such finished food products may only be labeled as 'made with organic ingredients,' but not as 'fully organic.'" (Such "made with" products, if they are further used as ingredients in other organic product formulations, will themselves have to have classifications as to whether they can be ingredients in "organic" product formulations, or only in "made with organic" products. An illustrative example of this might be corn syrup, or commonly-produced white grape juice concentrate.)

There are some potential drawbacks to considering the advantages in exclusion to other effects of use of these enzymes, both from a nutritional standpoint and from an organic foods perspective. The assumption given in the first paragraph in this section of the NOSB database for this part of the criteria, i.e. that so-called "non-nutritive" or "indigestible" food components serve no positive function as part of a human diet and can therefore be removed, is based on incomplete knowledge at best.

An alternative which also might serve organic principles is an itemization of enzyme use by food type (either substrate or final product), the allowances or restrictions for enzyme use being specific to each; such listing is more arduous to generate, but allows for more consistent application.

As some other points to consider, human safety can potentially be threatened by enzymes, either due to allergenic interactions or toxic by-products of microbial production of enzymes. Selection of appropriate strains, along with GMP's and HAACP plans can be used to minimize these dangers, usually with good results. Far less certainty on this point applies to those enzymes and microbes which are products of genetic engineering.

Finally, enzymes are often packed for industrial use with a number of carriers and preservatives, for convenience of both the enzyme manufacturer and the product user. All formulations, if they are to be used in organic systems at all, must have full disclosure as to all components in the formulation used, and only include components which are deemed acceptable materials on the National List for foods labeled as "organic." Processed products made with enzyme formulations which do not meet this requirement may or may not be labeled as "made with organic ingredients," depending on the formulation's component(s) in question. Carriers, standardization materials, and other commercial enzyme formulation ingredients should be listed on all product labels as ingredients, if they indeed end up in the final food product.

design of such trial experiments. Surprisingly, technological aspects in the utilization of such aqueous two-phase systems for the extraction and enzyme isolation on a large scale have not been investigated until very recently, in spite of the fact that earlier Albertsson had already pointed out the potential of such systems for large-scale work (Albertsson, 1971).

IV. CHOICE OF A SYSTEM

Albertsson (1971) described a number of different aqueous multiple-phase systems. In principle most of the hydrophilic polymers miscible with water will show phase separation in a mixture with a second polymer as discussed previously. The actual choice of a system will depend on the following points.

Obviously the most important factor is the question of whether the intended extraction can be accomplished with a particular system and under what conditions. Furthermore, the physical properties such as the viscosity and density difference of the phase system should be suitable so as to permit easy and fast operation during phase separation. The high intrinsic viscosity of certain polymers like methylcellulose or polyvinyl alcohol limits their application for such purposes. The last consideration relates to the possibilities of removal of the polymers from the enzymes and legislative requirements. In this respect, polyethylene glycol and dextran appear most suitable for enzyme extraction. Dextran is nontoxic and certified even for intravenous incorporation in man as a plasma expander. Polyethylene glycol has also been very thoroughly tested for pharmaceutical purposes and is included in the pharmacopoeia of most countries. It is also registered for food purposes in the United States (1975). Therefore, our first experiments were carried out using polyethylene glycol–dextran or polyethylene glycol–salt systems.

V. SELECTION OF PARAMETERS

For industrial purposes the yield of the extraction step and the proportional cost are the important factors in process development. In liquid–liquid separation the yield is determined by the partition coefficient and the phase ratio. The yield in the top phase is given by

$$Y(\%) = 100 \Big/ \left[1 + \left(\frac{V_b}{V_t} \frac{1}{K} \right) \right] \tag{5}$$

Our first aim, usually, is to find conditions that will allow extraction of a particular enzyme from broken cells in high yield in a single step by

selecting a high phase ratio V_t/V_b, e.g., ~5, presuming that cells and cell debris will partition in favor of the bottom phase. The partition coefficient of the enzyme is improved by changing the composition of the medium. This can be done by small-scale experiments; however, in most cases we had to conduct a series of experiments to achieve the desired high yield. The influence of the concentration of cells and cell debris in such systems has to be studied with regard to the yield of enzyme(s) as well as to operational parameters of the intended mode of separation.

VI. INFLUENCE OF THE POLYMERS

The partition coefficient of an enzyme in a two-phase system can be influenced by the average molecular weight and the concentration of the polymers. If it is desirable to shift the partition coefficient up, the most promising results are expected by lowering the average molecular weight of polyethylene glycol. In Fig. 2 some experiments are summarized that show the strong influence of this variable.

The influence of the average molecular weight of dextran is much smaller in comparism to polyethylene glycol (Fig. 3). This statement is restricted to dextran commonly used for partition having molecular

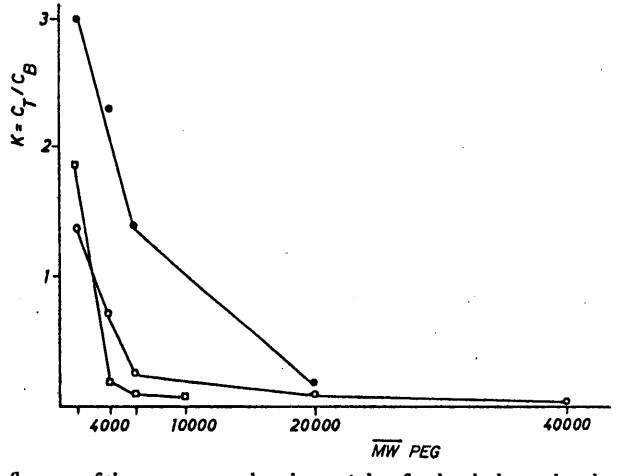

Fig. 2. Influence of the average molecular weight of polyethylene glycol on the partition of three enzymes: O—O pullulanase from *Klebsiella pneumoniae* (12% w/w polyethylene glycol, 1% w/w dextran T 500, 10 m*M* sodium phosphate, pH 7.5); □—□ 1,4-α-glucan phosphorylase from *K. pneumoniae* (9.3% w/w polyethylene glycol, 7% w/w dextran T 500, 50 m*M* potassium phosphate, pH 7.8); ●—● leucyl-tRNA synthetase from *Escherichia coli* (9.2% w/v polyethylene glycol, 6.3% w/v dextran T 2000, 73 m*M* potassium phosphate, pH 7.0).

It is possible to produce fruit juice without use of enzymes. Conventional food products on the market do not necessarily have to have an identical organic version, and in some cases, should certainly not, if we are to yield to the higher principle, which in this case is human nutrition. Enzymes should only be allowed in organic production if they serve the principle of maintenance of nutritional quality and truly are essential to the formation of the product. Being essential to achieving a desired technical effect which results in a product of degraded nutritive value should not be considered an essential need.

. . . [E]nzymes are compatible with principles of organic production, but only if they are placed in a larger perspective, not in all cases. It must be ensured that the ultimate nutritive value of foods is not robbed due to successive processing steps, where enzymes are an integral part in said processes, even if the enzymatic steps themselves do not result in the loss of nutritive value.

The discussion of enzymes for use in organic foods processing is complex, and several of the criteria discussed above overlap. Enzymes should be classified as a natural material, listed on the National List as being REGULATED, with the annotation being as proposed in the NOSB database, amended as discussed in this review (refer to section 2119(m)4 and NOSB processing criterion # 3, above). Otherwise, itemized decisions on individual enzymes (or types of enzymes) would be appropriate. Blanket acceptance of enzymes as processing materials is strongly discouraged.

Enzymes which are products of genetic engineering as defined by the NOSB should be classified as synthetic materials, and PROHIBITED for use in organic production systems.

Reviewer 2
Since pectin lyase is biosynthesized from Aspergillus or other fungal sources and is not chemically derived, I would classify this enzyme class as non-synthetic. This classification is predicated on the following criteria:
(i) Fungal organisms can not be derived from genetic engineered species and must be naturally occurring.
(ii) Extraction and manufacturing operations can not chemically modify or change the enzyme preparation.
(iii) All carriers, diluents and preservatives used in the final enzyme preparation shall be substances that appear on the National List of Ingredients allowed for use in foods labelled as organic.

I therefore agree with the OFPA status that this enzyme preparation if prepared from a non-GMO fungal source is non-synthetic. The risk to organic integrity depends on the isolation, purification and packaging (i.e., inclusion of stabilizers or preservatives).

Overall I found the NOSB materials database to be technically accurate. I agree with the proposed annotation inclusive of the three proposed criteria that qualify my recommendation that the NOSB consider that fungal enzymes (in this case, pectin lyase) are naturally derived enzymes but must be handled in a manner consistent with organic food processing criteria.

Reviewer 3
Fungal enzymes appear to be necessary for many types of food processing operations and the alternatives are either synthetic or less desirable. The use of fungal enzymes as described is compatible with organic production. Fungal enzymes, not produced through means of genetic modification should be added to the NOSB List of Allowed Non-organic ingredients.

Reviewer 4
Pectinase
This is a naturally occurring enzyme (or actually a class of them) and should not be considered synthetic. One note is that pectinase activity generally results in a loss of textural integrity; it's used to break down pectin for example in the juice industry. I don't think that there should be any question about the means by which pectinases are extracted or obtained from fungal cells. The enzyme database seems more straightforward to me. I don't think there have been any oversights in terms of information provided. There is nothing I disagree with here. I don't believe that enzymes could be considered "preservatives" (I always think microbial here!) but rather processing aids. The write-up on genetic engineering is well done; I agree that NOSB will probably not want to open that door again!

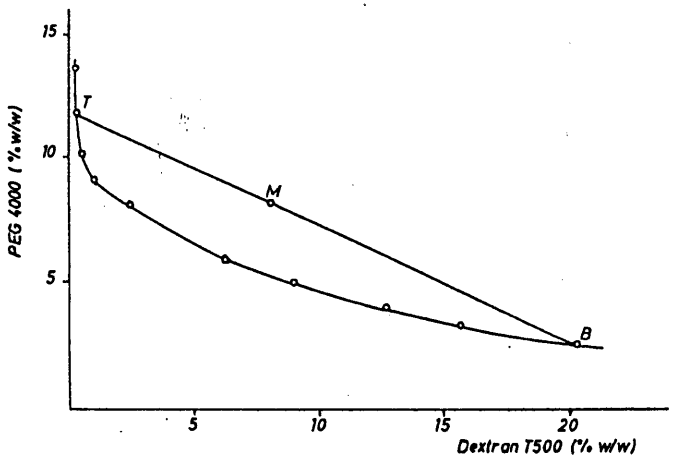

Fig. 1. Phase diagram of the polyethylene glycol 4000–dextran T 500 system at 20°C. (From Albertsson, 1971.)

polymers their total composition is represented by a point at or above the solid line in the diagram, phase separation occurs. The resulting phases have different compositions. Polyethylene glycol is concentrated in the upper phase and dextran in the lower phase. For a total concentration of 8.1% polyethylene glycol and 8.1% dextran, represented by point M in the diagram, the composition of the bottom phase is given by point B and the composition of the top phase is given by point T. All mixtures of polyethylene glycol and dextran with overall compositions represented by the so-called tie-line between T over M to B will yield phases with identical compositions but different volumes. It is important to note that the volume ratio of the phases is a complex function of the polymer concentration and other parameters. The volume ratio V_t/V_b can be estimated from the phase diagram. It is proportional to the ratio of the distance $(B - M)/(M - T)$, assuming that the density of the phases is not too far from 1. Also for such complex phase systems a partition coefficient K can be defined as

$$K = C_t/C_b \tag{3}$$

where C_t and C_b are the concentrations of the compound of interest in the top and bottom phases, respectively. K is constant in a large range of concentrations and can be used for the calculation of scale-up and performance. It is of special advantage that partition as a process does not depend on the absolute concentration of the desired product(s).

The partition coefficient K can generally be described by the Brønsted equation:

$$\ln K = \lambda M/kT \tag{4}$$

where M stands for the molecular weight of the compound, k for the Boltzmann constant, and T for the absolute temperature. λ is a parameter characterizing the phase system and the interaction with the compound of interest. Unfortunately, values for λ are not known for aqueous multiphase systems and the validity of the Brønsted equation for such systems has not yet been rigorously proven. Nevertheless some general aspects can be visualized from Eq. (4). For large values of M, small changes in λ will have a pronounced influence on K. For large molecules and particles, such as cells, phages, and high-molecular-weight DNA, partition coefficients of >100 to <0.01 are observed, while proteins in general show partition coefficients between 0.1 and 10, and small ions of around 1 (Albertsson, 1971).

The partition of a compound in aqueous two-phase systems is influenced by many parameters; the most important are listed in Table I. Any prediction is further complicated by the fact that most of these parameters influence each other, and no theoretical basis is available at present to analyze the complex systems in detail.

Occasionally, adsorption or precipitation of compounds takes place at the interface of a two-phase system. This has to be considered and checked if the yield is exceptionally low or not reproducible. Therefore, suitable if not optimal parameters for every extraction have to be found by trial experiments. This is comparatively easy if a fast analysis for the compound of interest is possible. The time needed for the determination of the concentration of the desired product in the upper and lower phase is the rate-limiting step in the development of a partition process. Numerous reports in the literature can guide the

TABLE I
SELECTED PARAMETERS INFLUENCING THE PARTITION OF PROTEINS IN AQUEOUS TWO-PHASE SYSTEMS

Polymers composing the two-phase system
Average molecular weight of the polymers
Concentration of the polymers
Kind of ions included in the system
Ionic strength
pH
Temperature

Conclusion

Enzymes are naturally occurring, widely used in food processing, and are currently used to process foods that are sold as organic without much controversy. The consensus is that enzymes from fungal and plant sources should be added to the National List of ingredients allowed for use in foods labeled as organic. However, there are some concerns that require annotations of what enzymes can be accepted. The reviewers appeared to all agree that not all enzymes are compatible with organic standards. The primary concern at present appears to be the degree that genetic engineering and recombinant DNA techniques are used and whether certain specific enzymes will be available in a non-genetically engineered form. Other concerns include extractants, preservatives, and incidental additives. While natural enzymes may be added to the National List, this does not imply to the TAP that all preparations that use natural enzymes will be formulated in a way that meets organic standards. Certifiers, processors, and suppliers are seeking clear, consistent industry guidelines on acceptable sources of enzymes. Finally, animal produced enzymes were not considered in this review and the NOSB may want to refer those to the TAP as well, or at least explicitly demur.

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somewhat surprising as the biochemical separation technique has reached a very high standard in the laboratory. In practice the scale-up of laboratory procedures beyond certain limits is difficult at best and impossible at times. Obviously, other and better methods are needed for the early steps in an isolation process when large amounts and volumes have to be handled.

For the disruption of cells, mechanical devices such as high-pressure homogenizers (Follows *et al.*, 1971; Brookman, 1975) and ball mills (Currie *et al.*, 1972; Marffy and Kula, 1974) have given satisfactory results even for large-scale work. The centrifugal sedimentation process employed for the removal of cell debris, however, is very difficult to perform on a large scale (Naeher and Thum, 1974). Centrifuges with the necessary capacities have low g forces, and one has to accept quite severe losses in clarification at this step, which in turn will disturb subsequent purification steps. The reason for the poor performance of the centrifuges becomes evident if one considers Eq. (1), which describes the throughput of a disk bowl centrifuge (Hemfort, 1970):

$$\dot{Q} = \left[\frac{d_{\text{llm}}^2 \Delta\rho}{18\eta} g\right] \left[\frac{2\pi}{3g} \omega^2 \tan\phi (r_1^3 - r_2^3) N\right] \qquad (1)$$

where $\Delta\rho$ is the density difference, η the dynamic viscosity, ω the angular velocity, g the earth acceleration due to gravity, d_{llm} the limit diameter of the smallest particle, N the number of the disk in the stack, $\tan\phi$ the angle of disk in the stack, r_1 the maximum radius of the disk, r_2 the minimum radius of the disk, and $\dot{Q}$ the volume of flow to the separator with time. An increase in the area equivalent—the second part of Eq. (1)—is limited by constructional parameters. The density between liquid and suspended matter is normally small, and the viscosity of extracts rather high; both parameters work in the same direction and make the process slow and inefficient. Filtration is also difficult to conduct under these conditions, as the density difference and viscosity are important parameters in such processes. The dimensions of broken cells and debris are expected to be even smaller than those of whole cells, which are already sometimes difficult to centrifuge due to their small size, as the diameter of the limit particle in Eq. (1) is of second order. It appeared to us that the extraction of intracellular enzymes and clarification of crude extracts could be accomplished much better on a large scale if the principle of separation was changed from a solid–liquid separation to a liquid–liquid separation with a concomitant increase of d_{llm}. This would require a two-phase system capable of suspending the cell debris and a partition of the desired product(s) in the opposite phase. Commonly used organic solvent systems are unsuitable for this purpose, as proteins are either insoluble or denature in organic solvents. But the use of aqueous two-phase systems appeared promising for the desired extraction.

II. INCOMPATIBILITY OF POLYMERS

Aqueous two-phase systems have been known since 1896, when Beijerinck described the formation of two liquid phases while mixing agar and soluble starch or agar and gelatin, (Beijerinck, 1896). Today the phenomenon is called "incompatibility of polymers," and is observed in organic solvents with synthetic macromolecules as well as in aqueous systems. The reason for the incompatibility is the inability of the polymer coils to penetrate into each other. Therefore, if one mixes polymer A with polymer B one does not obtain a homogeneous mixture but larger aggregates of the macromolecules with a strong tendency towards phase separation. As a result of the steric exclusion, the entropy difference between the two-phase state and a state of complete mixing becomes much smaller for macromolecules compared to that for low-molecular-weight substances. The entropy increase of mixing is a function of the number of molecules being mixed and not of their relative molmasses. If one considers a mixing process as an endothermic solution process, Eq. (2) can be applied:

$$\Delta F = \Delta H - T\Delta S \qquad (2)$$

Since ΔS becomes orders of magnitude smaller with macromolecules than with small molecules, a molecularly homogeneous mixture of polymers can only be expected when ΔH is negative. This will only be the case if the polymers show some interaction and the attraction between the different polymers is greater than that between the same kinds of polymers. Similar thermodynamic reasons can be discussed for the formation of polyethylene glycol–salt two-phase systems.

Aqueous multiple-phase systems have been extensively studied by Albertsson and his collaborators during the last 20 years (Albertsson, 1971; Albertsson, 1970; Albertsson, 1977). Our present understanding of the basic principles involved in partition of aqueous two-phase system is based heavily on his work.

III. GENERAL ASPECTS OF AQUEOUS TWO-PHASE SYSTEMS

Figure 1 represents a phase diagram of a polyethylene glycol–dextran system (Albertsson, 1971). Both hydrophilic polymers are miscible separately with water in all concentrations. If in a mixture of both

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Extraction and Purification of Enzymes Using Aqueous Two-Phase Systems

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I. INTRODUCTION

In 1973 Naeher and Thum quote in a paper on the production of enzymes for research and clinical use that "The purification of proteins is frequently more an art than a science" (Naeher and Thum, 1974). While methods and technology for screening of microorganisms and strain improvement are widely used to increase productivity, comparatively little work has been described regarding the optimization of fermentation and isolation processes for enzyme production. The last point is particularly true for intracellular enzymes. But with an increasing demand for enzymes as industrial catalysts, the production and recovery processes become important. Large-scale fermentation appears feasible today, but technology for the isolation of intracellular enzymes on a large scale is in part still lacking. The last observation is

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documents (MAFF, 1994) that indicate the thinking that is shaping legislation. Generally, the European Union (EU) guidelines are being implemented and the use of enzymes in foods will be regulated by considering each case using decision trees, the latest versions of which are described in the MAFF (1994) discussion document. Briefly, the decision tree defines a novel food as 'a food or food ingredient which has not hitherto been used for human consumption to a significant degree in the EC'. By asking a series of structured questions, the decision tree determines what information needs to be supplied by manufacturers who wish a novel food to be considered for general use. The key questions involve establishing the source of the enzyme, the presence and stability of any altered genetic material and whether the enzyme is active in the food. If an enzyme is used to effect a change during processing it is considered as a processing aid but if it has a function in finished product then it is considered as an additive. This shows the concepts behind any decision to permit the use of an enzyme in a food product and determines the typical information that must be supplied to satisfy concerns about safety. The situation is still under discussion and the guidelines remain flexible to allow new information or experience from other schemes to be included in future safety assessments. Up-to-date information on the current status can be obtained from MAFF who have a duty to consult interested parties and consider comments on the draft regulations. At the time of writing (August 1994), the MAFF telephone helpline was available on 01645 33 55 77. Advice can also be sought through trade associations like Leatherhead Food RA which offers help on legislation world wide. The industry seems generally satisfied with the current regulatory approach although there are other pieces of UK legislation that refer to particular foods (e.g. baked goods and cheese) which will need amendment to avoid conflict with any ACNFP recommendations.

The situation in other countries is different and has complicated the export and import of food between countries. Lists of national requirements tend to change quite quickly and manufacturers often rely on experts in trade associations or research associations for the latest information. At present, there is no evidence to link consumption of added enzymes in food with any deleterious effects in humans.

2.9 Use of enzymes

The specific applications of enzymes are considered in the following chapters but some general points can be made about the ways in which enzymes are used in the food industry. Examples from the brewing industry, where enzymes are an important part of the process, illustrate the ways in which enzymes are used. Since the enzyme activities for mashing are derived from the raw material malt, there are variations in the amount and activity of the enzymes

Enzymes

Livestock

Identification

Chemical Names:
Various. For bromelain: ananase, bromelin, extranase, inflamen, traumanase.
Other Names:
Many other enzymes are used in livestock production. The model enzyme used for the purposes of this review is bromelain. Other plant derived enzymes include amylase, ficin, and papain.

CAS Numbers:
9001-00-7; 37189-34-7
Other Codes:
IUB # s 3.4.22.32; 3.4.22.33

Recommendation

Synthetic / Non-Synthetic:	National List:	Suggested Annotation:
Non-synthetic (consensus)	*Allowed for use in livestock feed* *Allowed for livestock health care (consensus)*	*Must be derived from non-pathogenic bacteria, non-pathogenic fungi, or edible, non-toxic plants that are not genetically engineered as defined by the NOSB. Co-factors must either be organically produced or appear on the National List of ingredients allowed for use in foods labeled as "Organic." This includes water and substances that are insoluble in feed but removed from the feed after processing. (2-1)*

Characterization

Composition:
Enzymes are proteins composed of up to 20 amino acids (Nielsen et al., 1991). Plant enzymes are generally complex mixtures of proteins. Given their complexity, they have been difficult to either synthesize or isolate. For example, bromelain has a molecular weight of over 33,000 (Budavari, 1996).

Properties:
Enzyme preparations may consist of whole cells, parts of cells, or cell-free extracts from the source used. Individual preparations are generally characterized by functionality and activity rather than the properties of the product. Enyzmes may be in liquid, semi-liquid, or dry (crystalline) form. The color of preparations may vary from virtually colorless to dark brown (National Academy of Sciences, 1981). Bromelain is a combination of proteolytic (protein-digesting) enzymes that convert proteins into peptides by cleavage of peptide bonds (Budavari, 1996).

How Made:
Enyzmes may be extracted from bacteria, fungi, plants, or animals by a number of different methods (Nielsen, et al., 1991). The NOSB has previously considered bacterial enzymes for processing of food for human consumption (NOSB, 1995). Fungal enzymes are discussed in the TAP review for processing enzymes. Animal enzymes are not considered in this review. For the purposes of illustration, a plant-derived enzyme, bromelain, is used as a model enzyme for use in livestock production.

Bromelain can be isolated and purified from pineapple by several different methods. The stem tissue contains a different bromelain from the fruit (Collins, 1960; Budavari, 1996). Fruit bromelain was first isolated in 1891 (Budavari, 1996). Some methods involve the use of sodium chloride and ammonium sulfate (Collins, 1960). Other methods might involve the use of sodium cyanide and acetone (Tauber, 1949). Solvent extraction has been declining for a number of years (Pariza and Foster, 1983; a continuing trend confirmed by reviewers).

Bromelain can be extracted from plant tissue (pineapple) by salting out with sodium chloride followed by physically separating out the salt by physical methods such as dialysis or ultrafiltration. Tauber describes a process where alcohol is used to precipitate the bromelain (all enzymes are protein) from the plant extract

hazards in our diet are natural bacteria and fungal toxins... When a failure in processing occurred as in canned salmon in 1978, killing two people, sales were halved and the company lost £2 million. Motor vehicles kill about 100 people in Britain every week. Being run over by a lorry load of canned salmon is an acceptable hazard; being poisoned by it is not.' The consumers' current conception of food is that it should be absolutely safe. Furthermore, 'natural' foods are considered good and safe while processed foods have the 'goodness' taken out of them and are somehow perceived as inferior in safety and quality terms. While these perceptions are not backed by scientific evidence, manufacturers have to take note of consumer views and they have to adopt a pragmatic attitude, combining science and public opinion. Any discussion on safety testing is complicated by the many different types of legislation that vary country by country. Although the EC is working towards harmonisation in such matters, agreement has not yet been reached.

Enzymes may constitute a safety hazard on several grounds. Firstly, they are foreign proteins and may set off the immune response. Experience with biological washing powders highlighted this effect as certain individuals reacted when they inhaled the enzyme powder during the manufacturing process. It was recognised very quickly that powdered enzymes were a health hazard, thus most preparations are now in the form of solutions or suspensions. Minor allergenic responses associated with washing powders were also noted but it was established that enzymes were not the primary cause of this disorder (Denner, 1983). Generally, the consumer will not experience enzymes in the concentrated form but this only reduces the risk and does not completely remove it.

Secondly, the activity of enzymes may be injurious to humans. If enzymes remain active in the digestive tract, can they cause problems by attacking human tissue? The body's defences, however, are designed to cope with a wide range of active enzymes consumed in food and there is no evidence that enzymes added to food are an exception.

Thirdly, the source of enzyme has given some cause for concern as toxins may be incorporated into the crude enzyme preparations. Expert committees in the UK that considered the problem, reasoned that enzymes from plant and animal sources that are normally consumed by man did not require toxicological testing. Enzymes from microbial sources presented more problems as bacterial and fungal toxins are recognised as extremely toxic to humans and may also be carcinogenic. In the UK, minimum testing requirements have been defined together with guidelines for good manufacturing practice to avoid contamination with other pathogens or toxins.

In the UK, the Department of Health and the Ministry of Agriculture, Fisheries and Food (MAFF) have set up a committee to study the use of enzymes and the introduction of novel foods (Advisory Committee on Novel Food Products; ACNFP). ACNFP has produced several reports (e.g. Department of Health, 1991; ACNFP Annual Report, 1993) and consultation

(Tauber, 1949). The alcohol can be recovered and re-used. Organic bromelain could conceivably be manufactured from organically grown pineapple by at least three practical methods: the use of salt (sodium chloride), organic ethyl alcohol, or physical methods such as ultrafiltration or dialysis.

Because of the relative value of the fruit, the difficulty of processing the juice, and the improvements of methods to extract bromelain from stems, stem bromelain is the source of most bromelain. Because of the length of time it takes to grow plants relative to fermentation, there is interest in replacing plant enzymes with those from fermentation. Transgenic organisms are the likely source of such a fermentation product (Tucker and Woods, 1995).

Specific Uses:
Enzymes are used as feed components to improve digestibility, palatability and feed conversion; reduce mortaility; remove toxic substances from unconventional feed sources; and improve the consistency of manure or droppings and thus the ability to manage manure (Nielsen, et al., 1991). Bromelain in particular helps to make protein sources more digestible. Therapeutic uses of bromelain include as an anti-inflammatory, for obstetric manipulations, and to enhance the activity of drugs (Jayaran, Ahluwalia, and Cooney, 1991). Bromelain has been shown effective in reducing *E. coli* diarrhea in piglets (Mynott, Luke, and Chandler, 1996; Chandler and Mynott, 1998).

Action:
Enzymes act as catalysts. As such, they accelerate the rate at which various biochemical reactions achieve equilibrium, but are not themselves changed in the reaction. Protease enzymes such as bromelain remove various peptide bonds from proteins (Budaveri, 1996). Proteins that are in solution with protease enzymes like bromelain are more quickly broken down into proteins of lower molecular weight. In this way, bromelain makes protein from animal and vegetable sources more digestible by partially hydrolyzing protein into smaller peptides (Fennema, 1996).

Bromelain enhances serum fibrinolytic activity and inhibits fibrinogen synthesis (Lotz-Winter, 1990). Malignant cell growth appears to be inhibited by bromelain (Taussig and Batkin, 1988; Lotz-Winter, 1990). Application prevents or inhibits edema often associated with trauma (Lotz-Winter, 1990). When fed orally, bromelain inhibits the ability of enterotoxic diarrhea inducing *E. coli* to attach to the intestines of pigs (Mynott, Luke, and Chandler, 1996).

Combinations:
Enzymes often are included in whole cells or parts of the cells of the source (National Academy of Sciences, 1981). They are often packaged with various carriers that do not have catalytic activity that may or may not be synthetically derived (White and White, 1997). Synthetic preservatives are almost always added during processing, and may be present in the final preparation to prevent microbial growth and stabilize and maintained the desired enzymatic activity (Pariza and Foster, 1983). Bromelain is often combined with other enzymes, particularly the plant derived protease papain extracted from papaya fruit. Commercial preparations of bromelain may also include ammonium sulfate and monopotassium phosphate (White and White, 1997).

Status

OFPA
The substance is presumed to be used in handling and is non-synthetic but is not organically produced (7 USC 6517(b)(1)(C)(iii)).

Regulatory
Enzymes are regulated as feed additives under 21 CFR 573 and by state feed labeling laws. The association that develops uniform and equitable state feed labeling laws and regulations, the Association of American Feed Control Officials (AAFCO) recognizes that not all applications of enzymes used to process food for human consumption are directly transferable to animal applications (AAFCO, 1998). However, with a few specific exceptions, AAFCO recommends that the guidelines and annotations used for enzymes in food processing should be applied to enzymes used in animal feed milling. Bromelain is regulated for human food use under 21 CFR 184.1024.

Status among Certifiers

and medical use. The best-known food enzyme obtained from animals is rennin (EC 3.4.4.3) found in the stomachs of calves before they are weaned. The slaughter of young calves to produce rennet, however, is both emotional and economically wasteful. This has led to the development of a microbially derived alternative. In general, animals are poor sources of enzymes as they are slow-growing and expensive. Large-scale production of enzyme from animals therefore requires large numbers of animals and large capital outlay; and animal production lacks the flexibility if enzyme production needs to be suddenly decreased or increased. Extraction of enzymes from animal tissues can also be difficult, further adding to the production cost of the enzymes.

Plants grow more quickly than most animals and can be produced in quantity on an annual basis. Again, this time scale is too long for enzyme manufacturers and the only commercially important plant enzymes are proteases obtained from crops such as pineapple and papaya, which are important in their own right. For these reasons, enzyme production from microbes is preferred as they are fast growing, can be easily controlled during growth and produce enzymes that are easy to extract. In some cases, microbes produce extracellular enzymes making extraction and purification even simpler. The production and uses of microbial enzymes has been reviewed by Fogarty (1983).

2.8 Legal and safety implications

Enzymes are used in different ways in the processing of food, and their legal status depends on the application. In the manufacture of high-fructose syrup, hydrolysis is effected by free enzymes, whereas isomerisation is catalysed by immobilised enzymes. There is the possibility, therefore, that some amylase may find its way into the finished product but it is unlikely that any isomerase will be present. In the former case, the enzyme might be considered as an additive and subjected to the statutory additive safety testing programme. There is a subtle difference, however, in that an additive like a coal-tar dye shows colour properties over a wide range of conditions but an enzyme is more restricted and can be denatured in an irreversible manner. What tests are then appropriate and what labelling requirements are needed?

As a result of the consumers' view that food should be totally safe, these scientific and moral questions have to be addressed. The public's perception of food safety can be illustrated using analogies. Denner (1983) expresses it thus: 'When a traveller purchases an airline ticket he takes a positive decision to accept a small but quantifiable risk that the plane will crash, but when that same traveller enters the airport restaurant and purchases food, his expectation of the exposure to risk in consuming that food approaches zero.' A comparison between the relative dangers of motor vehicles and canned food was used by Angold *et al.* (1989) to illustrate the point: 'The major

Enzymes have historically been allowed by certifiers in feed labeled as organic.

Historic Use
Enzymes have been used by organic food processors. Because organic livestock has not developed as quickly as processing, enzymes appear to have been little used by organic livestock producers and feed mill operators.

International
In general, enzyme standards for international trade are set by the Joint FAO/WHO Expert Committee on Food Additives. Enzymes from non-GMO sources are allowed in both the current Canadian (Canadian General Standards Board, 1999) and European (European Union, 1999) organic standards. Enzymes are not specifically addressed in the most recent draft of the Codex Alimentarius organic standards. The most recent edition of the IFOAM *Basic Standards* considers enzymes acceptable for use in organic food processing provided they are based on the established Procedure to Evaluate Additives and Processing Aids for Organic Food Products (IFOAM, 1998). These standards are parallel to, but not exhaustively covered by the OFPA criteria.

OFPA 2119(m) Criteria

(1) The potential of such substances for detrimental chemical interactions with other materials used in organic farming systems.

Enzymes in the environment may accelerate the rate that pollutants are metabolized (Tinsley, 1979). This may be detrimental, beneficial, or have no net effect, depending on the substrate and metabolite.

(2) The toxicity and mode of action of the substance and of its breakdown products or any contaminants, and their persistence and areas of concentration in the environment.

This is discussed in the processing section below.

(3) The probability of environmental contamination during manufacture, use, misuse or disposal of such substance.

This is considered in the processing criteria below.

(4) The effect of the substance on human health.

This is considered in the context of the effect on nutrition and FDA GRAS status.

(5) The effects of the substance on biological and chemical interactions in the agroecosystem, including the physiological effects of the substance on soil organisms (including the salt index and solubility of the soil), crops and livestock.

Bromelain appears to have little acute toxicity for animals according to studies done in rodents and rabbits and yet possesses many of the therapeutic properties it demonstrates in humans (Lotz-Winter, 1989). It also has a protective effect against enterotoxigenic *E.coli* in pigs. (Chandler, 1998)

(6) The alternatives to using the substance in terms of practices or other available materials. See discussion of alternatives in (1) below.

(7) Its compatibility with a system of sustainable agriculture. This is considered more specifically below in the context of organic handling in (6) below.

NOSB Processing Criteria Used Here for Livestock Feed Processing

ture characteristics. It is worth testing a number of these to see if there are significant differences in performance or not. In the case of the proteases, there is a wide range available with pH optima from 2.5 to 9 although they do have different affinities for certain amino-acid bonds. Other types of enzymes generally have narrower ranges of optimum pH and some properties of common commercial enzymes are given in Chapter 1 and by Godfrey (1983). Recent advances in genetic engineering have provided the means for improving the stability of enzymes; this is achieved by altering the structure at vulnerable points by substitution of a different amino acid. These developments are discussed in Chapter 3.

Another factor that may limit the usefulness of an enzyme in the industrial context is product inhibition. In normal metabolism, this property is useful as it helps regulate metabolic pathways but if the enzyme is required to effect complete conversion of a substrate, the product needs to be removed to increase the percentage conversion. Enzyme processes need to be designed so that the desired changes can occur. The product may be removed to increase conversion and the design of enzyme reactors is critical.

When enzymes are used over relatively long periods and at elevated temperature, there is a decline in enzyme activity. In some applications, this is welcome as active enzyme may be unacceptable in the final food product. In other applications, it leads to decreased conversion rate and loss of efficiency. Again, design of the process can overcome these problems so that a constant degree of conversion is achieved.

2.7 Source of enzymes

Most organisms have certain 'core' enzymes in common. For instance, enzymes of the Embden–Meyerhof pathway can be found in microbes, plants and animals. Similarly, amylase activity is found widely in human saliva, in plant seedlings and in many microbes that use starch as an energy source. For enzymes like these, there are many potential sources. Other enzymes are specific to an organism or even give that organism its characteristic features. Examples are the specialised enzyme systems in nitrogen-fixing bacteria and the enzyme alliinase (EC 4.4.1.4), in onion and related plants, which catalyses the breakdown of a peptide precursor to liberate sulphur-containing volatiles that give the characteristic aroma. In cases like these, the source is limited as well as obvious. Techniques of genetic manipulation where genes can be removed from one species and transferred to a microbe, which then produces the protein (enzyme) on a commercial scale, have removed the technical problems of securing adequate sources of raw materials. The legal and safety status of engineered organisms is not totally clear, however, as discussed in Section 2.8.

Animals have traditionally produced some enzymes and products for food

A SYNTHETIC PROCESSING AID OR ADJUVANT may be used if;

1. An equivalent substance cannot be produced from a natural source and has no substitutes that are organic ingredients.

   Animals will secrete their enzymes as part of their digestive processes (Pond, Church, and Pond, 1995). Various physical, biological, and mechanical forms of processing can enhance the nutrient availability of feed, such as cooking (Sunde, 1973), fermentation (Oldfield, 1973), and pelleting (Slinger, 1973). Excessive processing can degrade feed quality, reduce nutrient content, and decrease nutrient availability (Morrison, 1951; Sunde, 1973). Higher quality organic feed may be a viable substitute for the use of enzymes to enhance inferior feed. Pineapple bran as a by-product of processing has historically been used as an animal feed, and was at one time shipped to the West Coast of the US for use as cattle feed (Morrison, 1951). Organic pineapple may at some point become a viable feed supplement.

   Other enzymes such as papain and rennet which are GRAS as feed additives may be considered alternatives from the viewpoint of a more acceptable regulatory status and perhaps equal status regarding sustainability. However, whether they are equivalent functionally as feed additives is debatable. Enzymes derived from bacteria can be used to substitute for plant-derived enzymes, with some limitations (Gallagher, et al., 1994).

   Bromelain's therapeutic uses may indicate it has a unique contribution to make to organic livestock production. For use as an anti-diarrheal in piglets, no acceptable non-chemical alternatives exist and conventional treatment is with antimicrobials / antibiotics (Aiello, 1998).

2. Its manufacture, use and disposal does not contaminate the environment.

   In general, enzymes are totally biodegradable (Nielsen et al., 1994). To be safe for human consumption, it is important for plant-derived enzymes to be extracted from plants that are both edible and non-toxic (Pariza and Foster, 1983). Food-grade and pharmaceutical-grade enzymes need to meet chemical and biological purity standards established by Food Chemicals Codex (National Academy of Sciences, 1981). Materials used to extract, concentrate, and standardize bromelain itself is non-toxic, but the production of the raw material and processing into purified form both may involve the use of toxic chemicals.

   Bromelain is derived from the fruit and stem of pineapples. Pineapples are edible, non-toxic plants. However, the production of conventional pineapples raise several concerns for contamination of the environment. Pineapple is a fruit crop with a history of intensive pesticide use. An increasing amount is grown outside the U.S. in coutries like Mexico, Honduras, and the Phillipines. The best data on pesticide use is from Hawaii. Targeted monitoring by the FDA has been conducted on pineapples throughout the 1980s and 1990s for N-methylcarbamates, organochlorines, organophosphates, atrazine and simazine. In 1991, a FDA survey of processed foods found that 17% of pineapple samples had residues of the carbamate pesticide - benomyl. In 1992, the food contamination database maintained by FDA found that Hawaii had pesticide residues in 72% of food samples tested and was the # 1 state for significant residues (14%) with a rate 2.5-fold greater than the next leading state (FDA, 1992).

   One source indicates that bromelain is manufactured by precipitation processes involving acetone and ammonium sulfate. Neither of these chemicals appear to have a significant toxicity profile. Ammonium sulfate is not considered a workplace hazard. Sodium chloride--table salt--is allowed in organic production. Acetone is moderately toxic (Sax, 1984). However, an earlier source indicates that sodium cyanide is involved in the precipitation process. (Tauber, 1949) Sodium cyanide has a toxicity rating of 6 (on a scale of 1 to 6 with 6 being highly toxic) and is one of the fastest poisons known to man (Gosselin, et al., 1984). Chronic exposure to low levels of cyanide is suspected to be responsible for various neuropathic and thyrotoxic conditions in humans (NTP, 1993).

3. If the nutritional quality of the food is maintained and the material itself or its breakdown products do not have any adverse effect on human health.

coli. These micro-organisms then synthesise prochymosin, an inactive precursor of the final enzyme. The prochymosin can be purified and converted to chymosin by treatment at low pH. The resultant enzyme is indistinguishable from natural calf chymosin and the cheese product is also identical to that manufactured using natural calf chymosin. For a general review of this work see Teuber (1993). The use of this micro-organism derived chymosin has been approved by the FAO and is now accepted in over 20 countries. The enzyme is marketed by several companies under the trade names of MAXIREN®, CHYMOGEN® and CHY-MAX®.

Maltose utilisation by bakers yeast. Normal strains of *Saccharomyces cerevisiae* used in baking exhibit repression of maltose utilisation when grown in the presence of glucose, fructose or mannose. This can be a problem when sweet doughs are required for the baking process. A novel strain of *S. cerevisiae* has been developed by Gist Brocades in which maltose utilisation is not subject to repression by these other sugars. This has been achieved by genetically manipulating the yeast with a DNA construct in which genes for the two enzymes required for maltose utilisation—maltase and maltose permease—are under the control of a strong promoter. This novel strain can thus grow and metabolise maltose efficiently on both lean and sweet doughs. The new strain was approved for use in Great Britain in 1990.

Improved tomato processing lines. High viscosity is an important quality parameter in tomato paste and ketchup products. This viscosity is due in part to the nature of the pectin derived from the tomato fruit. The fruit contain endogenous enzymes, in particular polygalacturonase and pectinesterase, the combined action of which results in the complete degradation of the fruit pectin and hence in a low viscosity product. Genetic engineering has been used to 'inactivate' genes for these two enzymes (Tucker, 1993) with the result that levels of these enzymes in transgenic fruit are less than 1% of normal. In the case of reduced polygalacturonase activity pectin degradation in the fruit is also reduced (Smith *et al.*, 1990) and the fruit as a result are more resistant to cracking and easier to transport (Schuch *et al.*, 1991; Tucker, 1993).

The paste produced from fruit with reduced polygalacturonase activity is significantly more viscous than that from normal fruit when analysed by an industry standard Bostwick test (Schuch *et al.*, 1991). Approval has been given for the sale of these genetically engineered fruit in America and it is envisaged that approval for their use elsewhere will follow shortly. Approval for the marketing in the UK of paste prepared from these genetically engineered fruit was given in January 1995.

It can be seen from the three examples above that this technology has much to offer the food processing industry. Fuller exploitation in many cases depends on a better understanding of the basic scientific principles of pro-

For all practical purposes, bromelain is non-toxic. Different studies have been unable to derive an LD_{50} that would induce death in rats or mice (Lotz-Winter, 1990). Most reported effects are beneficial, such as anti-carcinogenesis (Taussig and Batkin, 1988; Lotz-Winter, 1990). Bromelain improves digestibility of proteins, therefore it enhances nutritional quality (Chandrasiri, et al., 1990; Gallagher, Kanekanian, and Evans, 1994). Isolated soy protein treated with bromelain to remove bitterness peptides had over twice as much available lysine as acetylated soy protein (Yeom, Kim, and Rhee, 1994). By removing bitterness peptides, feed may be made more palatable.

Pigs given bromelain orally as an anti-diarrheal agent showed no adverse effects (Chandler and Mynott, 1998). Bromelains are used in human medicine in the treatment of soft tissue inflammation, edema from trauma and surgery, and as an aid to digestion. Adverse effects include nausea, vomiting, diarrhea, and menstrual flow abnormalities. However, the only precautions suggested were for cautious use in patients with impaired hepatic or renal function (Reynolds, 1996).

Like all proteins, enzymes carry with them the possibility of inducing allergies (Nielsen, et al., 1991). Its use as a food processing enzyme had not resulted in any reported cases of allergies in consumers as of the early 1980s, as might be expected due to its presence in foods at very low levels (Pariza and Foster, 1983). Allergies through other exposures may not be well-understood. Hypersensitivity reactions have occurred, including skin reactions and asthma, generally from occupational inhalation exposure or direct skin challenge allergy testing. There also, appears to be some cross-reactivity with papain manifested by skin reactions. Bromelain may also work synergistically in the presence of a given protein or free amino acid allergen, even when it is not an allergen *per se* (Pike, Bagarozzi, and Travis, 1997). Feed-mill workers who handle enzymes should wear proper clothing and respiratory equipment.

4. Is not a preservative or used only to recreate/improve flavors, colors, textures, or nutritive value lost during processing except in the latter case as required by law.

   Enzymes are not used for any of these purposes. However, enzymes may be used to improve the nutrient quality and digestibility of inferior feeds (Nielsen, et al., 1991). Bromelain is not used as a feed preservative.

5. Is Generally Recognized as Safe (GRAS) by FDA when used in accordance with Good Manufacturing Practices (GMP), and contains no residues of heavy metals or other contaminants in excess of the tolerances established by FDA.

   At present, bromelain is not specifically GRAS as a feed additive. The only two such enzymes are papain and rennet (21 CFR 582). For an interim period, FDA will accept as GRAS for use as animal feed additives those enzymes that are GRAS for human food (AAFCO, 1998). Bromelain qualifies as GRAS for such use under this provision per 21 CFR 184.1024:

   > "(a) Bromelain (CAS Reg. No. 9001-00-7) is an enzyme preparation derived from the pineapples *Ananas comosus* and *A. bracteatus* L. It is a white to light tan amorphous powder. Its characterizing enzyme activity is that of a peptide hydrolase (EC 3.4.22.32).
   >
   > "(b) The ingredient meets the general requirements and additional requirements for enzyme preparations in the Food Chemicals Codex, 3d ed. (1981) . . .
   >
   > "(c) In accordance with Sec. 184.1(b)(1), the ingredient is used in food with no limitation other than current good manufacturing practice. The affirmation of this ingredient as GRAS as a direct food ingredient is based upon the following current good manufacturing practice conditions of use:
   >
   > "(1) The ingredient is used as an enzyme as defined in Sec. 170.3(o)(9) of this chapter to hydrolyze proteins or polypeptides.
   > "(2) The ingredient is used in food at levels not to exceed current good manufacturing practice.
   >
   > "[60 FR 32910, June 26, 1995]"

with large-molecular-weight substrates such as proteins and complex carbohydrates. This problem can be overcome by the use of a specialised form of immobilisation support made from a so called 'soluble-insoluble' polymer. This type of support changes its solubility with respect to pH. Thus the processing reaction can be carried out at a pH at which the enzyme–support complex is freely soluble. On completion of the reaction the pH is altered to render the enzyme–support complex insoluble. The enzyme can then be recovered by precipitation and reused. Such enzyme–support systems have been described for protease (Fujimura *et al.*, 1987) and chitinase (Chen and Chang, 1994).

1.7 Genetic engineering

The application of genetic engineering techniques is already having an impact on food production and this is likely to increase in the future (OECD, 1992). There are several ways in which this technology can be applied to the manipulation of enzymes important in food processing. The ability to transfer genes between organisms can be used for cheaper and more efficient production of enzymes. The ability to carry out protein engineering can be used to alter the properties of an enzyme, e.g. pH optimum, temperature stability or substrate specificity, such that its efficiency is improved. The raw products can be manipulated so as to enhance the activity of beneficial, and reduce the activity of deleterious, endogenous enzymes. The enzymic complement of micro-organisms employed in food processing can be manipulated, again to improve their efficiency and range of applications.

Unless involved directly in product development the food processor in most cases need not understand the complex techniques involved in genetic engineering. As such these will not be covered in this chapter. For more information the reader is directed to Chapter 3, to the texts by Brown (1990), Glover (1984), Old and Primrose (1989), and for information more closely related to the use of enzymes in food processing, to Gerhartz (1990). Instead the importance of this technology will be examined more closely by considering three current commercial applications.

Production of chymosin in microorganisms. Chymosin (E.C 3.4.24.4) is used in the production of cheese, the industry as a whole requiring an estimated 56 000 kg per annum. The traditional source of this enzyme is calf stomach, the supply of which is gradually declining. There are microbial substitutes for chymosin but their use is restricted due to the production of adverse flavours. Genetic engineering has provided an alternative supply of this enzyme.

The cDNA for calf chymosin has been cloned into a range of micro-organisms including *Kluyveromyces lactis*, *Aspergillus niger* and *Escherichia*

6. Is compatible with the principles of organic handling.

   Enzymes appear to be compatible given their natural origin and specific mode of action. The two main questions are (1) should enzymes from plants be required to be derived from organically raised plants? and (2) which, if any, extractants should be allowed to isolate and concentrate the enzyme? The reviewers addressed these questions in the discussion.

7. There is no other way to produce a similar product without its use and it is used in the minimum quantity required to achieve the process.

   To assist in increasing the digestibility and palatability of certain foods and feeds, use of some enzymes may be beneficial. Enzyme concentrations in feed processing are effective at levels of less than 1% (Bedford, 1995). The minimum amount showing a positive effect in piglets was 250 mg/day. In hydrolyzing casein, the amount used is 1:500 bromelain:casein. Hydrolysis occurred with smaller amounts of enzymes derived from bacteria. However, bromelain has certain properties that are preferable to other bacterially derived protease enzymes screened (Gallagher, Kanekanian, and Evans, 1994).

Discussion

Condensed Reviewer Comments

None have a direct commercial or financial interest in enzymes in general or bromelain in particular. Reviewer 1 is a consultant in animal nutrition; reviewer 2 is a veterinarian and pharmacology researcher; reviewer 3 is a professor of food science.

Reviewer 1
(1) Should enzymes from plants be required to be derived from organically raised plants?
No strong feelings here but I would not think this would be necessary.

(2) Which, if any, extractants should be allowed to isolate and concentrate the enzyme?
The sodium cyanide and acetone method does not seem particularly compatible with organic standards and purposes, and therefore perhaps should not be allowed, but again no strong convictions on this question. However, [I] think it should be stated that production methods must be compatible with organic standards and purposes.

Enzymes derived from edible, non-toxic plants that have not been genetically engineered should be allowed as a non-organic ingredient, or if obtained in an organic manner from an organic plant as an organic ingredient, in food and as a feed additive for organic livestock production.

Reviewer 2
Based upon the literature, I would classify bromelain or any other plant derived enzyme as non-synthetic. However, the determinant is how it is isolated and manufactured. If the enzyme is manufactured using sodium chloride or organic ethyl alcohol followed by physical separation and concentration, then I believe that the enzyme should retain its non-synthetic status. Additionally, the source of all plant-derived enzymes must be from non-genetically engineered sources. Overall, I agree with the [information provided]. However, the official name for bromelain is spelled bromelain, not bromelin according to Fennema. I feel the most important issue outside the non-genetically engineered requirement, is how the enzyme is manufactured. Therefore the manufacturers of all commercial preparations of plant enzymes and microbial derived enzymes should be required to submit a process flow diagram showing use of all chemicals and process operations. This requirement is similar to the proposed annotation.

In summary, I agree with OFPA that bromelain as a plant derived enzyme is not synthetic and must meet the requirements of the proposed annotation to retain its use as an organic feed additive.

Reviewer 3

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The first question is whether it is synthetic or not. This may be a close question. The processes described in the literature for its derivation from pineapples appear to be more physicochemical processes, i.e., filtration, washing, dissolution, and precipitation, designed to separate the substance from its biological matrix in the natural state rather than processes implemented for the purpose of inducing chemical change through reaction and conversion. Nonetheless, it is extracted "by a chemical process" and therefore is presumptively synthetic unless a particular petitioner wants to put forth proof to the contrary for his or her particular product.
The next question is what is its use in organic livestock production - is it a medicine or a routine feed additive. It could be considered either one as continued research will probably add to its value as a "homeopathic" (using the word in the broad sense) therapeutic agent perhaps giving it wider scope for inclusion in organic livestock production. In either case, its use appears to be compatible with a program of sustainable agriculture. I would, however, concur with the proposed annotation for these substances in the NOSB Materials Database and add in the case of bromelain that it should be derived from organically grown pineapples (because of the particular environmental and health implications associated with the pesticidal regimens typically used in pineapple production) unless that is currently a commercial impossibility.

Conclusion

While enzymes may not be necessary in every situation, they appear to be naturally occurring substances that are compatible with organic principles. Enzyme treatment of some feedstuffs may improve amino acid availability and as a result reduce nitrogen pollution (Tamminga and Verstegen, 1992). Some plant enzymes may also have some anthelmintic properties (Tauber,1949). By improving feed efficiency, animals can meet their nutritional needs with less feed and produce less manure (Bedford, 1995). Enzymes derived from edible, non-toxic plants, and non-pathogenic bacteria or fungi that have not been genetically engineered should be allowed as a non-organic ingredient in food and as a feed additive for organic livestock. The suppliers of enzymes should report any co-factors used in the formulation. Carriers, diluents, and processing aids used in enzyme preparations for animal production must be of feed grade and be allowed for use in organic food processing or organic livestock production.

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TABLE 10
Higher alcohol content of wines (mg litre^{-1}) obtained from musts treated with the yeast pectinase and a commercial preparation

	Propanol	*Isobutanol*	*2-Methyl-1-butanol*	*3-Methyl-1-butanol*	*Total higher alcohols*
Control	8·6^{a}	20·9^{a}	69·6^{b}	282·2^{b}	380·0^{a}
Pectinex (2 g hl^{-1})	14·5^{b}	41·4^{b}	64·2^{ab}	259·9^{a}	379·0^{a}
Yeast pectinase (10 g hl^{-1})	7·8^{a}	43·1^{b}	62·7^{a}	265·0^{ab}	379·0^{a}
Yeast pectinase (20 g hl^{-1})	14·5^{b}	42·9^{b}	66·3^{ab}	270·0^{ab}	392·7^{a}

^{a,b} Column means followed by the same letter are not significantly ($P > 0·05$) different as determined by LSD test.

noticeable decrease in the colmability index, 223 and 30 for the control and the wine treated with the yeast pectinase, respectively.

Volatile compounds

The headspace GC analysis showed a definite ($P \leqslant 0·05$) increase in the ester content of the wines obtained by enzymic maceration (Table 9). The main volatile compounds involved in this increase were ethyl butyrate and ethyl decanoate. However, the increase was that recorded with higher alcohols, such as 1-propanol and 2-methyl-1-propanol (Table 10): these are very important compounds for flavour formation in red wines (Bertuccioli and Viani 1976).

This phenomenon is due to the lower availability of fats and to the greater availability of soluble nitrogen (Montedoro 1985).

CONCLUSIONS

The overall results obtained in this investigation indicate the different behaviour of the yeast pectinase. In olive oil extraction the process efficiency and sensorial qualities of the product are better. In red wine making it failed adequately to improve the monomeric and oligomeric anthocyanin concentrations and colour intensity as well as the turbidity of the wines.

The improved olive oil extraction efficiency, as well as the overall better quality achieved in this study, indicate that the endo-polygalacturonase produced by *Cr albidus* var *albidus* could have great potential in the olive oil extraction industry. Work is now in progress at pilot and commercial plant levels to further investigate this possibility.

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TABLE 8
Pectins, colloids, turbidity and filterability of untreated and enzyme-treated wines at the end of juice–skin contact

	Total pectins (*g litre*$^{-1}$)	*Pectins soluble in* H_2O (*g litre*$^{-1}$)	*Total colloids* (*g litre*$^{-1}$)	*Turbidity* (*NTU*)	*Filterability* (*CI*)
Control	3·10^{b}	0·85^{a}	7·8^{a}	410^{a}	223^{c}
Pectinex (2 g hl^{-1})	2·20^{a}	0·73^{a}	8·4^{ab}	600^{b}	12^{a}
Yeast pectinase (10 g hl^{-1})	3·00^{b}	0·90^{a}	8·8^{b}	750^{b}	130^{b}
Yeast pectinase (20 g hl^{-1})	2·92^{b}	1·03^{a}	10·1^{c}	1150^{c}	30^{a}

$^{a-c}$ Column means followed by the same letter are not significantly ($P > 0·05$) different as determined by LSD test.
CI, Colmability index.
NTU, Nephelometric turbidity units.

Phenols and colour stability

After 20 h of maceration in contact with the skins there was a marked increase in the phenolic content of the must treated with the enzymic preparations; total phenols, tannic phenols, tannic catechins, total anthocyanins and colour intensity were affected (Table 6).

In the enzyme-treated musts (yeast pectinase at 20 g hl^{-1}), the anthocyanin, tannic phenol and tannic catechin content increased but the colour intensity decreased. This contradictory effect is explicable by the increase in polymerised phenols that reduced the monomeric anthocyanin concentration.

After prolonged maceration in contact with the skins (Table 7) the enzyme-treated wines presented an increased content of polymeric phenols and total anthocyanins but not of colour intensity; only in the Pectinex-treated wine was the colour intensity greater.

The particular phenolic composition, as well as the colour development during skin contact, lead to the conclusion that the raw enzymic preparation from IMAT 4735 of *Cr albidus* var *albidus* was not enough to act consistently on the wall colloids and consequently, to induce a sufficient extraction of monomeric anthocyanins. Also, anthocyanase and/or phenoloxidase activities were probably present.

Colloidal fraction, turbidity and filterability

At the end of maceration in contact with skins, the musts obtained by enzymic treatment were richer in colloids and consequently more turbid than the untreated ones (Table 8). Moreover, the concentration of low molecular weight pectins (water-soluble) was greater in the wine treated with the yeast pectinase which implied better filterability compared with the control; in this case, however, the data were not statistically significant. That the filterability was better is also shown by

TABLE 9
Volatile compounds (% area) of the headspace of the wines obtained from musts treated with the yeast pectinase and a commercial preparation

	Isobutyl acetate	*Ethyl butyrate*	*Isoamyl acetate*	*Ethyl capronate*	*Hexanol*	*Total area* (*IU*)*
Control	1·4^{b}	11·7^{a}	77·3^{a}	2·9^{a}	6·7^{b}	1933532^{a}
Pectinex (2 g hl^{-1})	0·9^{a}	10·0^{a}	75·4^{a}	7·0^{b}	6·7^{b}	2601410^{b}
Yeast pectinase (10 g hl^{-1})	0·8^{a}	11·6^{a}	78·6^{a}	3·6^{a}	5·2^{a}	2485371^{b}
Yeast pectinase (20 g hl^{-1})	0·9^{a}	12·3^{a}	72·6^{a}	8·7^{c}	5·4^{a}	2945133^{b}

* IU = Integration unit.
$^{a-c}$ Column means followed by the same letter are not significantly ($P > 0·05$) different as determined by LSD test.

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... definite sensation of freshness and fruity flavour (Montedoro *et al* 1978).

... wine

General characteristics

As for the general analytical features of the finished ..., no marked differences ($P > 0.05$) between the two enzymes employed were observed except for their dry extract and methanol content (Table 5). Compared with the control (1·7 ml methanol litre^{-1} ethanol), the methanol content of the wine obtained by treatment with the commercial enzymic preparation increased substantially (2·16 ml litre^{-1} ethanol) while a very limited difference was induced by the yeast pectinase (1·72 ml litre^{-1} of ethanol).

TABLE 5

Chemical characteristics of untreated and enzyme-treated wines after 3 months' storage

	Ethanol (*ml litre*$^{-1}$)	*Total acidity* (*g litre*$^{-1}$)*	*Volatile acidity* (*g litre*$^{-1}$)†	*pH*	*Dry extract* (*g litre*$^{-1}$)	*Ash* (*g litre*$^{-1}$)	*Methanol* (*µl litre*$^{-1}$ *of ethanol*)
Control	126·3^a	7·80^a	0·20^a	3·17^a	27·4^b	1·94^a	1700^a
Pectinex (2 g hl^{-1})	125·1^a	8·10^a	0·22^a	3·24^a	27·4^b	1·61^a	2160^b
Yeast pectinase (10 g hl^{-1})	123·7^a	7·87^a	0·18^a	3·20^a	25·0^a	1·89^a	1720^a
Yeast pectinase (20 g hl^{-1})	122·0^a	7·87^a	0·18^a	3·17^a	26·0^a	1·90^a	1740^a

* As g of tartaric acid litre^{-1}.

† As g of acetic acid litre^{-1}.

‡ Column means followed by the same letter are not significantly ($P > 0.05$) different as determined by LSD test.

TABLE 6

Phenolic content and colour of untreated and enzyme-treated musts and wines after 20 h of juice–skin contact

	Total phenols (*mg litre*$^{-1}$)	*Tannic phenols* (*mg litre*$^{-1}$)	*Total catechins* (*mg litre*$^{-1}$)	*Tannic catechins* (*mg litre*$^{-1}$)	*Total anthocyanins* (*mg litre*$^{-1}$)	*Colour intensity*	*Hue*
...ntrol	935^a	230^a	340^a	80^a	512^a	21·6^b	0·39^a
Pectinex ... g hl^{-1})	1120^c	331·3^c	340^a	140^b	606^b	14·8^a	0·38^a
...east pectinase ... g hl^{-1})	950^a	230^a	340^a	140^b	540^a	12·8^a	0·39^a
...east pectinase ... g hl^{-1})	1060^b	250^b	395^a	150^b	614^b	14·7^a	0·38^a

Column means followed by the same letter are not significantly ($P > 0.05$) different as determined by LSD test.

TABLE 7

Phenolic content and colour of untreated and enzyme-treated musts and wines at the end of juice–skin contact

	Total phenols (*mg litre*$^{-1}$)	*Tannic phenols* (*mg litre*$^{-1}$)	*Total catechins* (*mg litre*$^{-1}$)	*Tannic catechins* (*mg litre*$^{-1}$)	*Total anthocyanins* (*mg litre*$^{-1}$)	*Colour intensity*	*Hue*
...ntrol	2035^a	810^b	900^a	400^a	713·3^b	13·4^a	0·35^a
Pectinex ... g hl^{-1})	2280^c	745^a	1050^b	450^b	783·0^c	15·6^a	0·36^a
Yeast pectinase ... g hl^{-1})	2200^b	830^c	1050^b	450^b	650·0^a	12·7^a	0·35^a
Yeast pectinase ... g hl^{-1})	2325^d	893^d	1050^b	450^b	660·0^a	13·2^a	0·35^a

‡ Column ... followed by the same letter are not significantly ($P > 0.05$) different as determined by LSD test.

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TABLE 2

Physicochemical characteristics and autoxidation stability of the oil obtained by treatment with the yeast pectinase and a comm... preparation

	Turbidity (NTU)	*Free acidity (g kg^{-1} as oleic)*	*Peroxide number*	*Total chlorophyll (mg kg^{-1})*	*Total phenols (mg kg^{-1})*	*Autoxidation stability (h)*	
Control	110^{b}	4·2^{a}	12·70^{a}	24·5^{a}	67·0^{a}	4·75^{a}	
Olivex (200 g t^{-1})	50^{a}	3·6^{a}	15·10^{b}	28·6^{b}	68·0^{ab}	4·80^{a}	
Yeast pectinase (100 g t^{-1})	43^{a}	4·4^{a}	11·06^{a}	30·1^{b}	71·0^{bc}	5·15^{ab}	
Yeast pectinase (200 g t^{-1})	49^{a}	4·2^{a}	10·45^{a}	29·0^{b}	75·0^{c}	5·30^{b}	

$^{a-c}$ Column means followed by the same letter are not significantly ($P > 0·05$) different as determined by LSD test.

and increased the oxidation induction time. This is in agreement with previous findings (Montedoro and Servili 1989, 1990b). Differences in free acidity were observed.

The same treatment also increased the content of some phenolic acids such as *p*-hydroxyphenylethanol, 3,4-dihydroxyphenylethanol, ferulic acid etc (Table 3). It is interesting to note that the increase in the content of 3,4-dihydroxy-phenethyl alcohol and *p*-hydroxy-phenethyl alcohol was significantly ($P \leq 0·05$) correlated with the enzyme concentration employed.

With the exceptions of turbidity and chloro... content, no significant differences in the overall ... pearance of the oil were observed (Table 2). How... the increased limpidity and chlorophyll content ... to indicate a positive effect of the enzyme treatm...

Furthermore, as shown in Table 4, the oils prod... by enzymic treatments (both Olivex and yeast pectin... were characterised by a lower content of ... compounds and a more favourable ratio ... aldehydes (*n*-hexanal and *trans*-2-*n*-hexenal) ... cohols (hexanol). Characteristically, this resulted ...

TABLE 3

Phenolic composition (mg kg^{-1}) of the oils obtained from pastes treated with the yeast pectinase

	3,4-Dihydroxy-phenylethanol	p-*Hydroxy-phenylethanol*	*Catechin*	*Vanillic acid*	*Syringic acid*	p-*Coumaric acid*	*Ferulic acid*	*Cin... ac...*
Control	0·174^{b}	1·248^{a}	0·686^{a}	0·056^{a}	0·28^{a}	0·025^{a}	0·074^{a}	0·0...
Yeast pectinase (100 g t^{-1})	0·143^{a}	1·825^{b}	1·253^{b}	0·068^{a}	0·44^{a}	0·031^{a}	0·133^{b}	0·0...
Yeast pectinase (200 g t^{-1})	0·245^{c}	2·507^{c}	1·079^{ab}	0·082^{a}	0·42^{a}	0·035^{a}	0·104^{ab}	0·05...

$^{a-c}$ Column means followed by the same letter are not significantly ($P > 0·05$) different as determined by LSD test.

TABLE 4

Volatile compounds (% area) of the headspace of the oils obtained from pastes treated with the yeast pectinase and a comm... preparation

	Pentanal	*Isobutyl acetate*	*Hexanal*	*1-Penten-3-ol*	*Heptanal*	trans-*2-Hexenal*	*3-Hexenyl acetate*	*Hexanol*	*Nonanal*	*To... ac... (IU...*
Control	26·7^{b}	24·9^{b}	11·0^{a}	7·2^{a}	2·9^{a}	20·8^{a}	1·5^{a}	2·9^{a}	1·80^{a}	112...
Olivex (200 g t^{-1})	13·8^{a}	9·6^{a}	16·2^{b}	12·9^{b}	3·3^{a}	34·2^{b}	2·8^{b}	3·7^{ab}	2·97^{b}	563...
Yeast pectinase (100 g t^{-1})	14·7^{a}	8·2^{a}	14·9^{b}	13·7^{b}	4·7^{a}	32·5^{b}	3·5^{b}	4·7^{b}	2·80^{b}	470...
Yeast pectinase (200 g t^{-1})	22·5^{ab}	5·7^{a}	11·5^{a}	13·8^{b}	4·4^{a}	31·4^{b}	3·0^{b}	5·0^{b}	2·70^{b}	599...

NOSB NATIONAL LIST
FILE CHECKLIST

PROCESSING

MATERIAL NAME: **# 9 Cultures, Dairy**

✓	**NOSB Database Form**
✓	**References**
_____	**MSDS (or equivalent) .**
_____	**FASP (FDA)**
✓	**TAP Reviews from: Rich Theuer**

precipitation with methylcellulose (Montedoro and Fantozzi 1974). Anthocyanins were assayed according to the method described by Somers and Evans (1974). Total catechins were assessed colorimetrically with a vanillin reagent (Pompei and Peri 1971); tannic catechins were evaluated using the same reagent, measuring the colorimetric differences before and after precipitation with methylcellulose (Montedoro and Fantozzi 1974). Colour intensity was quantified as the sum of the absorbances at 420 nm and 520 nm; hue was measured as the ratio of the absorbances at 420 nm and at 520 nm (Ough and Amerine 1988). Reducing sugar, total acidity, volatile acidity, ash, dry extract, ethanol and methyl alcohol were determined according to AOAC (1984) methods.

The aromatic constituents of the wine were analysed using a Varian Model 3700 gas chromatograph; both the volatile constituents of the headspace (Bertuccioli and Montedoro 1974) and those determined by direct injection (Bertuccioli 1982) were examined.

Statistical analysis

This was performed by analysis of variance; the test of least significant difference (LSD; Snedecor and Cochran 1969) was used to compose the means of five repetitions for the olive oil and three repetitions for the must and wine.

RESULTS AND DISCUSSION

Olive oil

Extraction yield

As shown in Table 1, the data demonstrate that the addition of the raw enzyme preparation from the yeast *Cryptococcus albidus* var *albidus* IMAT-4735 to the olive paste resulted in a significant increase ($P = 0.05$) in extracted oil with respect to the untreated and Olivex-treated olives (228, 228 and 236 kg Mg^{-1} of olives, respectively). In previous studies (Fantozzi *et al* 1977; Siniscalco and Montedoro 1988; Siniscalco *et al* 1989), the use of Olivex generally resulted in an increase in oil yield. In our work, however, it is possible that the peculiar rheological state of the olive pulp, due to the particularly dry season, required an enzyme preparation more active in the depolymerisation of the pectic structure of the mesocarp.

Quality

The quality of the oil is determined on the basis of the free acidity (Reg CEE No 1915/87, 2 July 1987), the compounds responsible for colour, smell and taste and the resistance to autoxidation (Montedoro *et al* 1978; Servili and Montedoro 1989).

Various compounds have been indicated as responsible for the sensory characteristics of the oil (Guitierrez *et al* 1977; Montedoro *et al* 1978; Montedoro 1985; Solinas *et al* 1988; Montedoro *et al* 1990). The 'fruity' sensation depends on the concentration of unsaturated C_5 and C_6 aldehydes and alcohols and phenolic compounds; in particular, 3,4-dihydroxy-phenethyl alcohol is a very important antioxidant (Chimi *et al* 1988; Servili and Montedoro 1989).

On this basis we have determined the headspace volatile compounds, phenolic compounds and oxidation induction time, as well as other parameters.

Free acidity, peroxide number, total phenols and induction time values are reported in Table 2. The treatment with the yeast pectinase increased the phenolic content and consequently reduced the peroxide number

TABLE 1

Comparative technological evaluation of the enzyme produced by *Cryptococcus albidus* var *albidus* (yeast pectinase) and a commercial preparation in mechanical olive oil extraction

	Extracted oil		*Husk*				*Vegetation waters*		
	(*kg Mg*$^{-1}$ *olive paste*)	(*g kg*$^{-1}$ *FW olive*)	(*kg Mg*$^{-1}$ *olive paste*)	*Moisture* (*g kg*$^{-1}$)	*Oil* (%)	*Oil* (*g kg*$^{-1}$ *FW olive*)	(*kg Mg*$^{-1}$ *olive paste*)	*Oil* (*g kg*$^{-1}$)	*Oil* (*g kg*$^{-1}$ *FW olive*)
Control (water)	228^{a}	850^{a}	564^{b}	568^{b}	45·3^{a}	102^{b}	1313·0^{a}	9·0^{a}	48^{a}
Olivex (20 g t^{-1})	228^{a}	850^{a}	514^{a}	518^{a}	54·0^{b}	102^{b}	1450·3^{b}	8·5^{a}	48^{a}
Yeast pectinase (20 g t^{-1})	236^{b}	876^{b}	550^{b}	554^{b}	42·0^{a}	90^{a}	1390·3^{ab}	5·8^{a}	34^{a}
Yeast pectinase (30 g t^{-1})	234^{b}	870^{b}	544^{b}	548^{b}	43·0^{a}	95^{ab}	1360·0^{ab}	6·5^{a}	35^{a}

Column means followed by the same letter are not significantly ($P > 0.05$) different as determined by LSD test.

(44)

TAP REVIEWER COMMENT FORM for USDA/NOSB

Use this page or an equivalent to write down comments and summarize your evaluation regarding the data presented in the file of this potential National List material. Complete both sides of page. Attach additional sheets if you wish.

This file is due back to us by: Sept 8, 1995

Name of Material: Cultures, dairy - YOGURT CULTURE

Reviewer Name: RC Theuer

Is this substance Synthetic or non-synthetic? Explain (if appropriate) YOGURT CULTURES - NON-SYNTHETIC

If synthetic, how is the material made? (please answer here if our database form is blank)

YOGURT CULTURES ARE NATURAL, NON-SYNTHETIC, NON-BIOENGINEERED, TO MY KNOWLEDGE.

This material should be added to the National List as:

____ **Synthetic Allowed** ____ **Prohibited Natural**

or, ____ **Non-synthetic (Allowed as an ingredient in organic food)**

____ **Non-synthetic (Allowed as a processing aid for organic food)**

or, ____ **this material should not be on the National List**

Are there any use restrictions or limitations that should be placed on this material on the National List?

NONE (YOGURT CULTURES)

Please comment on the accuracy of the information in the file:

NEED SPECIFIC PRODUCTION INFO ON EACH CULTURE OR ENZYME

Any additional comments? (attachments welcomed)

IT IS ESSENTIAL TO ADDRESS EACH MATERIAL SINGLY, SINCE SOME MAY BE BIO-ENGINEERED AND THUS SYNTHETIC WITHIN MEANING OF THE ACT.

Do you have a commercial interest in this material? ____ **Yes;** ✓ **No**

Signature RC Theuer **Date** 8/28/95

galacturonide glycanhydrolase, EC 3.2.1.15) activity into the culture medium (Federici 1985; Petruccioli *et al* 1988). A previous preliminary study has shown the suitability of a raw pectic preparation from this yeast for use in some food processes (Federici *et al* 1988b).

The aim of the present work was to investigate the possibility of using the endo-polygalacturonase produced by *Cr albidus* var *albidus* strain IMAT 4735 in the mechanical olive oil extraction process and in the production of red wines; special attention has been given to the quality of the finished products.

EXPERIMENTAL

Materials

The polygalacturonase from *Cryptococcus albidus* var *albidus* IMAT 4735 (yeast pectinase) was produced and recovered as previously reported (Federici *et al* 1988b). The final enzymic activity of the crude preparation was approximately 9·0 viscosimetric units (VU) mg^{-1}; the viscometric activity was determined as indicated by Federici (1985). Two commercial preparations, Olivex and Pectinex Ultra Sp-L (8·6 VU mg^{-1} and 86·0 VU mg^{-1}, respectively), kindly supplied by Novo Enzymes (Bagsvaerd, Denmark), were used for comparison. These commercial enzymes had already been tested in mechanical olive oil extraction (Siniscalco and Montedoro 1988; Siniscalco *et al* 1989) and in wine making processes (Montedoro and Bertuccioli 1976). The olives used were of the Moraiolo cultivar. Due to the particularly dry season, the drupes had a very low moisture content (*c* 31 %). The red grapes used were of the Ciliegiolo cultivar.

Experimental procedures

Olive oil

Olive drupes (100 kg) were crushed with a hammer mill; malaxation (the olive paste mixing process) was performed for 60 min at 35°C following the addition of water to give a total moisture content of *c* 400 g kg^{-1}. The paste was then diluted with an equal weight of warm water and extracted at 35°C using an NX 306 Alfa Laval horizontal centrifuge. The oil was separated from the must by means of a PX 3400 Alfa Laval vertical centrifuge. The enzymic treatment was performed during malaxation. The following enzyme concentrations were employed: 100 and 200 g t^{-1} yeast pectinase, and 200 g t^{-1} Olivex. These concentrations were chosen on the basis of the viscometric activity of the preparations. For each enzyme preparation there were five replicates.

Red wine

The grapes, crushed and destemmed by a roller-crusher-stemmer, were put into special 10-litre glass tanks and SO_2 (50 mg $litre^{-1}$) was added. Three tanks utilised for each preparation. The enzymic maceration skins was performed after addition of either the pectinase (10 and 20 g hl^{-1}) or Pectinex Ultra (2 g hl^{-1}). Also in this case, the concentrations chosen according to the viscometric activity preparations. Fermentation was performed with *Saccharomyces bayanus* strain CH-158 Getrankeschuntz, Germany); the inoculum active dried yeast hl^{-1}. When the reducing sugar of the wines was around 40 g $litre^{-1}$ and increase in the total phenolic content was ob wines were separated from the solids which pressed with a hydraulic press. All wines were complete the fermentation at 20°C. Samples were at 10°C until analysed.

Analytical methods

Olive oil

Some analytical parameters responsible for nut and sensorial qualities (Montedoro *et al* 197 tedoro 1985; Montedoro and Servili 1990) examined.

The stability of oil to oxidation, expressed as ind time (h), was determined by the Swift acc automatic test (Laubli and Bruttel 1986) Rancimat apparatus (Matrohn Company, Base zerland) at 120°C and an air flow of 20 litres h and Montedoro 1989). Turbidity expressed elometric turbidity units (NTU) was determined Ratioturbidimeter (Hach); a silica gel suspension was used as a standard. Total phenols were det in the oil methanolic extract using Folin–Cioc reagent, as previously reported (Montedoro an tarelli 1969). Phenolic compounds were measur HPLC (Varian Model 5000) on the methanolic (Servili and Montedoro 1989). Free acidity, per number and chlorophyll were assayed accordi AOAC (1984) methods. Volatile compounds wer sayed by gas chromatography (Varian Model 3 the 'head-space' (Montedoro *et al* 1978).

Must and wine

Filterability, expressed as colmability inde (IC = $T_2 - T_1$ where T_2 and T_1 are the filtration ti two subsequent wine fractions expressed as 0·0 was determined with the Millipore equipment des by Descourt *et al* (1976). The operating condition wine fractions 200 ml; filtration membrane 0·65 working pressure 2 bar.

Pectins and total colloids were tested accord procedures described by Montedoro and Angelini Total polyphenols were assayed colorimetrically the Folin–Ciocalteau reagent; tannic polyphenol evaluated using the same reagent before and

Please address the 7 criteria in the Organic Foods Production Act:
(comment in those areas you feel are applicable)

(1) the potential of such substances for detrimental chemical interactions with other materials used in organic farming systems;

NONE

(2) the toxicity and mode of action of the substance and of its breakdown products or any contaminants, and their persistence and areas of concentration in the environment;

NONE - YOGURT FERMENTATION HAS A LONG HISTORY AS A WAY OF "PRESERVING MILK"

(3) the probability of environmental contamination during manufacture, use, misuse or disposal of such substance;

NONE

(4) the effect of the substance on human health;

GREAT. YOGURT HAS DOCUMENTED BENEFITS ON COLONIC BACTERIAL BALANCE

(5) the effects of the substance on biological and chemical interactions in the agroecosystem, including the physiological effects of the substance on soil organisms (including the salt index and solubility of the soil), crops and livestock;

GOOD

(6) the alternatives to using the substance in terms of practices or other available materials; and

BIOLOGICAL ACIDIFICATION REDUCES LACTOSE CONTENT; CHEMICAL ACIDIFICATION (LACTIC ACID) IS NOT AS GOOD

(7) its compatibility with a system of sustainable agriculture.

EXCELLENT AND HAS BEEN FOR THOUSANDS OF YEARS.

J Sci Food Agric 1992, 58, 253–260

Utilisation of a Yeast Pectinase in Olive Oil Extraction and Red Wine Making Processes

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Abstract: The possibility of using an endo-polygalacturonase produced by the yeast *Cryptococcus albidus* var *albidus* (yeast pectinase) in the mechanical olive oil extraction process and in the production of red wine was investigated.

Compared with the control and olive pastes treated with a commercial enzyme preparation, an increase in oil yield was achieved by treatment with the yeast pectic preparation. Also, the finished oil quality (turbidity, oxidation induction time, chlorophyll, and the content of polyphenols and aromatic compounds) was generally better.

Treatment of red musts with yeast pectinase resulted in an improved aromatic profile in the wines, even in the absence of an increase in colour intensity. Moreover, the use of the yeast pectinase did not cause any particular increase in the methanol content of the finished wines.

Key words: *Cryptococcus albidus* var *albidus*, yeast pectinase, olive oil extraction, red wine making.

INTRODUCTION

Pectic enzymes of microbial origin have been used for years as technological additives by the food processing industry (Neubek 1975; Baron and Drilleau 1982; Pilnik 1982; Sheppard 1986). Their use in the olive oil and wine sectors has recently been reviewed (Montedoro 1988). While research has sufficiently resolved the red wine making process by means of enzymes, at least two aspects of olive oil extraction remain unclear, the irregular yields from the olive oil extraction process and the factors that cause variations in the chemical composition of the extracted oil (Montedoro *et al* 1978).

The most common pectinase commercially available is a complex preparation produced from *Aspergillus niger* in which pectolytic enzymes such as polygalacturonase, pectinesterase etc, as well as hemicellulases, cellulases and proteases, are all present (Fogarty and Kelly 1983; Lambert 1983; Priest 1984). However, the new and improved technologies and the trend towards the use of pectinases in more specific sectors of the food-processing industry have led to renewed demands for enzyme preparations free from contaminating activities (Montedoro and Bertuccioli 1976; Szajer and Szajer 1982; Mendoza *et al* 1987; Montedoro 1988; Siniscalco and Montedoro 1988). Therefore there is a continuing search for microorganisms endowed with powerful and highly specific enzymic capabilities (Federici 1982; Fellows and Worgan 1984; Federici *et al* 1988a).

In this context, *Cryptococcus albidus* var *albidus* strain IMAT 4735 appears to be very promising. In fact it grows well on pectic substrates (eg various industrial pectins and complex pectic materials such as sunflower calathides and olive oil vegetation waters) and releases high levels of endo-polygalacturonase (poly 1,4-α-D-

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NOSB Materials Database

Identification

Common Name **Cultures, dairy** **Chemical Name**

Other Names cultures for butter, yogurt and cheese

Code #: CAS **Code #: Other**

N. L. Category Non-agricultural **MSDS** ○ yes ● no

Chemistry

Family

Composition See attached for list of organisms.

Properties rapid growth, usually in a specific temperature range. Many species produce flavor compounds, such as acetaldehyde, acetoin and diacetyl, which create the characteristic flavors of dairy product.

How Made Several methods of starter culture production are in use, the simplest being to use a sample of fermented food to inoculate the following batch. In commercial use, what is used is either a Daily Propagated Culture in liquid or freeze dried form, using frozen cultures or concentrates which are thawed as needed, or using Direct Vat Inoculation cultures. Most dairy plants have their own starter room where the starters are incubated and then provided for inoculation. Each type of dairy process has specific requirements for single strain or blend of culture species, time and conditions of inoculation.

Use/Action

Type of Use Processing

Specific Use(s) Used in making yogurt, cheese, some butter, and milk-derived products such as kefir.

Action In general, bacteria in cultures aid the preservation of food by utilization of carbohydrate in conversion to lactic acid. The resulting pH drop helps protect against spoilage organisms. Yogurt is formed by the synergistic relationship between the 2 strains of bacteria used: *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. These grow together in milk, with *S. thermophilus* releasing carbon dioxide to stimulate *L. bulgaricus*, and *L. bulgaricus* in return releasing amino acids from the milk proteins which speed the development of *S. thermophilus*.

Combinations

Status

OFPA

N. L. Restriction

EPA, FDA, etc FDA-GRAS

Directions

Safety Guidelines

State Differences

Historical status

Internationl status

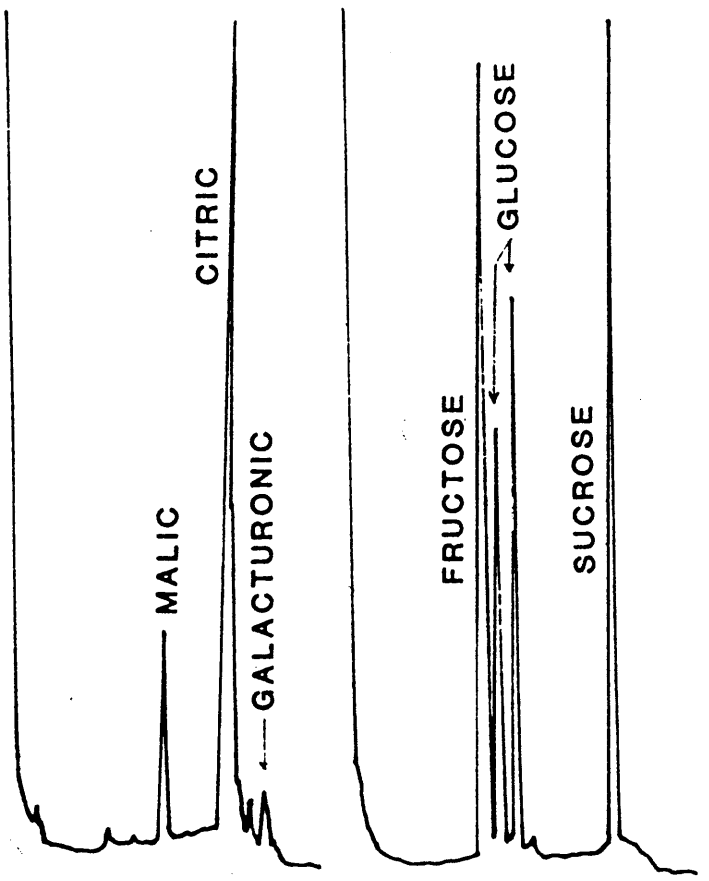

Fig. 2. Representative GLC peaks of acids and sugars from reconstituted 60°B orange concentrate prepared from either control or enzyme-treated raw juice samples.

Table 2. Quantitative GLC analyses of major acids and sugars in orange juices reconstituted from 60°B concentrates.[z]

	Control	Treated
Acids (% of total peak area)		
galacturonic	0.9,0.1	1.2,0.1
malic	7.4,0.2	7.0,0.2
citric	91.3,0.3	91.8,0.3
Sugars (% w/w)		
fructose	2.68,0.4	2.83,0.5
glucose	1.93,0.2	2.22,0.5
sucrose	3.92,0.7	4.08,1.1

[z]Values are for N = 2 experiments, duplicate analyses reported as $\bar{x}$, standard deviation.

of 0.1 M glycine) were highest for pectinic acid degraded by H_2O_2, decreasing in the order galacturonic acid, glucuronic acid, ascorbic acid and common monosaccharides.

Nitrogen content (Table 1) was not affected by enzyme treatment, the quantity being similar to previously reported values (11). There was also a slight decrease observed in vitamin C content of the enzyme-treated juice when compared to the control.

A non-trained taste panel of 15 persons was presented the samples in a multiple comparison (R = control, sample 1 = control, sample 2 = treated) difference analysis on 2 consecutive days. T[illegible]elists one day and 2 panelists the second day were abl[illegible] identify the pectinase-treated reconstituted concentrate. From an analysis of variance, the mean square F ratio was significant for $\alpha = 0.05$. Panelists were asked to compare 2 samples coded with random numbers to the "R" sample. The major reasons given for the difference were comments like "watery," "thin," "beverage-like," "different mouth-feel." These comments indicate that mouth-feel or texture was a major reason the enzyme-treated samples could be identified.

Treatment of heat-stabilized juice. Additional data was needed comparing pectinase treatment to an enzyme-stabilized control, particularly concerning the previous discussion about cloud loss and pulp flocculation. The question to be considered is whether pulp and cloud differences are related to action of native juice pectinesterase or to commercial pectinase. Enzyme treatment of raw, unpasteurized juice was performed because of time limitations involved with using the pilot evaporator. This necessitated eliminating a 1 hr control (no enzyme) juice.

To compare cloud loss and pulp sedimentation using samples of heat-stabilized orange juice, control concentrate was diluted to 12.5°B and treated with commercial pectinase. Values in Table 3 indicate that cloud loss (measured by serum turbidity in centrifuge pulp test) was not significant during the 2.0 hr treatment of pasteurized juice with pectinase. Results in Table 3 showed that pectinase treatment also reduced the amount of pulp sedimentation, another measure of cloud loss.

Table 3. Quality factors of 12.5°B pasteurized orange juice treated with 500 ppm commercial pectinase for various time periods.

Parameter Measured[z]	Enzyme Reaction Time (hr) 0[x]	0.5	1.0	1.5	2.0
Viscosity (cps)	1.7,0.1	1.6,0.1	1.5,0.1	1.5,0.1	1.5,0.1
Sediment (%)[y]	24.6,2.9	21.0,1.1	20.2,1.8	19.8,1.6	19.5,1.0
Cloud (% T)	11.6,0.2	12.7,0.6	12.9,0.8	12.9,0.8	12.9,0.8
Vitamin C (mg/100 ml)	40.6,1.8	39.7,1.9	40.5,1.7	41.1,1.9	40.3,1.4

[z]Values are for N = 5 experiments reported as x, standard deviation.
[y]Sediment is % height of sediment from sample in 100 ml graduated cylinder after standing undisturbed for 72 hr.
[x]0 hr is a no enzyme control.

Krop and Pilnik (7) explain the beneficial effects of pectinase in stabilizing orange juice cloud as resulting from lowering the MW of pectate fractions below that MW which can be precipitated by calcium ions in the juice. They (7) also treated juice with oxalate (to bind calcium), showing significantly less pulp sedimentation than juice containing active pectinesterase but no oxalate.

Results of Tables 1 & 2 for °B, sugars and acids would imply that flavor changes might be slight. Also, the GLC data (Figure 2, Table 2) for acids (discussed above) and for individual sugars showed no significant differences. It was suggested from the data that a reduction in viscosity could be detected by sensory evaluation. Other results indicate the importance of inactivating the native juice enzymes, prior to any treatment with commercial pectinases. It is hoped that the above results and discussion will be useful, when considering the potential application of pectinases as processing aids for treating orange juices. As shown, there are some changes resulting from pectinase treatment of the juice, the most significant of which is reduction of viscosity. It should also be mentioned that standards of identity for concentrated orange juice do not include pectinase treatment as proposed.

OFPA Criteria

2119(m)1: chemical interactions **Not Applicable**

2119(m)2: toxicity & persistence **Not Applicable**

2119(m)3: manufacture & disposal consequences

2119(m)4: effect on human health

Fermented dairy products are claimed to be more nutritious because some ingredients are partly predigested. Heat treatment of milk is known to reduce the availability of lysine and damage Vitamin C, some B-complex, and sulfur amino acids. However the beneficial changes to the milk caused by the growth of lactic acid bacteria are considered to be of more nutritive value.

2119(m)5: agroecosystem biology **Not Applicable**

2119(m)6: alternatives to substance

ambient air starters: not reliable or controlable.

2119(m)7: Is it compatible?

References

Encyclopedia of Food Science, Food Technology and Nutrition. 1993. Academic Press, Ltd., San Diego, CA

See also attached.

140°C, 4 minutes, then 8°C/minute to 220°C, hold 4 minutes; duplicate samples, duplicate injections. Sugars—same as for acids, except column oven, 90°C, 4 minutes, 8°C/minute to 220°C; triplicate samples, duplicate injections. Injections were automatically performed, peak areas were integrated by computer and compared with 0.001 mg/ml standarde solutions of acids and sugars.

Results and Discussion

The cost and inconvenience of treating fruit juices with pectinases can be weighed against advantages like viscosity reduction. For orange juice to be concentrated to greater than 60°B, the cost and inconvenience of using pectinase can be estimated or calculated, after effects on quality have been defined. The following results define some of these quality effects and should assist a processor to decide whether using pectolytic enzyme treatment as a juice processing aid is justified.

Treatment of raw juice. As stated before, the major action of pectolytic enzymes in orange juice is to reduce viscosity (by fragmenting pectin molecules—see ref 9). The 50% viscosity reduction illustrated in the 60°B concentrate of Figure 1 and Table 1 was achieved by pre-determining enzyme reaction conditions in small scale laboratory experiments. Other levels of viscosity reduction could be achieved by similarly adjusting enzyme concentrations and/or reaction times. These procedures involve applying levels of enzyme to the raw juice and concentrating to about 3-fold using a lab-size vacuum evaporator. Viscosity is then measured on the obtained concentrate. The low viscosity of the control concentrate (Table 1) could be accounted for by the fact that the fruit used in the study were of good quality (Author's opinion).

Closer examination of Table 1 indicates some other differences between treatments. There was a slight increase in titratable acidity and a corresponding decrease in the ratio.

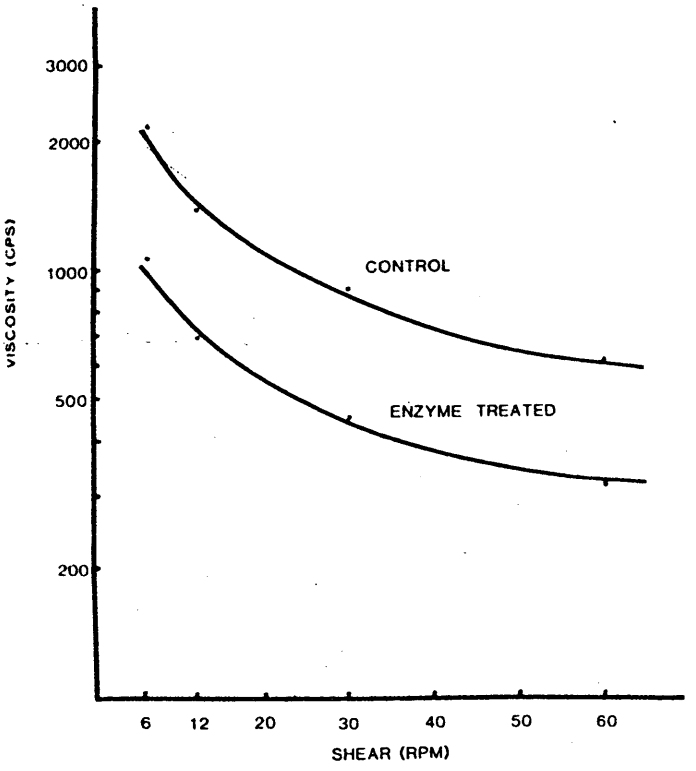

Fig. 1. Viscosity curves of 60°B orange concentrate from control and pectinase-treated raw juices. Enzyme was 500 ppm of soluble solids, 1 hr at 25°C.

Table 1. Quality factors of pectinase treated and control Valencia juices and concentrates.

Factor	Control	Treated
Concentrate:		
°Brix	60	60
acid (%)	4.10	4.17
ratio (°B/a)	14.6	14.4
nitrogen (%)	0.6	0.6
viscosity (cps at 60 RPM)	600	322
Reconstituted conc:		
°Brix	12.5	12.5
acid (%)	0.86	0.87
serum viscosity (cps at 60 RPM)	1.58	1.38
pH	3.79	3.79
pulp (%)	7.4	5.6
color Cr	38.2	37.6
Cy	82.5	82.5
N	38.0	37.9
pectinesterase (P.E.U.)[y]	.035	.030
vitamin C (mg/100 ml)	36.8	36.0
hesperidin (ppm)	540	457
cloud (% T at 650 nm)[x]	13.1	13.3

[z]Values are the means of triplicate analyses.
[y]See Praschan (8).
[x]See text for raw juice cloud.

Because of the importance of ratio to flavor quality evaluation, major organic acids in the juices were analyzed more closely. Results in Table 2 show increases in free galacturonic acid in the enzyme-treated juice compared with the control. However, as can be seen in Figure 2, the galacturonic acid fraction is small (1.2% of total acid) compared with the other 2 major juice acids.

The centrifuge pulp content of the enzyme-treated sample (Table 1) was less than the control. This is probably a result of the viscosity decrease, since destruction of the pectin would allow larger sized suspended matter in the juice to precipitate more easily and form a more compact pellet in the centrifuge tube. The pulp content of these juices was lower than the 12% allowed for commercial Florida concentrate. The reconstituted juice cloud did not seem to be affected by enzyme treatment (Table 1). However, the cloud in the raw juice serum (prior to heat stablization in the evaporator) was considerably reduced, 14.5% T for control vs 24% T in the treated sample. This result could be explained by the clarifying effect of the native pectinesterase in the raw juice over the 1 hr holding time (2.5 units control vs 2.4 units treatment).

When the reconstituted juices were allowed to stand for a couple of hours, a 20 to 25% increase in pectate-type flocculation occurred in the enzyme-treated sample. Again, this was probably a function of native juice pectinesterase actions in the raw juice during holding prior to heat stabilization, as well as reduced viscosity.

Color was not significantly affected in this study by enzyme treatment, but caution should be exercised at this point. One should recognize that pectinase treatment will allow easier concentration to very high brix. Above 60°B, it could be assumed that the additional concentration of juice browning constituents, as well as a requirement for more processing (higher heat, longer evaporator residence time, etc.), could easily result in more browning in the evaporator pump-out. Increasing the free galacturonic acid content by enzymatic hydrolysis of the pectin in the juice has potential to promote browning during manufacture of concentrate. For example, Seaver and Kertesz (10) showed that rates of colored polymer formation (during heating in the presence

OFPA Criteria

2119(m)1: chemical interactions **Not Applicable**

2119(m)2: toxicity & persistence **Not Applicable**

2119(m)3: manufacture & disposal consequences

2119(m)4: effect on human health

Fermented dairy products are claimed to be more nutritious because some ingredients are partly predigested. Heat treatment of milk is known to reduce the availability of lysine and damage Vitamin C, some B-complex, and sulfur amino acids. However the beneficial changes to the milk caused by the growth of lactic acid bacteria are considered to be of more nutritive value.

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2119(m)6: alternatives to substance

ambient air starters: not reliable or controlable.

2119(m)7: Is it compatible?

References

Encyclopedia of Food Science, Food Technology and Nutrition. 1993. Academic Press, Ltd., San Diego, CA

See also attached.

Proc. Fla. State Hort. Soc. 94:2[illegible] 1981.

PECTINASE TREATMENT OF RAW ORANGE JUICE AND SUBSEQUENT QUALITY CHANGES IN 60°BRIX CONCENTRATE[1]

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Abstract. **Comparisons of pectinase-treated raw juices and 60°Brix concentrates with untreated controls indicated little or no changes in standard citrus juice quality tests, i.e., °Brix, acid, ratio, pH, pulp, color, pectinesterase activity, % oil, vitamin C, cloud and hesperidin. A significant decrease in concentrate viscosity resulted from a 1 hr/25°C treatment of raw juice with 500 ppm of the soluble solids as pectinase. The enzyme treated-concentrate contained 33% more free galacturonic acid than the control; however, this acid was only 1.2% of the total acid in the juice. Concentrations of fructose, glucose and sucrose in the concentrates and raw juices were similar, regardless of treatment. Pasteurized juice, subsequently treated with enzyme had lower viscosity and a very slight loss of cloud with increasing enzyme reaction time. Enzyme treatment improved juice pulp settling characteristics as measured by 20% less sediment in juice allowed to stand undisturbed for 72 hr.**

Modifying the nature of food products by enzymatic reactions has exciting potential. Besides traditional uses of enzymes (cheese production, fruit juice clarification, alcohol production, meat tenderization, etc.), newer uses such as corn syrup processing, vegetable protein hydrolysis, flavor modification and fruit juice processing are finding wide application (6).

Pectinases are useful during juice recovery from berries and fruits, hydrolyzing pectin to lower molecular weight (MW) fractions. This results in viscosity reduction and less gel formation via a mechanism which has been described in detail (9). The obvious advantage of viscosity reduction is that it allows a high degree of concentration for juices containing significant quantities of pectin.

Pectinase treatment of citrus juice liquids such as pulp wash is common, and concentrates over 70°Brix (°B) have been produced (3). Most quality changes resulting from pectinase treatment of pulp wash liquids are minimal, the large decreases in viscosity excepted (4). Besides viscosity reduction in pulp wash liquids, use of pectinases has been shown to improve cloud stability of orange juice (2).

Although pectinase use for pulp wash liquid is well established (5), there are concerns that such treatment of raw juice to facilitate concentration and handling at high °B might result in quality deterioration. This study was designed to compare standard citrus industry quality factors in untreated and pectinase treated orange juice and 60 °B concentrate.

Experimental

Juice. Approximately 30 boxes (1,235 kg) of mature Valencia oranges (harvested May 14, 1981) were washed and the juice extracted using an FMC Model 391 (Lakeland, FL) extractor. Finished juice yield was 49.8% (615 kg). The juice was thoroughly mixed and divided into 2 equal portions, one tank serving as a control, the other for enzyme treatment. While the control juice was being concentrated in the evaporator (pilot T.A.S.T.E. Gulf Machinery, Safety Harbor, Fla.), enzyme reaction was commenced in the other sample. Upon concentration of juice in the first tank, the evaporator was rinsed briefly with water and without interruption, the enzyme-treated juice was concentrated. Evaporation rate was approximately 182 kg H_2O/hr.

Enzyme (Biopectinase 700, Biocon, Lexington, Ky.) was added at a level of 500 ppm of the total juice soluble solids in the tank (approx. 18 g enz/tank juice). Average enzyme reaction time (1 hr) was based on evaporation rate and was calculated to include emptying the feed tank. Juice temperature was approximately 25°C. General enzyme temperature and pH optima are described in the manufacturer's technical data. For the enzyme (Biopectinase 700) used in this study, the polygalacturonase activity as supplied was approximately 3000 units (1 unit = 1 μmole galacturonic acid/g enzyme/min).

In other experiments, the above mentioned evaporator heat-stabilized control concentrate was used to study effects of enzyme addition on juice viscosity, pulp sedimentation, vitamin C, and cloud. For these studies, experiments were performed using 5 separate 500 ml samples and controls for each analysis. Enzyme (500 ppm as above) was added to the juice diluted to 12.5 °B (the original corrected °B of the raw finished juice). The enzyme was allowed to react for 0 (no enzyme control), 0.5, 1.0, 1.5, and 2.0 hr. After reaction, added pectinase was inactivated by heating the samples in a stainless steel pan with lid to 74°C, then cooled to room temperature in an ice bath. This process took about 8 minutes.

Analyses. Routine citrus juice quality tests of °B, acid, ratio, pH, sinking pulp, color score, pectinesterase activity, Scott oil analyses, cloud (% T), and hesperidin (Davis test) were performed as described in Praschan (8). Nitrogen content of 60°B concentrates was determined by micro-Kjeldahl analyses (1). Viscosities were determined with a viscometer (Brookfield Model LVF) equipped with a UL adaptor for single strength juices and spindle 2 for the 60°B concentrate (300 ml sample at 25°C in a 500 ml Berzelius beaker, 6, 12, 30, 60 rpm). Pulp sedimentation was determined after 72 hr by measuring the sediment height of juice in a 100 ml graduated cylinder (7).

Sugars and organic acids were determined by gas-liquid chromatography (GLC) of the silylated derivatives (12). The 60°B concentrates were diluted to single strength (12.49°B), 1 ml brought to 10 ml volume with 95% ethanol, mixed, 0.1 ml sat'd lead acetate added and centrifuged 1500 x g to obtain ppt. The supernatant fluid was saved. The ppt was washed 2 X 95% ethanol, 1 X acetone, 1 X ether and dried to constant weight at 75°C in an oven. The silylating reagent (1 ml TriSil, Pierce Chemical Co.) was added to the dry ppt, reacted for 30 minutes and centrifuged to obtain a clear sample for GLC injection to measure organic acids.

The supernatant fluid (1 ml) from the lead acetate ppt was evaporated to dryness under vacuum. The silylating reagent was added as before to the dry residue. This procedure prepared the sugar fractions for GLC analyses.

GLC (Hewlett Packard 5730 A with 3385 A automation, Avondale, PA) conditions were as follows: acids—2.5 μl injection; 1.8 m x 0.5 cm glass SE 30 column; column oven at

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Table 2—**Advanced Maceration** *of pomace from first press*

Treatment		
Enzyme dose/ ton pomace (mL)		Yield* (lbs)
2 hr at 48–52°C		
120		70.5
100		72.8
85		75.0
75		77.2
2 hr at 20–30°C		
140		75.0
115		77.2
100		79.4
90		81.6

** Extraction yield expressed in pounds (lbs) of apples necessary to obtain one gallon of concentrated juice at 70–72° Brix. In all cases, midseason fruit at 12° Brix, has been considered*

tems have become a critical part of the juice production process. Today, there is a growing interest in enzymes such as polyphenol oxidase, rhamnogalacturonase, galactananse, proteases, glucose oxidase, xylanase, and pectinacetyl esterase among others for use in juice applications. Continued research efforts to better define and understand enzymatic activity on specific substrates as they apply to juice processing will mean the successful creation of unique enzyme formulations in the future tailored to fit the specific needs of the processor.

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—Edited by James Giese, Associate Editor

Table 1. Organisms used in the dairy industry

Group	Organism	Specific application
Mesophilic lactic acid bacteria	*Lactobacillus casei*	Flavour production and texture improvement; cheese
	Lactococcus lactis subsp. *cremoris*	Acid production; cheese
	Lactococcus lactis subsp. *lactis*	Acid production; cheese
	Lactococcus lactis subsp. *lactis* var. *diacetylactis*	Flavour production; cheese, fermented
	Leuconostoc lactis	Flavour production; cheese
	Leuconostoc mesenteroides subsp. *cremoris*	Gas production and flavour production
	Micrococcus varians	Enhances activity of thermophiles
	Pediococcus spp.	'Eyes' in Swiss cheese
	Propionibacterium shermanii	'Eyes' in Swiss cheese
Thermophilic lactic acid bacteria	*Bifidobacterium* spp.	Mild yoghurt, fermented milks
	Lactobacillus acidophilus	Yoghurt, fermented milks
	Lactobacillus delbrueckii subsp. *bulgaricus*	Flavour production; yoghurt
	Lactobacillus delbrueckii subsp. *delbrueckii*	
	Lactobacillus delbrueckii subsp. *lactis*	Acid production; cheese
	Lactobacillus helveticus	Acid production; cheese
	Streptococcus salivarius subsp. *thermophilus*	Acid production; yoghurt, cheese
Yeasts	*Debaromyces hansenii*	Deacidification of curd to encourage growth of *Brevibacterium linens*
	Kluyveromyces lactis	Flavour production and texture improvement; cheese
	Torulopsis spp.	Flavour production in soft cheese by proteases and lipases
	Rhodosporidium infirmominiatum	Pigmented surface coloration on cheese
Surface-ripening bacteria	*Arthrobacter globiformis*	Surface smear on cheeses
	Brevibacterium linens	Pigmented surface smear on cheeses
Moulds	*Aspergillus flavus*	Yellow-grey surface-ripened cheeses
	Chrysosporium merdarium	Sulphur-yellow surface-ripened cheeses
	Fusarium solani	White surface-ripened cheeses
	Geotrichum candidum	White surface-ripened cheeses
	Penicillium album	Grey-blue surface-ripened cheeses
	Penicillium camemberti	White surface-ripened cheeses
	Penicillium caseicolum	White surface-ripened cheeses
	Penicillium cyclopium	White surface-ripened cheeses
	Penicillium nalgiovensis	White surface-ripened cheeses
	Penicillium roqueforti	Blue-veined cheeses
	Scopulariopsis fusca	Beige surface-ripened cheeses
	Sporendonema casei	Red surface-ripened cheeses

Deep-frozen Cultures

Frozen cultures eliminate the need for daily propagation by the food manufacturer. A bottle is supplied containing inoculated, but unincubated, reconstituted skimmed milk which has been frozen. The cultures are shipped in an insulated box and need to be transferred to a freezer at −20°C as soon as they are received at the production site. Bulk starter is prepared by thawing and then clotting the bottle overnight and inoculating the clotted culture into the bulk starter medium.

Deep-frozen Concentrated Cultures

Frozen concentrated cultures are packed in aluminium ring-pull cans of 70 or 125 ml capacity. The supplier ship them in insulated boxes containing solid carbon dioxide to maintain their temperature at approximately −70°C. On receipt they have to be transferred either to a special low-temperature freezer operating at −40°C or to a liquid nitrogen refrigerator. The shelf life at −20°C is between 1 and 3 months but in liquid nitrogen it is

tank is filling. Light maceration processes usually require no agitation of the mash tank, or if so, it is very gentle.

After a 1–2 hr hold time, the action of the light maceration enzyme promotes the transformation of protopectin into soluble pectin, which then becomes only partially hydrolyzed during the process due to the relatively short action time and lower temperatures that may be used. This level of action is beneficial for reducing the viscosity of the mash, but still maintaining its pressing integrity, and increasing the overall juice volume. Horizontal or belt presses are then used to separate the juice, which is more easily released, from the mash or pomace. The resulting juice then undergoes conventional clarification and further downstream processing.

Successful trials carried out on this product for light maceration have shown increased fruit juice yields (Figs. 4 and 5), with improved pressibility and throughput.

• **Advanced Maceration Product.** Advanced maceration can be classified as an intermediate type process that results in medium to high extraction yields. A food grade enzyme complex from the controlled fermentation of fungal strains (Macerex™, Solvay Enzymes, Inc., Elkhart, Ind.) is designed to give increased hydrolytic action on the polysaccharides that form the cell walls of fruit tissue. The standardized formulation is composed of accurately controlled combinations of polymethylesterase, polygalacturonase, pectin lyase, arabinase, cellulases, hemicellulases, and other enzyme activities. These activities promote a more advanced maceration and extraction of pulp or pomace, especially in processes carried out at low (20–30°C) and medium (48–52°C) temperatures. The enzyme system demonstrates optimal activity within the pH range of 3.2–5.3, and has an effective temperature range of 10–55°C.

A typical application of the advanced maceration product would be in the treatment of pomace resulting from a first extraction (Fig. 6). The action of the enzyme decreases the insoluble solids content and facilitates separation by centrifuges and decanters. This product can also be adapted for belt and horizontal presses.

Typical dosage rates and yield averages are shown in Table 2 for processes run at medium or low temperatures. The enzyme should be diluted with cool clean water prior to use and can be added directly or via a feed pump. Moderate agitation of the treated pomace is recommended during the normal two hour treatment time. With an adequate dosage and contact time, it is often possible to reach a high degree of depectinization during pomace treatment, thus permitting lower pectinase dosages during the clarification stage.

This advanced maceration product optimally improves yields by releasing soluble solids from the lysed cells and incorporating the products of hydrolysis into the juice so interferences in downstream processing do not occur. As a result, clear and stable concentrates with excellent flavor can be obtained.

Looking To The Future

Whether it be to increase yields, solve a specific processing problem, or enhance the quality of the product, enzyme sys-

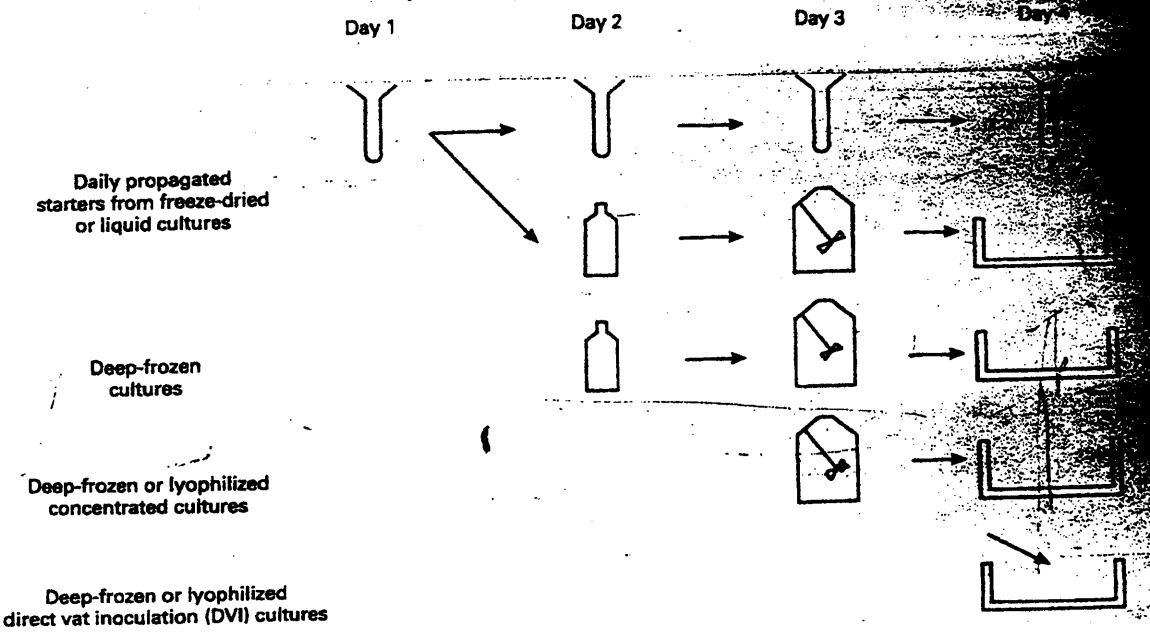


Fig. 1 Culture systems.

Table 4. Summary of the advantages of various culture systems

	Daily propagated starters from freeze-dried or liquid cultures	Deep-frozen cultures	Liquid-nitrogen-frozen concentrates	Lyophilized concentrates	Liquid-nitrogen- or low-temperature-frozen DVI cultures	Lyophilized DVI cultures
Cost of cultures per vat	Low	Medium	Medium	Medium	High	Medium
Level of technical skill required	High	High	Medium	Medium	None	None
Cost of culture storage	Low	Low	High	Low	High	Low
Level of phage relationship data available	None	High	High	High	High	High
Amount of planning required to manufacture starter (h)	72	48	24	24	None	None
Amount of culture performance data generated prior to use (fresh starter)	Fully tested	None	None	None	None	None
Amount of culture performance data generated prior to use (stock starter)	Fully tested	Fully tested	Fully tested	Fully tested	None	None
Level of technical support provided for system	None	High	High	High	High	High
Range of cultures available	Good	Good	Good	Adequate	Good	Adequate

DVI, direct vat inoculation.

risk of bacteriophage attack. Bacteriophage is a virus which attacks healthy bacteria by first adsorbing onto the surface of the bacterial wall, then injecting genetic material (deoxyribose nucleic acid; DNA) into the cell in which it multiplies to produce several dozen new virulent 'phages. These are liberated in the medium by the break-up (lysis) of the infected bacterium and are capable of infecting new cells. The latent period between the first 'phage–bacterium contact and liberation varies between 20 and 30 min and Fig. 2 shows how potent one 'phage particle can be.

It is for this reason that single-strain cultures are always used in pairs or triples. Suppliers offering these starters have carried out extensive work on the 'phage

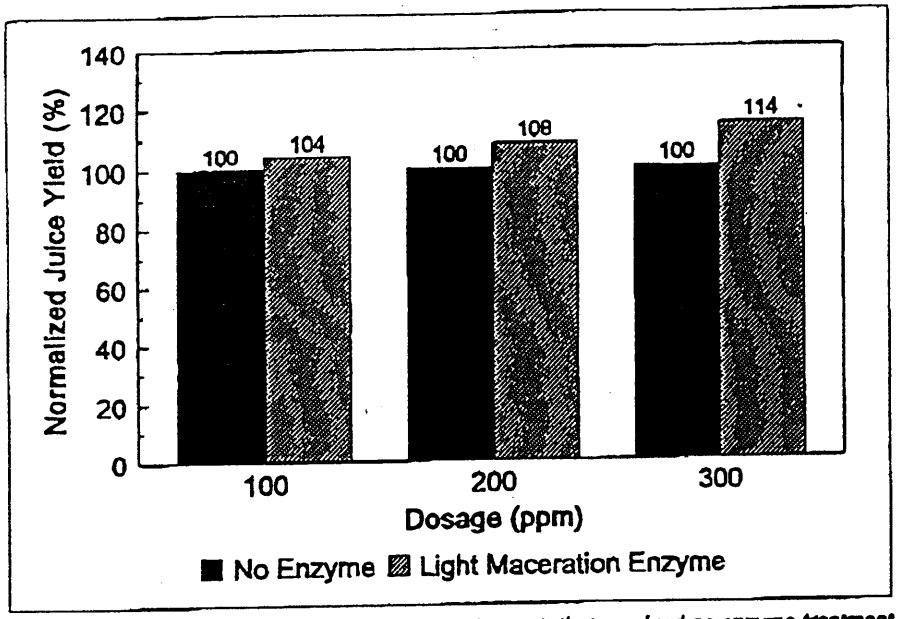


*Fig. 4—**Comparison of Juice Yields** from apple mash that received no enzyme treatment (black bar) and apple mash that was treated with the light maceration enzyme product (slashed bar) at varying dosages for 2 hr at 50°C, then pressed. Normalized yields were based on the percent (w/w) of juice obtained as related to the amount of mash that was enzyme treated*

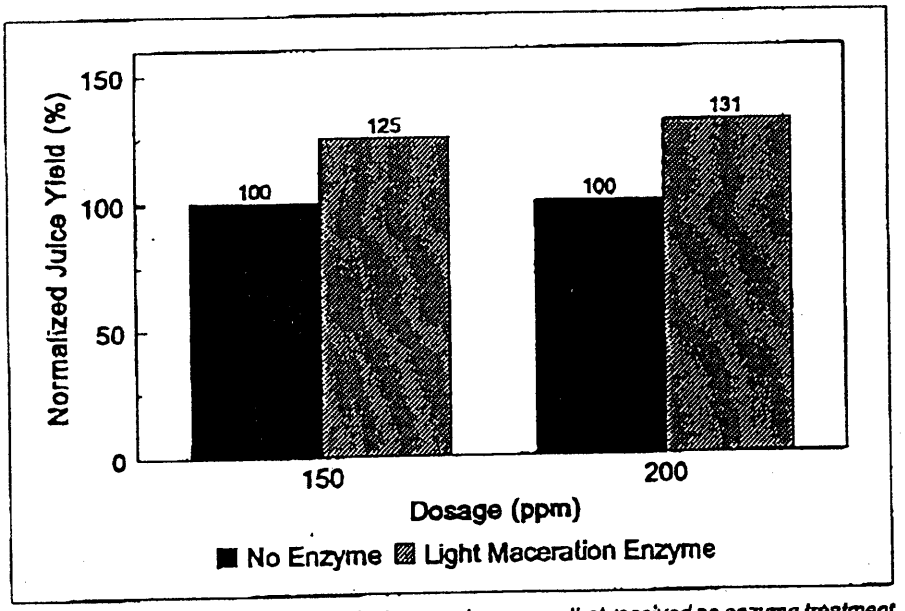


*Fig. 5—**Comparison of Juice Yields** from apple pomace that received no enzyme treatment (black bar) and apple pomace that was enzyme treated with the light maceration enzyme (slashed bar) for 1.5 hours at 25°C, then pressed. Normalized yields were calculated as the percent (w/w) of juice obtained as related to the amount of apples pressed*

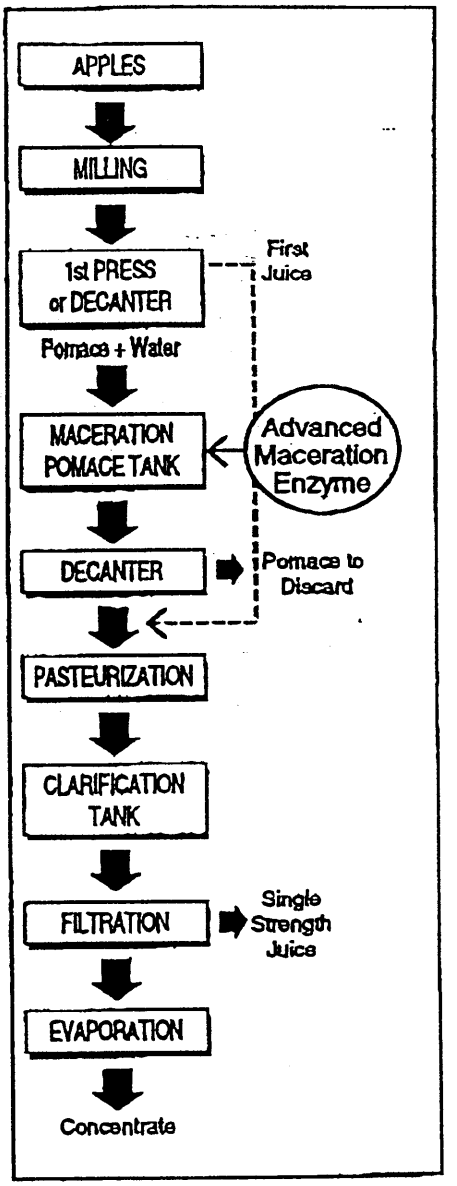


*Fig. 6—**Flow Process** for the production of clear apple juice demonstrating advanced maceration enzyme treatment of the pomace*

cesses in order to optimize fruit juice yields and soluble solids extraction. The product is standardized on pectinase activity and contains other carbohydrase side activities which are important for degrading other polysaccharides present in the lysed cell. The enzyme system demonstrates optimal activity over the pH range of 3.5–5.0 and has an effective temperature range of 2–60°C.

This enzyme formulation can be used to treat fruit mash prior to separation of pomace and juice (Fig. 2), or to treat the pomace which is crushed fruit remaining from an initial press where 60–70% of the juice has already been extracted (Fig. 3). The pomace is normally mixed with water in a 1:1 ratio and then enzyme treated. The light maceration product is especially suited for processes employing belt or horizontal presses to achieve separation of juice from the enzyme treated fruit.

Dosage levels of the enzyme can vary depending upon the type of equipment, desired level of maceration, age of the fruit, and processing conditions such as temperature and contact time. Typical usage levels range from 150–300 mL per ton of fruit. A 10–20% solution of the enzyme should be made with cool clean water just prior to use to facilitate the dispersion. This solution can be added to the whole fruit at the mill, or to the mash/pomace hold tank either by metering into the feed line or by direct addition as the

Table 1—Comparison of extraction yields with different apple processes

Process	Yield[a] (%)	Yield[b] (lbs)
Classic	75–80	83.8–88.2
Light	80–92	77.2–83.8
Advanced	92–96	72.8–77.2
Liquefaction	96–103	63.9–72.8

[a] Extraction yield expressed in percent (%) recovery of soluble solids
[b] Extraction yield expressed in pounds (lbs) of apples necessary to obtain one gallon of concentrated juice 70–72° Brix

tein in suspension. Pectinase preparations produced primarily from *Aspergillus* are added to clarify the juice. These preparations are usually mixtures of pectin esterase, polygalacturonase, and pectin lyase. Additional side activities such as arabinase, rhamnogalacturonase, cellulase, and galactomannanase may be present (Pilnik and Voragen, 1993). Starch degrading enzymes may also be added during the clarification stage. The addition of depectinizing enzymes reduces the viscosity by hydrolyzing the soluble pectin and causing the electrostatic charges of particles in suspension to neutralize. This causes the insoluble solids in the juice to agglomerate and sediment. Filtration becomes much easier to accomplish and the juice can be further concentrated without gel or precipitates forming.

In contrast to classic extraction, liquefaction processes are very intensive, giving maximum extraction. Enzyme requirements are usually high, requiring formulations that cause extensive cell wall degradation. At the same time, these enzyme formulations must eliminate the formation of haze precursors during the operation and facilitate downstream processing during ultrafiltration. Normally, strong agitation, high temperatures (50–55°C), and lengthy treatment times (3–5 hr) are required to enzymatically liquefy fruit pulp (with or without seeds, peels, and cores) to soluble solids and liquid, so pressing is not necessary.

Separation of solids from the liquefied fruit pulp is totally dependent upon high efficiency separation equipment such as decanters rather than presses. Liquefaction and clarification can occur simultaneously in this process. Juices obtained by liquefaction exhibit a large quantity of extracted soluble substances, which require the use of enzymes with activity to adequately eliminate certain substances that could pose problems with regard to the juice meeting specification values.

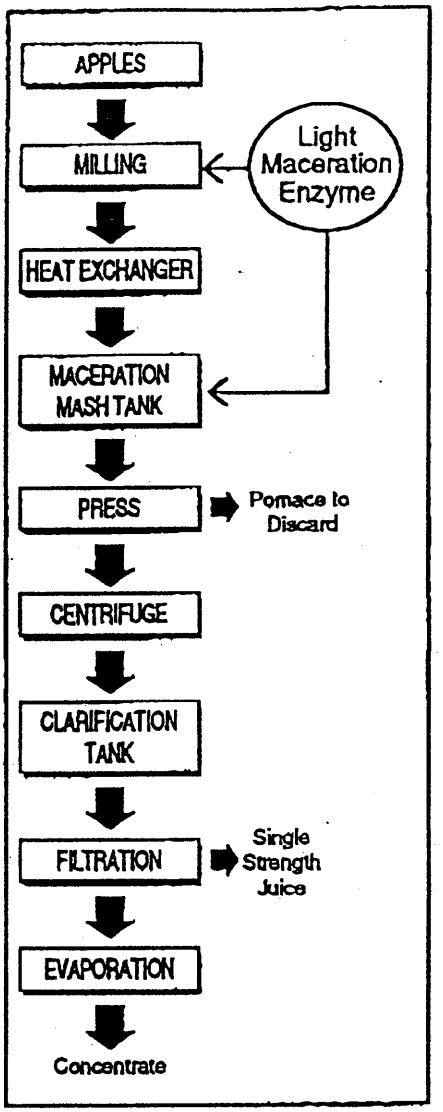


Fig. 2—Flow Process for the production of clear apple juice demonstrating light maceration with enzyme treatment of the mash

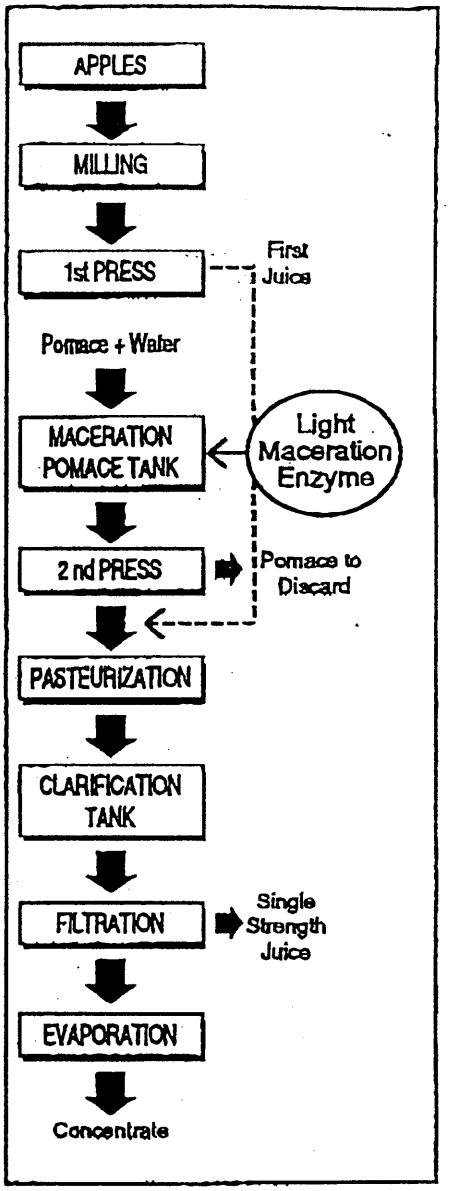


Fig. 3—Flow Process for the production of clear apple juice demonstrating light maceration with enzyme treatment of the pomace

Yielding levels of extraction in-between classic extraction, where no macerating enzymes are utilized, and the aggressive extraction of liquefaction, are the light maceration and advanced maceration processes, Based upon our knowledge and work with fruit juice processors, light maceration or advanced maceration which employ enzymes to treat the fruit pulp or pomace to achieve a partial breakdown of the fruit tissue, are the predominant types of processes being used for domestic fruit juice production.

Enzyme Formulations

Focused efforts by research and process development on light and advanced maceration processes have recently lead to the development of new enzyme formulations optimized for these processes.

- **Light Maceration Product.** A food grade enzyme system (Clarex® B, Solvay Enzymes, Inc., Elkhart, Ind.) from *Aspergillus niger* has been developed to work effectively under the milder extraction conditions of light maceration pro-

AUG 25 1999

TECHNICAL ADVISORY PANEL
REVIEW ON
ENZYMES, FUNGAL (PROCESSING CLASSIFICATION)

NOTE: The following review covers enzymes as a general class of materials, using pectin as an example in parts of the discussion.

NATURAL AND SYNTHETIC FORMS:
Enzymes are complex proteins which are produced by cellular anabolism. The source organism may be a microbe or a higher plant or animal. Isolation of the enzymes from their sources generally involves use of materials and processes which are not allowed on organic foods themselves (such as strong alkalis or acids, and ion exchange columns), and as such, are not available as organic ingredients themselves. The isolated material is molecularly the same as produced by the functioning cell. As such, the materials can be deemed non-synthetic, in accordance with earlier NOSB determinations. Until recently, all enzymes produced and used by mankind were these naturally-sourced biological products.

Recent technological advances in genetic engineering have made it possible to alter cellular genetic content, resulting in new production capabilities of the cell. The results of such genetic engineering (as defined by the NOSB) are not completely understood, and may have serious consequences heretofore unforeseen by mankind; such effects may be hazardous to human health and the environment, and have been described as being outside the realm of naturally-occurring biological processes. Thus, in accord with previous NOSB reasoning, all enzymes produced by genetic engineering as defined here should not be considered natural, but rather as synthetic.

CRITERIA:
2119(m)(1) - Chemical interactions in organic farming systems:
Not applicable for purposes of classification as a processing material.

2119(m)(2) - Toxicity and persistence in the environment:
Not applicable for purposes of classification as a processing material.

2119(m)(3) and NOSB processing criterion #2 - Consequence of manufacture, misuse, disposal:
Production of enzymes is generally conducted in controlled, closed environments. Materials necessary for their manufacture generally do not in and of themselves constitute an environmental hazard. Good manufacturing and handling practices are sufficient to protect workers from any negative effects of exposure, although inhalation or other ingestion of enzymes can have irritating or allergenic effects on some people.

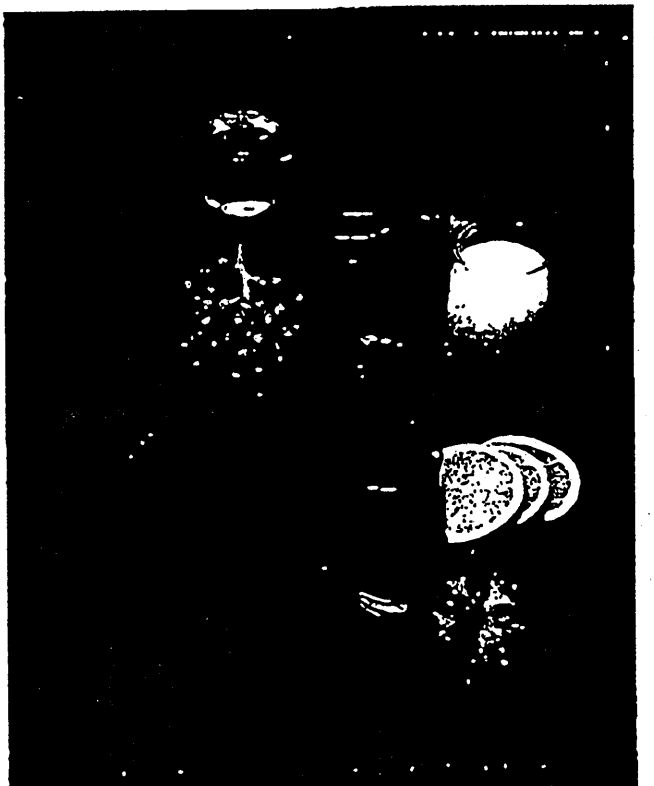
Escape or deposit into the environment of enzymes is generally not a concern. They are active in very low concentrations, and each enzyme's action is specific to a very narrow range of substrate(s). They can be relatively stable molecules, but are generally degradable by heat or other environmental factors.

There is not a concern regarding escape of the enzyme-producing organism into the environment, except in the case of genetically-engineered organisms (especially microbes, but perhaps others as well). Wild-type producing strains have shown a fair ability to be controlled in open ecosystems by natural competition. Genetically-engineered strains, on the other hand, may have far-reaching consequences if released into the environment; there is insufficient data and experience with such strains to regard their potential interactions as safe, in anything but a very controlled environment, and even then this may not be a certainty.

Enzyme Formulations for Optimizing Juice Yields

Enzymes have become a necessary aid in fruit juice production. Two new enzyme products increase juice yield in light and advanced maceration processes

JILANE G. FAIGH



□ THE POTENTIAL FOR STRONG continued growth of fruit juice products in the future appears assured for the juice processor. There is an increasing consumer awareness that fruit and fruit beverages are healthy, nutritious types of food that can beneficially affect health.

Research studies continue to cite evidence that there are connections between eating fruit and preventing certain diseases. Cranberry juice is said to help prevent urinary infections (Sobota, 1984); while pectin, a soluble fiber from fruit can help in the prevention of heart disease (Sable-Amplis, 1983). Antioxidants like vitamins C and A found in fruit are said to give cancer protection by scavenging free radicals (Diplock, 1991).

Other trends that have recently influenced the growth of the processed fruit category have been the boom in popularity of "blended juice" products being heavily marketed by giants in the food and soft drink industries and the introduction of new food products utilizing fruit juice concentrates as sweeteners rather than refined sugar.

As the demand for fruit juice beverages continues to grow, juice production will remain a highly competitive activity. The primary objective of the juice processor is to obtain the highest possible yield with maximum productivity, while maintaining or improving the quality and stability of the final juice product. To achieve this goal, fruit processors rely on continuous technological support with respect to equipment, processes, and processing aids such as enzymes.

Since pectinases were first used by the fruit industry in 1930 (Kertesz, 1930) to clarify pressed juice, the use of enzymes as processing aids to produce clear and cloudy juices has become standard practice (Neubeck, 1975; Rombouts and Pilnik, 1978; Voragen and Pilnik, 1989). The last two decades have seen advances in enzyme technology with the development of enzyme preparations containing pectinases, cellulase, and hemicellulase activities (Voragen et al., 1980; Voragen et al., 1982) that synergistically act on fruit tissue to macerate and further liquefy in order to extract additional soluble solids.

Today, enzyme manufacturers are able to offer preparations containing carefully balanced levels of enzyme activities optimized for specific types of fruits and/or specific processes (Fig. 1). These preparations have become indispensable for hydrolyzing plant cell walls, increasing yields and capacities, reducing viscosities, and improving separations. Two recently developed enzyme formulations facilitate the extraction of greater yields of juice from the fruit, especially in the production of clear apple juices. This article will describe the application and properties of these two enzyme formulations.

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Extraction Processes

Juice extraction processes will vary depending upon consumer use of the final product, the quality and quantity of available fruit, the type of equipment in the plant, and current manufacturing regulations. Applicable processes can be broadly classified, as shown in Table 1, according to their degree of extraction as: classic extraction, light maceration, advanced maceration, and liquefaction (Solvay, 1995). There can be variations to all of these processes because of plant-to-plant differences in operating conditions, processing aids, and equipment. The particular requirements of each process determine what type of enzyme preparations are necessary.

In classic extraction, using the production of clear apple juice as an example, fruit is pressed mechanically after milling without the addition of macerating enzymes to aid in further breaking open fruit cell walls. This process results in minimal yields as compared to the other extraction processes. Enzymes are used only in the clarification stage to depectinize turbid juice and eliminate starch.

The pressed juice from classic extraction is cloudy and viscous due to the presence of soluble pectin which acts as a protective colloid to hold small particles of cell wall fragments and cytoplasmic pro-

2119(m)(4) and NOSB processing criterion #3 - Effect on human health, nutritional quality: Enyzmatic activity on foods is specific and transformational, usually resulting in a significant change in the characteristics of the substrate. The new food product may have significantly different effect on the human system when ingested. For example, consider the difference between corn meal and corn syrup, or milk and cheese. That a transformation occurs is not by itself enough to say whether the ultimate effect on human health is positive or negative.

Research included by OMRI for this review suggests that processing of foods with enzymes can enhance the nutritive value of foods by breaking down "indigestible" food components, thereby making certain nutrients more available in the final product. One example given is that pectinase activity on plums in juice manufacture can result in greater availability in the final juice product of antioxidant components, which otherwise might not have been yielded during processing. How such laboratory trials correlate to human nutrition is not clear from the information presented, and not completely known - that is, whether such enzymatic treatments would be necessary to make said antioxidants as available in the human gut, or whether the altered food overall is definitely better than the original whole food. (This question/example could be extended to other fruits and nutritional components thereof, which are processed similarly with pectinases.) Generally speaking, processing yields of total juice from various fruits is increased when pectinases are used, as more of the fruit can be liquefied and separated from the seed and fiber; this is the primary reason enzymes are used in fruit processing.

There are some potential drawbacks to considering the advantages in exclusion to other effects of use of these enzymes, both from a nutritional standpoint and from an organic foods perspective. The assumption given in the first paragraph in this section of the NOSB database for this part of the criteria, i.e. that so-called "non-nutritive" or "indigestible" food components serve no positive function as part of a human diet and can therefore be removed, is based on incomplete knowledge at best. For example, the pectin in an apple, and the overall nutritional value of an apple, is much greater to the consumer than is pectinized, filtered apple juice. In which instances an isolated food component is desirable or valuable and which cases it is not is subject to variance from one commodity to another. We do not fully understand the complex balance of nutrients and how they interact on human nutrition for any agricultural product, and we should therefore be careful in choosing which components we deem appropriate to keep in the product and which to discard. Research continually shows how previously unidentified or poorly-understood food components can play significant roles in human health and nutrition. The value of food fiber is a good case in point.

A purely materials-based approach to processing of organic foods is flawed, in that the effects of individual processing steps and their associated materials can be cumulative. Analysis of each component step in the process does not necessarily reveal the total effect of all processes combined to make the final product. For this reason, although use of enzymes may not in and of themselves be seen as negative, use may be an integral part of a negative outcome as regards one of the goals of organic foods production, namely wholesome foods of high nutritional value. Fruit and juice processing is sometimes a good example of this; although initial yield of juice from the fruit may be higher than without use of the enzyme(s), the final products often are only a shadow of the original material. The nutritive values of corn meal and corn syrup are starkly different from each other; this is partly due to the action of enzyme, and partly due to the subsequent isolation of the product of the enzyme's activity. In such cases, it could be said that the nutritional value of the organic agricultural commodity has largely been lost.

Allowance of use of enzymes on organic foods therefore poses a potential danger as regards the nutritional value of the finished product. Non-specific allowance of all enzymes (or allowance of a specific class of enzymes used non-specifically, i.e. on any commodity), can lead ultimately to production of organic food products which lack much of the nutritive value of the original agricultural component(s).

Sugars

Juice from Au Red plums had the highest glucose, fructose and sorbitol of any cultivar, but was lowest in sucrose (Table 3). Differences in cultivar, geography, season, maturity, postharvest conditions, and processing can affect sugar composition (Wrolstad and Shallenberger, 1981). The sugar contents of plums have been reported by Richmond et al. (1981), Wrolstad and Shallenberger (1981) and Robertson et al. (1991) where glucose, fructose, sucrose and sorbitol were in the range 1.26–5.22, 0.74–4.93, 0.02–5.68, and 0–5.22 g/100g plums, respectively. Juice from all cultivars, except Abundance, had glucose/fructose ratios > 1.0. Enzyme-extracted plum juice was higher in total sugar content than control samples because of the release of soluble solids from the cell walls. Glucose and fructose contents of juice were higher than average and the sucrose content was lower than average in enzyme-extracted juice. According to Wrolstad and Shallenberger (1981) and Gorsel et al. (1992), invertase activity may be a prime factor in sucrose decrease and simple sugar increase. Gorsel et al. (1992) also reported that fresh plum juice was always higher in sucrose. Juice from Au Red was an exception because it had low concentrations of sucrose, even in control juices. The sugar composition of Au Red changed very little during processing but its total sugar was increased slightly by pectinase.

Sensory evaluation

For sensory evaluation, the plum juice was adjusted to a °Brix/acid ratio of 13.5–16.6, as determined in preliminary taste tests. Juices from different cultivars differed significantly ($p<0.05$) in astringency, color, sweetness, flavor and overall acceptance (Table 4). Juices from Pobeda, Abundance and Peach Plum cultivars were considered by the panel as sweetest, least astringent, most red, best in flavor and overall acceptability. Correlation coefficients among sensory attributes and overall acceptability were also determined. For Peach Plum juice, flavor seemed to have a significant influence ($p<0.01$) on judges' perceptions of astringency, with less astringency related to better flavor. Flavor had the strongest effect on overall acceptability of juice from all cultivars ($r=0.724-0.819$, $p<0.01$). Sweetness significantly ($p<0.05$) correlated with acceptance for Au Red ($r=0.399$), Abundance ($r=0.334$) and Early Golden ($r=0.414$) juice, and the higher the sweetness the greater the acceptability. Sensory evaluation of acceptable plum juices indicated that those juices could be characterized by sweetness, color and astringent sensations but the preference rating for plum juice could be adequately predicted by flavor preference ($r=0.75$, $p<0.01$) with the provision that Brix/acid ratio was suitable.

CONCLUSIONS

THE USE OF PECTINASE ENZYME increased juice yield, clarity, soluble solids, titratable acidity, content of total anthocyanins and total phenolics, but decreased pectin contents in most plum juices. Fining agents effectively removed pectin, anthocyanins and phenolics, thus increasing juice clarity. Juice from Pobeda had the highest overall acceptability followed by that from Peach Plum and Abundance. The °Brix/Acid adjustment had a significant effect on sensory ratings. To obtain the best product with a consistent flavor, juice from several cultivars may be needed in blends to provide the best balance between acidity, sweetness, aroma, and astringency.

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What is needed, therefore, is a broader principle on which to base decisions as to whether or not materials such as this are appropriate for particular foods. In this discussion, nutritive value is the determining criterion. The annotation as proposed in the NOSB database file should therefore be amended with a statement similar to the following: "Use of enzymes in any given process is subject to overall evaluation of the final nutritional value of the finished product compared to its initial ingredient(s). Such evaluation shall take into account all processing steps involved, not just those involved with the use of the enzyme(s). In cases where the nutritional profile of the raw ingredient(s) is deemed to have been substantially weakened, such finished food products may only be labeled as 'made with organic ingredients,' but not as 'fully organic.'" (Such "made with" products, if they are further used as ingredients in other organic product formulations, will themselves have to have classifications as to whether they can be ingredients in "organic" product formulations, or only in "made with organic" products. An illustrative example of this might be corn syrup, or commonly-produced white grape juice concentrate.)

An alternative which also might serve organic principles is an itemization of enzyme use by food type (either substrate or final product), the allowances or restrictions for enzyme use being specific to each; such listing is more arduous to generate, but allows for more consistent application.

As some other points to consider, human safety can potentially be threatened by enzymes, either due to allergenic interactions or toxic by-products of microbial production of enzymes. Selection of appropriate strains, along with GMP's and HAACP plans can be used to minimize these dangers, usually with good results. Far less certainty on this point applies to those enzymes and microbes which are products of genetic engineering.

Finally, enzymes are often packed for industrial use with a number of carriers and preservatives, for convenience of both the enzyme manufacturer and the product user. All formulations, if they are to be used in organic systems at all, must have full disclosure as to all components in the formulation used, and only include components which are deemed acceptable materials on the National List for foods labeled as "organic." Processed products made with enzyme formulations which do not meet this requirement may or may not be labeled as "made with organic ingredients," depending on the formulation's component(s) in question. Carriers, standardization materials, and other commercial enzyme formulation ingredients should be listed on all product labels as ingredients, if they indeed end up in the final food product.

2119(m)(5) - Agroecosystem biology:
Not applicable

2119(m)(6) and NOSB processing criteria #1 and #7 - Alternatives to substance, essential need:
In certain food products, enzymes are the only way to produce the desired product, such as barley malt or rice syrup, or for certain cheeses. In others, such as production of invert certain starches, the alternatives (such as sulfuric or phosphoric acid) would be less desirable for use in organic systems, and might at the same time result in products of lower quality. There are some cases in which microbial fermentation can replace the role of isolated enzymes (such as with using *mucor mehei* to produce certain kinds of cheeses), and these avenues deserve to be explored further; this is likely not a satisfactory alternative in all cases, however. In summary, in certain cases there are no better alternatives to enzymes.

Although classifiable as a non-synthetic material, enzymes are highly refined, and the spirit of NOSB Processing Criterion #7 should apply, namely that they can be used if "There is no way to produce a similar product without its use..." It is possible to produce fruit juice without use of enzymes. Conventional food products on the market do not necessarily have to have an identical organic version, and in some cases, should certainly not, if we are to yield to the higher principle, which in this case is human nutrition. Enzymes should only be allowed in organic production if they serve the principle of maintenance of nutritional quality and truly are essential

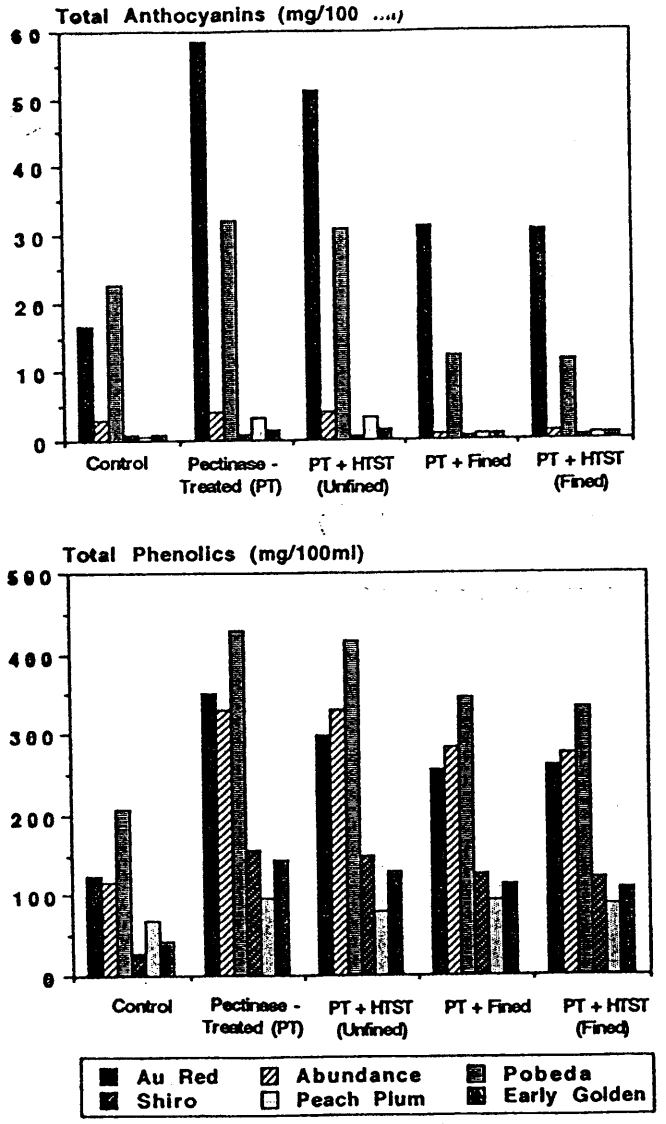

Fig. 3—Effect of processing conditions on total anthocyanins and total phenolics of juice from plum cultivars.

Table 3—Effect of processing conditions on content of juice sugars (g/100 mL juice) from plum cultivars

	Au Red	Abundance	Pobeda	Shiro	Peach Plum	Early Golden
Glucose						
Control	6.03[a]	1.84[a]	1.90[a]	2.70[a]	2.15[a]	3.18[a]
Pectinase treated (PT)	6.57[b]	3.17[b]	3.42[b]	5.13[b]	3.73[b]	4.05[b]
PT + HTST[2] (unfined)	6.13[c]	3.31[c]	3.97[c]	5.23[c]	4.03[d]	4.22[c]
PT + Fined	6.64[d]	3.27[c]	3.99[c]	5.17[b]	3.99[c]	4.42[d]
PT + HTST (fined)	6.42[e]	3.31[c]	4.09[d]	5.55[d]	3.79[b]	4.56[e]
Fructose						
Control	5.91[a]	2.50[a]	1.21[a]	1.64[a]	1.29[a]	2.67[a]
Pectinase Treated (PT)	5.71[b]	3.75[b]	2.73[b]	4.08[b]	2.83[b]	3.55[b]
PT + HTST (unfined)	5.30[c]	3.87[c]	3.18[c]	4.17[c]	3.09[e]	3.75[c]
PT + Fined	5.72[b]	3.84[c]	3.18[c]	4.14[c]	3.03[d]	3.89[d]
PT + HTST (fined)	5.56[d]	3.89[c]	3.31[d]	4.41[d]	2.91[c]	4.07[e]
Sucrose						
Control	0.10[a]	2.41[c]	3.73[c]	4.73[d]	7.45[e]	3.80[b]
Pectinase Treated (PT)	0.22[a]	0.65[ab]	0.69[b]	1.22[b]	4.09[d]	1.99[a]
PT + HTST (unfined)	0.42[b]	0.57[a]	0.75[b]	1.45[c]	3.48[a]	1.99[a]
PT + Fined	0.53[b]	0.77[b]	0.48[a]	0.59[a]	3.88[c]	1.86[a]
PT + HTST (fined)	0.88[c]	0.57[a]	0.46[a]	0.59[a]	3.70[b]	1.82[a]
Sorbitol						
Control	1.61[a]	0.05[a]	0.05[a]	0.43[a]	0.04[a]	0.01[a]
Pectinase Treated (PT)	1.53[b]	0.05[a]	0.34[d]	0.50[b]	0.14[c]	0.01[a]
PT + HTST (unfined)	1.58[c]	0.07[a]	0.31[c]	0.50[b]	0.13[c]	0.01[a]
PT + Fined	1.39[d]	0.14[b]	0.21[b]	0.43[a]	0.12[c]	0.02[a]
PT + HTST (fined)	1.37[e]	0.14[b]	0.20[b]	0.41[a]	0.11[b]	0.01[a]

[a-e] Values with the same letters in columns not significantly different at $P \leq 0.05$.
[2] HTST-High Temperature Short Time treatment.

Table 4—Means of sensory attributes of juice from plum cultivars

Sensory attribute	Au Red	Abundance	Pobeda	Shiro	Peach Plum	Early Golden
Astringency	3.95[b]	3.61[ab]	2.64[a]	3.56[ab]	2.78[a]	3.37[ab]
Color	9.02[d]	7.78[d]	8.76[d]	1.55[a]	7.63[c]	4.59[b]
Sweetness	4.69[a]	5.69[abc]	6.51[c]	5.26[ab]	5.82[abc]	6.12[bc]
Flavor	4.61[ab]	5.88[cd]	6.07[d]	4.82[bc]	5.28[bcd]	3.63[a]
Overall acceptability	4.61[ab]	5.39[bcd]	5.80[d]	4.39[ab]	5.54[cd]	4.01[a]

[a-d] Means in horizontal rows with same letters not significantly different at $P \leq 0.05$.

were very low, juice color from that cultivar was not significantly affected by pectinase treatment. However, pasteurization (HTST), which caused browning, affected color in Shiro plum juice as well as juice from all other cultivars. Non-enzymatic browning due to heating may not have been as extensive as browning from polyphenol oxidase, which is inactivated by heating (Sapers, 1992; Siddiq et al., 1994). Hunter L*, a* and b* values were decreased by fining. The color value changed less by HTST + fining because the fining agents removed particles susceptible to browning. Sapers (1992) reported that the capacity of raw apple, grape and pear juices to undergo browning was associated with particulate fractions that could be removed by filtration with bentonite and diatomaceous earth.

Plum juice from Abundance and Pobeda had higher a* values than that of other cultivars, indicating more redness while Shiro gave (−)a* values. Au Red and Early Golden plum juices gave the lowest b* values, while Abundance, Shiro and Peach Plum juices showed higher b* values. Plum juices treated with enzyme and fining agents were less yellow than untreated juices. Shiro plum juice gave negative hue angle values. A significant ($p<0.05$) decrease in hue angle values was observed in the enzyme-extracted juice samples than in untreated ones, with maximum decreases in unfined juice.

As expected the red-colored cultivars, Au Red and Pobeda, had higher anthocyanin content (16 and 22 mg/100 mL juice, respectively) than the yellow Shiro (0.33 mg/100 mL juice) cultivar. Unfined enzyme-extracted juice had higher total anthocyanins than the control (Fig. 3). This increase in total anthocyanins was believed to be due to preferential release of anthocyanins into the liquid phase by the action of enzyme (Rommel et al., 1992). Loss of heat sensitive anthocyanins in unfined plum juice ranged from about 4 to 13%.

Phenolic compounds contribute to color, flavor, astringency, enzymatic and nonenzymatic browning of horticultural products. To consumers, the most evident properties of phenolic compounds are colors and astringent taste they impart to foods (Spanos et al., 1990). The total phenolics of plum juice from different cultivars ranged from 27 to 417 mg/100 mL (Fig. 3). Plum juices made from Pobeda and Au Red had higher total phenolics than juice from Shiro and Early Golden plums. Processing also had an effect on total phenolics. Enzyme treatment increased levels of phenolics in juice while fining decreased phenolic contents. Enzyme treatment resulted in a two- to fourfold increase in total phenolics in juice from all cultivars with exception of Peach Plum. Pectic enzyme added to apple juice for clarification has been shown to release phenolics from cell walls (Spanos et al., 1990). Addition of fining agents, like gelatin, has been related to a decrease (about 50%) in total phenolics in prune juice (Bannach, 1984). In our results, addition of gelatin brought about a 25% decrease in phenolic content of juice samples.

to the formation of the product. Being essential to achieving a desired technical effect which results in a product of degraded nutritive value should not be considered an essential need.

NOSB criterion #4 - Preservative, recreating qualities lost during processing:
Enzymes in and of themselves generally would not be considered preservative materials. The products of enzyme activity could conceivably act as preservatives, but these would be from the breakdown of the food material itself, not from an outside source. Food qualities are changed by enzymatic activity, but this change should not necessarily be construed as a means of re-creating qualities of the original product which get lost in processing; rather the product is substantially different from the raw ingredient(s).

NOSB criterion #5 - GRAS, residues:
Enzymes are not changed by their action on their substrates; they remain as they are, and active, until denatured by heat or other factors, or until the substrate is exhausted. Depending on the process, enzymes may be removed from the final product, or denatured and left in, or may even be potentially active. How they are labeled in final product formulations should be dependent on the specific outcome for the product in question. As mentioned above, carriers, preservatives, or other commercial enzyme formulation components must also be considered as potential residues in finished goods.

Many enzymes are classified as GRAS, although such determination has not been universally made. Quality control measures (GMP's and HAACP plans, analytical testing, etc.) should be in place by enzyme manufacturers to minimize the risk of mycotoxins being included in fungal enzyme formulations as by-products of the manufacturing process.

2119(m)(7) and NOSB processing criterion #6 - Compatibility:
As discussed in depth in the sections above, enzymes are compatible with principles of organic production, but only if they are placed in a larger perspective, not in all cases. It must be ensured that the ultimate nutritive value of foods is not robbed due to successive processing steps, where enzymes are an integral part in said processes, even if the enzymatic steps themselves do not result in the loss of nutritive value.

SUMMARY AND RECOMMENDATION:
The discussion of enzymes for use in organic foods processing is complex, and several of the criteria discussed above overlap. Enzymes should be classified as a natural material, listed on the National List as being REGULATED, with the annotation being as proposed in the NOSB database, amended as discussed in this review (refer to section 2119(m)4 and NOSB processing criterion #3, above). Otherwise, itemized decisions on individual enzymes (or types of enzymes) would be appropriate. Blanket acceptance of enzymes as processing materials is strongly discouraged.

Enzymes which are products of genetic engineering as defined by the NOSB should be classified as synthetic materials, and PROHIBITED for use in organic production systems.

COMMERCIAL/FINANCIAL INTEREST:
I unequivocally claim that I have no personal, commercial, or financial interest whatsoever in the this material or the decisions regarding it.

23 August, 1999

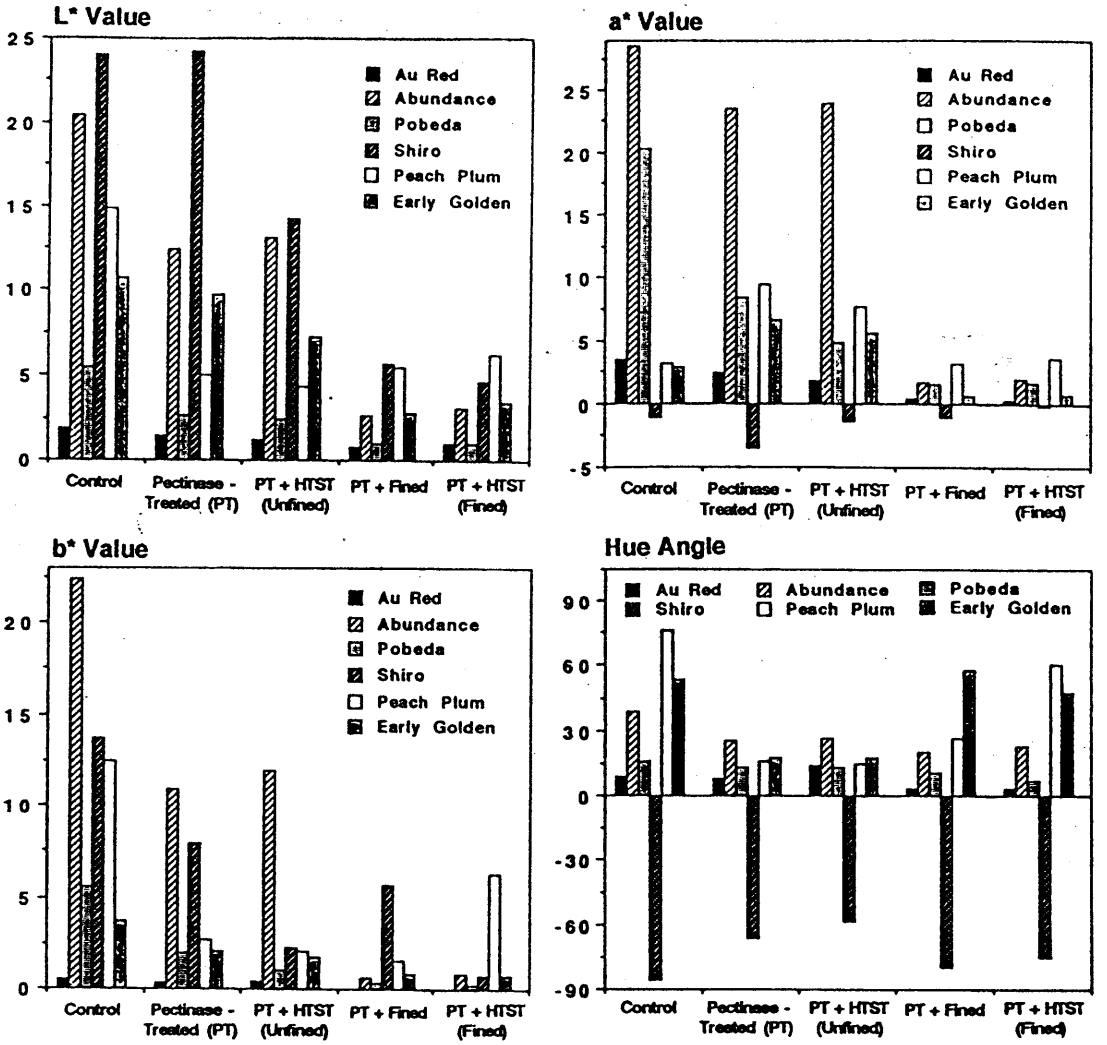

Fig. 2—Effect of processing conditions on the Hunter L*, a* and b* values and hue angle of juice from plum cultivars.

the least clarity (23% T) and Clarex® L, gelatin and bentonite had little influence on juice clarity from Au Red. Juices treated with fining agents are usually clearer than those treated only with enzymes, as confirmed in our results. The clarity of fined juices was highest in all cultivars. This was followed by samples which were fined and HTST-pasteurized. HTST treatment of juice resulted in a decrease in % T for juice from all cultivars. Heat induced dissociation of protein-phenolic complexes of fruit cell wall during pasteurization has been shown to influence clarity of fruit juices (Hsu et al., 1989).

Pectin contents of control plum juice ranged from 0.15 to 0.39 g galacturonic acid/100 mL (Fig. 1). Enzyme treated juice had an average 54% lower pectin than control. The fining process further reduced pectin content. For most treatments, Early Golden and Au Red had higher pectin contents ($p<0.05$) than other cultivars. Pectin content correlated negatively with %T for all cultivars, indicating that clarity of juice was in direct proportion to degree of pectin breakdown by the Clarex® L enzyme system.

Soluble solids, pH, titratable acid and °Brix/acid ratio

°Brix values of juice from different plum cultivars ranged from 9.9 to 18.9 (Table 2). Au Red had the highest soluble solids followed by Early Golden and Shiro. Abundance had the lowest °Brix reading. Addition of Clarex® L increased soluble solids of all plums by 2.2% to 20.8%. Commercial pectinase has been shown to release about 80% polysaccharides from apple cell wall, in addition to degrading pectic material, thus increasing soluble solids content (Pilnik and Voragen, 1991). The pH of juice, which ranged from 3.01–3.53, was cultivar specific and was not affected by processing conditions (data not shown).

The % titratable acidity of plum juice ranged from 1.10 (Shiro) to 1.83 (Pobeda). Plum juice contains predominantly malic acid (Meredith et al., 1992). The acidity of pectinase-extracted juice was 24% higher, on average, than controls. However, subsequent processing techniques (HTST or fining) had no significant effects on juice acidity. A similar increase in acidity has been reported for blackberry juice (Rommel et al., 1992).

Au Red plum juice had the highest °Brix/acid ratio (15.18), while juice from Pobeda plums had the lowest (6.09). This indicates that Pobeda may not be advisable for fresh use although it has desirable red color. However, it might be processed into acceptable quality juice (or juice drink) by modifying the °Brix/acid ratio through addition of sugar, sugar syrup or blending with other juices. Fellers et al. (1988) reported that grapefruit juice with °Brix/acid ratios of 7.0 had lower consumer preference scores than juice with °Brix/acid ratios above 11.0.

Color, total anthocyanins and total phenolics

The Hunter CDM (Color Difference Measurement) values, L*, a* and b*, and color hue angle for all plum juice samples varied with cultivar and processing (Fig. 2). The dark red cultivars, Au Red and Pobeda, produced juice with lower L* values while the yellow Shiro variety gave the lightest juice. Enzyme treatment decreased Hunter L* values of juices by about 30%, due to increased release of pigment from the cells (Pilnik and Voragen, 1991). Since the total anthocyanins in Shiro cultivar

Common Name: Enzymes, Fungal Class: Processing

Chemical Name: Pectin Lyase

Introduction

Pectic enzymes have been available commercially for about fifty years in the US and Germany and for a somewhat less time in other countries. A large variety of pectic enzymes are available both in liquid or solid forms and in various strengths (i.e. ability to hydrolyze the glycosidic bond between the biopolymer pectin of repeating chains of the sugar galactose or galacturonic acid), the amount of pectin in fruits depends on the maturity, degree of ripeness, variety, and subsequent storage conditions of harvested fruit (1).

Specific applications of pectin lyase is in juice clarification, extraction, wine clarification and production, cloud stabilization of citrus juices, extraction of citrus juices, use in production of vegetable and fruit puree's and other uses as noted on the NOSB materials database.

Review

Since pectin lyase is biosynthesized from *Aspergillus* or other fungal sources and is not chemically derived, I would classify this enzyme class as non-synthetic. This classification is predicated on the following criteria:

Table 1—Effect of commercial pectinase, ® L, on juice yield from plums

Cultivar	Juice yield (% wt/wt) Control	Enzyme treated	% Increase
Au Red	25.30[a]	79.48[e]	214.15
Abundance	55.87[c]	82.07[f]	46.89
Pobeda	59.95[d]	84.76[g]	41.38
Shiro	36.21[b]	81.84[ef]	126.01
Peach Plum	34.61[b]	84.70[g]	144.73
Early Golden	53.79[c]	83.63[fg]	55.47

[a-g] Values with the same letters in columns are not significantly different at the 5% level of significance.

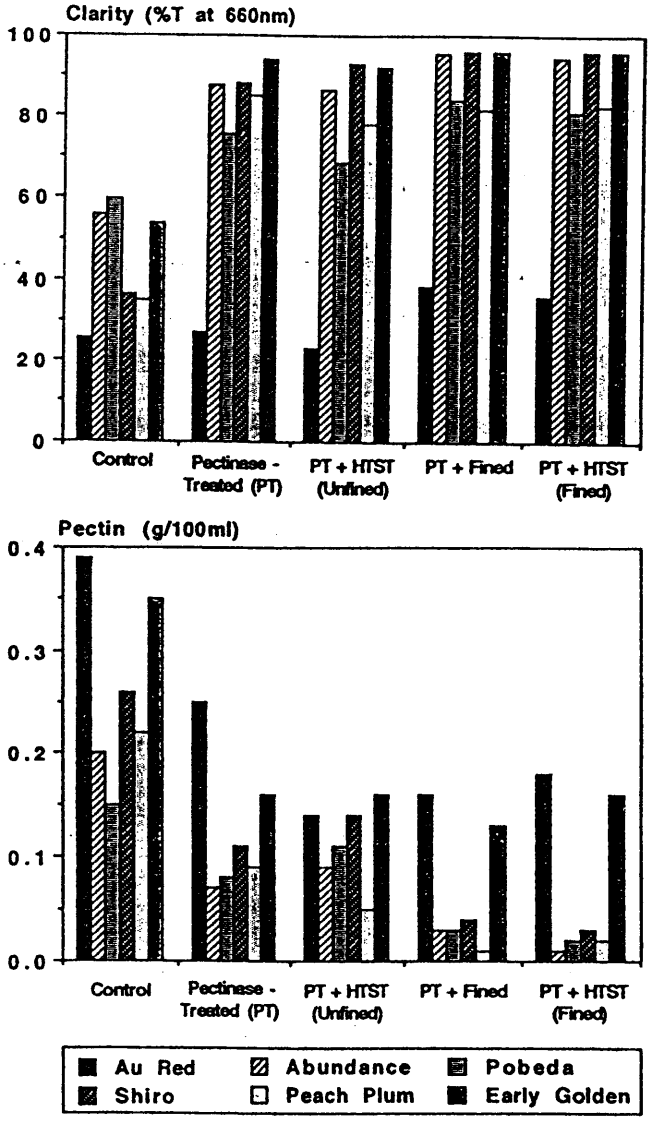

Fig. 1—Effect of processing conditions on clarity and pectin content of juice from plum cultivars.

cose+sorbitol. The YSI analyzer was then used to determine the content of glucose. Sorbitol content was estimated by subtracting glucose content, determined by YSI, from HPLC measurements of glucose+sorbitol. The relative precision of YSI analysis and agreement with AOAC methods was very good (Weetal and Hersh, 1974; Mason, 1983).

Sensory evaluation

Preliminary sensory trials were done with 8–10 panelists from our lab to determine the optimum level of °Brix for each of the juice samples. Based on these preliminary trials, pectinase-extracted, and HTST-treated juice was used for sensory evaluation. The °Brix of samples was adjusted with sucrose. Samples were tested by a larger panel for tartness, bitterness, color, flavor and acceptability using an unstructured 10 cm hedonic scale (with anchors, "extremely poor" as zero to "extremely good" as 10). Each panel consisted of 40–45 untrained panelists from the faculty, staff and students in the Food Science & Human Nutrition Dept. Judges were asked to mark the horizontal scale at the point that most closely corresponded to their judgment of the intensity of each attribute. These points were then measured in cm and translated into numerical values for statistical analysis. All tests were conducted in the sensory evaluation laboratory of the Department of Food Science & Human Nutrition, Michigan State Univ., under cool white fluorescent lighting.

Table 2—Effect of proce. g conditions on soluble solids (°Brix), titratable acidity and °Brix/acid ratio of juice from plum cultivars

	Au Red	Abundance	Pobeda	Shiro	Peach Plum	Early Golden
Soluble solids (°Brix)						
Control	18.5[a]	9.9[a]	11.1[a]	12.9[a]	12.7[a]	13.0[a]
Pectinase Treated (PT)	18.9[b]	11.9[c]	12.3[c]	14.3[b]	14.4[b]	15.0[b]
PT + HTST[d] (unfined)	18.9[b]	11.8[bc]	11.8[b]	14.2[b]	14.4[b]	15.0[b]
PT + Fined	18.4[a]	11.6[bc]	12.1[bc]	14.0[b]	14.1[b]	14.9[b]
PT + HTST (fined)	18.5[a]	11.5[b]	12.2[c]	14.2[b]	14.2[b]	14.8[b]
Titratable Acidity (% malic acid)						
Control	1.23[a]	1.22[a]	1.83[a]	1.10[a]	1.13[b]	1.52[a]
Pectinase Treated (PT)	1.33[a]	1.59[b]	2.18[b]	1.55[b]	1.17[a]	2.00[b]
PT + HTST (unfined)	1.37[a]	1.59[b]	2.17[b]	1.57[b]	1.19[ab]	1.96[b]
PT + Fined	1.33[a]	1.59[b]	2.18[b]	1.55[b]	1.17[a]	2.00[b]
PT + HTST (fined)	1.25[a]	1.50[b]	1.96[ab]	1.44[b]	1.11[a]	1.66[a]
°Brix/Acid Ratio						
Control	15.18[a]	8.09[b]	6.09[a]	11.67[b]	11.23[a]	8.53[a]
Pectinase Treated (PT)	14.16[a]	7.49[ab]	5.66[a]	9.24[a]	12.31[b]	7.51[a]
PT + HTST (unfined)	13.85[a]	7.44[ab]	5.45[a]	9.01[a]	12.08[b]	7.63[a]
PT + Fined	13.81[a]	7.30[ab]	5.54[a]	9.09[a]	12.01[b]	7.42[a]
PT + HTST (fined)	14.86[a]	7.68[b]	6.24[a]	9.84[ab]	12.77[b]	8.92[a]

[a-c] Values with same letters in columns not significantly different at $P \leq 0.05$.
[d] HTST—high temperature short time treatment.

Statistical analysis

The experiment was designed as a three factor (replication × cultivars × processes) randomized model. All determinations were made in duplicate, except for the value for color, which was determined in triplicate. Mean, standard errors, mean square errors, one factor ANOVA, two factor ANOVA, correlation and interaction of main effects were done using SuperANOVA software (Abacus Concepts, Inc., Berkeley, CA). Mean separations were performed using LSD with the mean square error term at the 5% level of probability.

RESULTS & DISCUSSIONS

Juice yield, clarity and pectin content

As a result of Clarex® L addition, a 41–214% increase in juice yield was obtained from different plum cultivars (Table 1). Highest yield increase was obtained in Au Red (214%), followed by Peach Plum (145%) and Shiro (126%). The average yield of plum juice from these varieties was about 20% higher than Stanley plum (Chang, 1993). Wani and Saini (1990) reported juice yields ranging from 17% to 23% from different plum cultivars using a pectinase. In contrast, yields of plum juice using Clarex® L, were much higher in our current results. Although the increase in juice yield was cultivar specific, Clarex® L enhanced liquefaction of plums resulting in higher juice yields, an important economic consideration.

Clarity, as % transmittance (% T), of Clarex® L extracted juice samples was higher than the control in all cases except unfined Au Red (Fig. 1), indicating the enzyme system was effective in removing constituents which affect clarity. A higher % T indicates clearer juice. Early Golden plum juice had the highest % T values, (except for the unfined sample), followed by Shiro, Abundance and Peach Plum juice. Au Red juice had

(i) Fungal organisms can not be derived from genetic engineered species and must be naturally occurring.

(ii) Extraction and manufacturing operations can not chemically modify or change the enzyme preparation.

(iii) All carriers, diluents and preservatives used in the final enzyme preparation shall be substances that appear on the National List of Ingredients allowed for use in foods labelled as organic.

I therefore agree with the OFPA status that this enzyme preparation if prepared from a non-GMO fungal source is non-synthetic. The risk to organic integrity depends on the isolation, purification and packaging (i.e., inclusion of stabilizers or preservatives).

Overall I found the NOSB materials database to be technically accurate. I agree with the proposed annotation inclusive of the three proposed criteria that qualify my recommendation that the NOSB consider that fungal enzymes (in this case, pectin lyase) are naturally derived enzymes but must be handled in a manner consistent with organic food processing criteria.

I have no commercial or financial interest in any form of glycine.

References:

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Plum Juice Quality Affected by Enzyme Treatment and Fining

TUNG-SUN CHANG, MUHAMMAD SIDDIQ, NIRMAL K. SINHA, and JERRY N. CASH

ABSTRACT

Juice from six plum (*Prunus domestica* L.) cultivars, Au Red, Abundance, Pobeda, Shiro, Peach Plum and Early Golden, was extracted using 0.2% Clarex® L pectinase. The juice was processed as high temperature-short time (HTST)-unfined juice, fined juice or HTST-fined juice, and analyzed for yield, clarity, pectin content, soluble solids, pH, titratable acidity, color, sugars, total anthocyanins and total phenolics. Pectinase treatment resulted in 41–214% increase in juice yields, with highest increase for Au Red. Enzyme-extracted juice averaged 54% lower pectin than controls, and fining further reduced pectin. Enzyme treatment also improved juice soluble solids and color. A consumer type sensory panel indicated juice from Abundance, Pobeda and Peach Plum had best flavor and acceptability.

Key Words: plums, fining, color, flavor, acceptability

INTRODUCTION

PLUMS (PRUNUS DOMESTICA L.) have potential as a fresh market and/or processing crop which can be harvested between cherry and apple crops in many areas. About one-half of the plums are consumed fresh while the rest are processed (Espie, 1992). The major processed plum products are whole canned plums, prunes and prune juice. Other processed forms, such as paste, sauce, juice, concentrates and prune bits have not been developed and marketed on a scale similar to apples, cherries, citrus, pears, apricots, etc. A major need within the plum industry is development of new processed products. One such market may be the beverage industry. Since the mid-1980s, packaged soft drink consumers have increased purchases of nutritious healthy beverages. Chief among these has been fruit juices, with their consumption increasing steadily (Kortbech-Olesen, 1991). Several industries market juices exclusively and trends in beverage flavors appear to be shifting toward flavors such as plum and raspberry (Kortbech-Olesen, 1991; Sfiligoj, 1992).

Previous research on plum juice production has reported various methods of extracting and clarifying juice (Komiyama et al., 1977; Ismail et al., 1981; Liou and Wu, 1986). Fining agents are added during juice and wine processing to remove particulate matter such as grape tissue, yeast, or colloidally suspended particles. Such particles may be present in the form of proteins, pectins and gums, metallocolloids, and degradation products of polyphenols (Zoecklein et al., 1990). Removal of pectins and degradation products of polyphenols is critical for improving clarity and color of certain fruit juices. According to Zoecklein et al. (1990), enhanced filterability, due to absorption/adsorption of colloidal and suspended material by the fining agent complex, is a frequent benefit of using fining agents.

Much work has been done to develop plum juice and paste from Stanley plums (Arnold et al., 1992; Wang, 1993; Siddiq et al., 1994). Chang (1993) reported that among five commercial pectinases, Clarex® L at 0.2% produced optimum quality plum juice. Our objective was to investigate the effect of a commercial pectinase treatment on physicochemical and sensory characteristics of juice from selected plum cultivars.

MATERIALS & METHODS

SIX PLUM CULTIVARS, Au Red, Abundance, Pobeda, Shiro, Peach Plum and Early Golden, grown at Michigan State University's Northwest Horticultural Experiment Station at Traverse City, MI were used. Plums were harvested in 1992 at optimum maturity and stored whole at −20°C in a commercial freezer until processed.

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Plum juice extraction

One kg plums were processed into juice, in duplicate, with some modification to the procedure previously described (Siddiq et al., 1994). Plums were thawed, crushed and 0.2% Clarex® L (Solvay Enzymes, Inc., Elkhart, IN) was added. The macerate was held at 49°C for 3 hr before pressing. Bentonite (sodium form @ 5.0%; Sigma Chemical Co., St. Louis, MO) and gelatin (1.0% solution @ 0.05%, wt/wt of juice; Difco Labs., Detroit, MI) were added to clarify juice. After adding bentonite or gelatin, juice was held overnight at 2–3°C before racking and filtration. Pasteurization (high temperature short time, HTST) of the juice was carried out at 85°C for 90 sec using a Spirotherm (Cherry-Burrel, Inc.). Plum juice samples were frozen until analyzed in duplicate for different physicochemical and sensory characteristics.

Juice clarity and total pectin

Juice clarity or turbidity measurements were done according to the methods of Krop and Pilnik (1974) and Ough et al. (1975). Ten-mL portions were shaken and centrifuged at 360 × *g* for 10 min to remove pulp and coarse cloud particles. Percent transmittance was measured at 660 nm with a Spectronic-70 spectrophotometer (Milton Roy Co., Rochester, NY). Extraction of pectin was according to the method of McCready and McComb (1952). Colorimetric measurement of galacturonic acid was done using the method of Kintner and Van Buren (1982). The concentration of pectin was calculated from a standard curve of galacturonic acid.

Soluble solids, pH and titratable acidity

Percent soluble solids, expressed as °Brix, were determined with an Abbe-3L refractometer, sensitivity 0.1% (Bausch & Lomb Optical Co., Rochester, NY.) at 20°C. The pH of samples was measured using a digital pH meter (Model 601A, Corning Glass Works, Medfield, MA.). For titratable acidity, a 10g sample of juice in 100 mL distilled water was titrated to pH 8.0 with a 0.1N NaOH solution. Titratable or total acids of the sample were expressed as % malic acid (% malic acid = mL NaOH × N NaOH × 0.067 meq × 100/wt of sample).

Color, total anthocyanins and total phenolics

Color was measured using a Hunter Color Difference Meter (D25 DP-9000 System, Hunter Associates Lab., Reston, VA). Fifty mL of juice were placed in a standard optical cell for measurement after standardization with a pink tile ($L^*=73.49$; $a^*=17.34$; $b^*=10.28$). This system was based on the Hunter L^*, a^* and b^* coordinates, L^* representing lightness and darkness, $+a^*$ redness, $-a^*$ greenness, $+b^*$ yellowness and $-b^*$ blueness. Hue was calculated as the angle whose tangent equals b^*/a^* (Little, 1975).

Total anthocyanins in the plum juice were measured according to the method of Skalski and Sistrunk (1973). A 5 mL sample of juice was mixed with 45 mL acidified ethanol (1.5N HCl; 95% ethanol = 15:85, v/v). The pH of the solvent was adjusted as required to obtain a final pH of 1.0 in the plum extract. The diluted extract was stored in the dark for 2 hr and filtered (#2 paper) before measuring absorbance at 535nm. Total anthocyanins were calculated using the formula: total anthocyanins = [(absorbance × dilution factor)/E] × (100/5 mL). Factor E was 98.2 for acidified ethanol (Fuleki and Frances, 1968; Francis, 1982). Total phenolics, as tannic acid, were determined by the method of Singleton and Rossi (1964). Results are reported as µg tannic acid/100 mL juice.

Sugars analysis

Sugars were analyzed according to the procedure described by Guyer et al. (1993). A combination of HPLC and YSI glucose analyzer (Yellow Springs Instrument Co., Inc., Yellow Springs, OH) was used to analyze sugars. HPLC was used to separate fructose, sucrose, and glu-

TAP Review of Fungal Enzymes

September 14, 1999

Synthetic or Non-synthetic

Fungal enzymes are non-synthetic substances produced by fermentation.

Properties and Uses

The information about the properties, sources and usage of the materials appears to be complete.

Criteria for Processing

The criteria for Processing, both OFPA and NOSB, appear to be complete and accurate.

Fungal enzymes appear to be necessary for many types of food processing operations and the alternatives are either synthetic or less desirable.

Compatible with Organic Production

The use of fungal enzymes as described is compatible with organic production.

Commercial Interest

I have no commercial or financial interest in this material.

Summary

Fungal enzymes, not produced through means of genetic modification should be added to the NOSB List of Allowed Non-organic ingredients.

The NOSB Materials Database on this issue appears to be complete.

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News and Events

9th Annual Food Microbiology Research Conference

The 9th Annual Food Microbiology Research Conference will be held November 2-4, 1983 in Chicago, Illinois. For more information contact: Dr. J. M. Goepfert, Canada Packers, Ltd., 2211 St. Clair Avenue West, Toronto, Ontario, Canada M6N 1K4.

Classified Ads

For Sale

Single Service milk sample tubes. For further information and a catalogue please write, Dairy Technology Inc., PO Box 101, Eugene, OR 97401.

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Pectinase

This is a naturally occurring enzyme (or actually a class of them) and should not be considered synthetic. One note is that pectinase activity generally results in a loss of textural integrity; it's used to break down pectin for example in the juice industry. I don't think that there should be any question about the means by which pectinases are extracted or obtained from fungal cells. The enzyme database seems more straightforward to me. I don't think there have been any oversights in terms of information provided. There is nothing I disagree with here. I don't believe that enzymes could be considered "preservatives" (I always think microbial here!) but rather processing aids. The write-up on genetic engineering is well done; I agree that NOSB will probably not want to open that door again!

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I hope this is what you had in mind. I haven't done a review previously so let me know if more information is needed. I noticed on both databases that there are words missing in places....please re-check it!

Nonmicrobial enzymes. As indicated previously, meat animals, e.g., cattle, swine and sheep, and edible and nontoxic plants, e.g., papaya, pineapple, barley and fig, have long histories as sources of enzymes used in food processing (*3, 4, 16, 45, 47*). These traditional sources need not be subjected to toxicity testing.

For the purposes of this paper, it is assumed that only animals commonly regarded as food will be employed in enzyme manufacture. As long as CGMPs are followed during manufacture, enzymes derived from food animals may be assumed to be safe for use in food processing. Animal testing for possible toxicity is not warranted.

With regard to new plant enzyme sources, it is assumed that only edible plants will be considered. If the edible plant has been well-studied, is widely consumed without apparent harm, and does not produce toxic substances, then no animal testing should be required. However, if the plant is known to produce toxins, then care should be taken not to concentrate the toxic substances during enzyme manufacture. The final enzyme preparation should not contain toxic substances in quantities that might represent a hazard to health.

ACKNOWLEDGMENTS

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Identification

MSDS Yes

Common Name Enzymes, Fungal

Class Processing

Other Names The model amino acid is pectinase. See the attached table of other amino acids commonly used in food processing.

Code #: CAS

Code #: Other

Chemical Name pectin lyase

Characterization

Composition Biologically active proteins which are sometimes conjugated with metals, carbohydrates and/or lipids. Whole cells, parts of cells, or cell-free extracts. The model enzyme for this review, pectinase, actually refers to a combination of at least six different enzymes (Wingard, Katchalski-Katzin, and Goldstein, 1979), the principle ones are pectin methylesterase, pectin lyase, and polygalacturonase (Food Chemicals Codex, 1981). These are off-white to tan amorphous products, and tan or dark-brown liquids. It is practically insoluble in alcohol, in chloroform, and in ether. The active components consist of the biologically active proteins. These proteins have highly complex structures and may be conjugated with metals, carbohydrates and / or lipids. Active components have known molecular weights that range from 12,000 to several hundred thousand (Food Chemicals Codex, 1981).

Properties Activity measured according to reaction catalyzed by individual enzymes. For example, pectinase hydolyzes the pectin molecule.

How Made Pectinase and other amino acids can be produced by a wide number of methods. One source of commercial pectinase is the mold *Aspergillus niger* grown by controlled fermentation. The substrate will contain various grains and synthetic nutrients. The organisms are separated from the media, usually by physical means such as centrifuging and sorting by specific gravity. The cell walls of the organisms are then burst through a mechanical process of homogenization, similar to that used on milk. The specific enzymes are then extracted by means of either precipitation or absorption by the use of a variety of chemical constituents and / or ion exchange columns. These may be polymers, such as methylcellulose or polyvinyl alcohol, or glycol ethers such as polyethylene glycol (PEG), or salts, such as sodium phosphate. Because extraction is pH dependent, the pH may be adjusted through the use of various acids and bases that are food grade, but not necessarily naturally occurring.

Specific Use(s) Enzymes have a wide variety of uses.See the attached table. In particular, pectinase is used primarily to depolymerize and esterify plant pectins in fruits such as apples, lemons, cranberries, oranges, cherries, grapes, and tomatoes, to name a few. The application of pectinase enables the entire fruit to be liquified. This has the effect of improving saccharification and thus sweetness, reducing waste and energy use per unit of juice produced, improving aroma and color; enhancing clarity, removing haze, preventing gel formation, and increasing fruit juice yield (Neilsen et al., 1994; White and White, 1995).

Action Enzymes are proteins that increase the rate at which chemical reactions will reach equilibrium. They are not consumed in the chemical reactions. As such, their action is catalytic. For example, two constituents of pectinase are pectin methylesterase and polygalacturonase. Pectin methylesterase demethylates pectin; polygalacturonase hydrolyzes the a-1,4-galacturonide bonds in pectin (Food Chemicals Codex, 1981).

Combinations Enzymes are usually used in combination with other enzymes. For example, pectinase is often used with cellulases, hemicellualases, and proteases. Several of these are also produced by *A. niger* (White and White, 1997). May be combined with a number of different carriers, stabilizers, preservatives, humectants, and diluents. Some of these materials are on the recommended National List.

Status

OFPA 2118(b)(1)(C)(iii) the substance is presumed to be used in handling and is non-synthetic but is not organically produced (7 USC 6517(b)(1)(C)(iii)).

Regulatory Status Enzymes are considered food addtives under the Food, Drug, and Cosmetic Act. See 21 CFR 184.

34.

TABLE 3. *Guidelines for determining the safety of microbial enzymes*[a].

A. *Decision Tree*	If yes --proceed to--	If no
1. Is the test material free of antibiotics?[b]	A,2	D
2. a. *For bacteria and yeast*, is the test material: i. Free of toxins[c] known to be produced by other strains of the same species?	A,3	D
ii. If there are no known toxins[c,d] produced by other strains of the same species, is the no-adverse effect level in a single oral challenge at least 100 times greater than the estimated mean human consumption level?[e,f]	B	D
b. *For molds*, is the test material free of detectable levels of aflatoxin B_1, ochratoxin A, sterigmatocystin, T-2 toxin, zearalenone and any other toxins known to be produced by strains of the same species?[g]	C	D
3. Is the no-adverse effect level in subchronic (90-d) feeding sutdies at least 100 times greater than the estimated mean human consumption level?[f,h]	ACCEPT	D

B. *Special considerations for certain yeasts and bacteria*

1. If the source culture is well-known, widely distributed, nonpathogenic yeast, e.g., certain species of the genus *Saccharomyces*, or if it belongs to a bacterial species that is well-characterized, commonly present in foods, has a history of safe use in food enzyme manufacture, and has never been implicated in foodborne disease, e.g., *Bacillus coagulans*, *Bacillis licheniformis*, *Micrococus lysodeikticus*, and *Bacillus subtilis* (*17*), the test material can be ACCEPTED at this point.
2. Test material from other bacteria and yeasts must be considered under part A,3.

C. *Special considerations for certain molds*

1. If the source culture is well characterized, commonly present in food, has a history of safe use in food enzyme manufacture, and has never been implicated in foodborne intoxication or disease, e.g., *Aspergillus oryzae*, *Apergillus niger* and *Rhizopus oryzae* (*16,23,36,41,42,43,45,47,50*), the test material can be ACCEPTED at this point.
2. Test material from all other species of molds must be considered under A,3.

D. *Disposition of materials that fail any Decision Tree requirement*

A negative answer to questions 1, 2 or 3 signifies the presence of an undesirable substance and the material is not acceptable for use in food. If the undesirable substance can be removed, the purified material must be passed through the system again beginning at the point of the original negative answer.

[a]These guidelines are intended for crude culture extracts, for whole cultures, and for concentrated enzyme fractions which, when diluted, become enzyme preparations suitable for marketing.

[b]As determined by (*4*) or comparable methods.

[c]For the purposes of these guidelines, the term "toxin" refers to a substance which is regarded by experts as a cause of food poisoning, intoxication or illness when ingested. Examples are staphylococcal enterotoxins, botulinal neurotoxins and mycotoxins.

[d]Certain cultures in this category are acceptable on the basis of a single acute oral toxicity test, as explained in part B,1. Cultures that fall under part B,2 can go directly to part A,3 without an acute oral toxicity test. This is permissible because the subchronic feeding specified in part A,3 is more rigorous and more meaningful than the acute oral toxicity test embodied in part *A,2,aii*.

[e]Expressed as mg/kg body weight and determined using two appropriate animal species.

[f]Estimated mean consumption level is calculated from the sum of the intakes for each food category in which the material is expected to be used. An example of such determination is: (USDA mean portion size) × (Maket Research Corporation of America eating frequency for the entire population) × (the usual level of use expressed as TOS for the enzyme in question)(*2,14*).

[g]As determined by (*38*) or comparable methods.

[h]Expressed as mg/kg body weight/day, and determined using two appropriate animal species.

known microbial toxins active via the oral route and present at effective levels will be detectable by these procedures. It should be pointed out that preparations will be tested in these proposed feeding studies only after first being assayed for toxins which might reasonably be expected, using chemical, biochemical or biological methods. For example, all test material from fungal sources should be assayed for certain known mycotoxins (*4, 38*).

4. In establishing an Acceptable Daily Intake for microbial enzymes based on the animal feeding studies which we have proposed, there should be no adverse effect at a dose which is 100 times the estimated mean human exposure (based on TOS). This criterion applies to the single oral challenge and to the subchronic feeding study, and is based on the traditional 100-to-1 safety factor for food chemicals (*26*).
5. The only test materials which can pass through the Decision Tree without a subchronic feeding study are those which satisfy the criteria of B.1 or C.1, i.e., certain bacteria, yeast and molds, which are well-known and have never been associated with foodborne illness or disease. However, as stated above, bacteria and yeast that meet these criteria sti must pass the single oral challenge test, and molds must give negative test results for a battery of known mycotoxins.

Historic Use	Enzymes in various forms have been to prepare foods used for centuries. The use of enzymes in organic processing appears to be
International	IFOAM has published criteria for certifiers to use to evaluate the compatibility of enzymes used in organic handling. This same criteria is applied to microorganisms used for processing.
Existing Restrictions	Most US certifiers have allowed the use of fungally derived enzymes documented to not be from genetically engineered sources.

Proposed Annotation

Must be derived from organisms that are not genetically engineered as defined by the NOSB. If synthetic or genetically engineered substances are used in the growth media or the extraction process, then the supplier must demonstrate appropriate measures to ensure that the products do not contain those contaminants. The carriers, diluents, and processing aids used in the production of enzyme preparations shall be substances that appear on the National List of ingredients allowed for use in foods labeled as "Organic." This includes water and substances that are insoluble in food but removed from the foods after processing.

OFPA Criteria for Processing

2119(m)1: chemical interactions Not Applicable 2119(m)2: toxicity & persistence Not Applicable

NOSB Processing Criteria #2 2119(m)3: manufacture & disposal consequences

The fermentation process is relatively efficient and closed. Because of their catalytic nature, enzymes theoretically can react indefinitely, and relatively small amounts are effective in performing their functions. Enzymes need to be replaced when they are degraded by physical conditions (e.g. heat) or removed with the processed food.

NOSB Processing Criteria #3 - 2119(m)4: effect on human health
Also "Nutritional quality of the food is maintained."

Most studies show that nutritional quality as measured by vitamin and mineral content, as well as other parameters, is maintained (Braddock, 1981). In some cases, because of the enzyme's role in the removal of the non-nutritional part of the food and making the nutrients of the food more digestable, enzymes can measurably improve the nutritional quality of food. Other indicators of quality are also improved (Chang et al., 1994).

There is the potential for enzymes to pose a threat to human health and safety. As proteins, enzymes can cause allergic reactions in sensitive individual (Tucker and Woods, 1995). Enzymes can remain active after digested and there is concern that novel enzymes--particularly some of the more potent ones being developed by genetic engineering--will attack human tissues in some instances (Tucker and Woods, 1995). Perhaps the greatest concern with fungal enzymes is the presence of mycotoxins from either the source organism or a competing organism that invades the fermentation media. Many of these organisms are capable of producing antibiotics. While Good Manufacturing Practices require that non-pathogenic strains be used, quality control and hazard analysis critical control point (HACCP) need to be sufficient to ensure that both the strains and the media avoid contamination with pathogens and toxins. The organism used as a case study for this review, *A. niger*, provides a good example. *A. niger* is capable of producing low levels of toxins, but most strains are considered non-toxigenic because the levels (Pariza and Foster, 1983).

Finally, after processing and packaging, the enzyme may be prone to spoilage by a microbial contaminant. For this reason, preservatives are almost always added during processing and after final preparation (Pariza and Foster, 1983).

2119(m)5: agroecosystem biology Not Applicable

NOSB Processing Criteria #1 2119(m)6: alternatives to substance

Enzymes frequently offer the only way to achieve a desired technical effect. Nearly all commercially prepared foods contain at least one ingredient that has been made with enzymes. Acceptance of lower yields and lower quality. In a number of cases, the alternatives would be prohibited for use in organic production (e.g. sulfuric acid); in other cases, the alternatlive would be chemical modification (e.g. sodium hydroxide used to produce starch). Some enzymes are essential for the production of certain foods, for example amylase to produce barley malt or rice syrup; or various coagulents used to produce cheeses.

is no basis for concern that the enzymes under consideration in this report are themselves toxic. Acute and subchronic oral toxicity studies (to be proposed) should be conducted with two animal species (*24*). This is necessary to compensate for possible species variation in toxic response. For example, rats are much more sensitive to aflatoxin B_1 than mice, whereas dogs are more sensitive than rats to ochratoxin A (*42*). There are also species variations in response to the protein/peptide enterotoxins and neurotoxins of bacteria (*41*). Additionally, some animal species are capable of emesis, e.g., dogs and pigs, whereas others are not, e.g., rodents. Selection of appropriate test animals should be based on two criteria: (a) which toxins could be produced by the source organism and (b) which toxins have already been eliminated from further consideration by the use of specific chemical/biochemical assays. In many instances, rats and dogs may be the most appropriate test animals (*24*).

Guidelines for determining enzyme safety

Basic premises. In developing guidelines to assure the safety of enzymes used in foods, we have adopted the following basic premises to guide our thinking. The rationale for each of these premises can be found in preceding sections.

1. Enzymes are naturally occurring proteins. Only a very few, highly unusual enzymes are toxic and they would not be used in foods.
2. There is no basis for concern that enzymes acting on otherwise wholesome food constituents will generate harmful products. Hence, there is no reason to test enzyme-treated foods for toxicity.
3. New enzymes could be derived from animals, plants or microorganisms. However, for technical reasons it is likely that most new enzyme preparations will be derived from microbial sources, in many instances new microbial species or strains.
4. Enzymes are added to food at very low levels. Failure to demonstrate harmful materials in, or toxic effects from, concentrated enzyme fractions, which when diluted yield finished enzyme preparations for marketing, gives reasonable assurance of their safety. Alternatively, failure to demonstrate harmful materials in, or toxic effects from, cultures or crude extracts of a proposed source microorganism, gives reasonable assurance of safety for any enzyme preparation which may be produced from that source organism using CGMPs.
5. If a microbial culture does not produce known toxins and if its metabolites are nontoxigenic in the sense that they do not produce food poisoning, intoxication or illness when ingested, then enzymes derived from that culture using CGMPs will be safe for use in food processing.
6. If there are toxigenic strains of the species to which the new culture belongs, then growth conditions under which those strains produce toxins should be tested. The condition(s) to be used for enzyme manufacture would, of course, be included. It is also prudent to test mutants for toxins produced by other *strains* of the same *species* even if the parent culture is negative for such substances.
7. Certain microbial species produce antibiotics, which are detectable in appropriate bioassays.
8. Some of the filamentous fungi and *Actinomycetales* produce toxins. A few of these substances are carcinogenic, e.g., aflatoxin, and some also possess antitumor and antimicrobial activity, e.g., azaserine. Such metabolites may be detected with specific chemical, biochemical or biological assays.
9. Bacteria other than *Actinomycetales* may also produce acute toxins. Of specific concern are the peptide/protein toxins that act via the oral route, e.g., enterotoxins and certain neurotoxins. Toxins associated with foodborne illness can be detected with serological or animal assays.
10. Bacteria as a group (other than *Actinomycetales*) are not known to produce carcinogens or mutagens when grown in ordinary culture medium which does not contain nitrite and secondary amines.
11. Yeasts as a group are not known to produce toxins, although some yeasts are pathogenic. The carcinogen urethan may form at very low levels in yeast fermentations. Urethan can be detected by chemical assay.

Microbial enzymes. Guidelines for determining safety of microbial enzymes are shown in Table 3. These guidelines may be applied to concentrated enzyme fractions which are diluted to produce finished enzyme preparations. Alternatively, the guidelines may be applied to crude culture extracts or whole cultures from which enzymes are manufactured. If the crude culture extracts or whole cultures are judged to be safe, then enzymes can be manufactured from these sources without further testing.

It is important to note the following features concerning the guidelines in Table 3.

1. All test materials must be evaluated for antibiotic activity.
2. No test material can pass through the Decision Tree without being tested for toxic constituents.
3. Two animal bioassay systems are proposed. The first is a single oral challenge. The purpose of this assay is to evaluate the test material for food poisoning toxins, specifically enterotoxins and certain neurotoxins, which are protein or peptide toxins produced by a few bacterial species. The second proposed bioassay is a subchronic feeding study in two appropriate animal species. The purpose of this procedure is to detect mycotoxins and other toxic substances which might not produce acute toxicity. All

NOSB Processing Criteria #6 2119(m)7: Is it compatible?

Enzymes have been used in organic processing for as long as organic processed food has been marketed. They are currently being used in certified organic processing. Pectinase is a good example. The first use of pectinase in fruit juice processing dates back to the 1930s (Nielsen et al., 1994). A survey of organic processors about the compatibility of various processes did not ask a general question about enzymes or a specific question about pectinase. In general, enzymes were rated between 2.5 and 2.7 on a scale of 1 to 5 as compatible with organic processing (Raj, 1991).

NOSB Criteria for Processing

NOSB Proc Criteria #4: Preservative?

While enzymes can be used to transform food into a more stable product, these processed foods are generally identified as different from their raw ingredients. For example, raspberry jelly is considered to be different from raspberries. The use of pectinase neither increases nor decreases the shelf life of a raw product. In a natural situation, various enzymes are produced by either the plant itself (Kays, 1991) or various organisms to accelerate decay, decompose cell walls, increase sugar content, and release the nutrients contained in the fruit and other plant organs in the senescence process.

NOSB Proc Criteria #5: GRAS & residues?

A number of fungal enzymes are generally and specifically considered GRAS. The Enzyme Technical Association has made a self-declaration of GRAS for a number of enzymes: See the attached table. The Food Chemicals Codex places the following limits on residues: Arsenic (as As) not more than 3 ppm; coliforms: not more than 30 per g. Heavy metals as lead: not more than 0.004%. Lead (Pb): not more than 10 ppm. Salmonella spp: Negative by test. The *Food Chemicals Codex* also states that "[a]lthough tolerances have not been established for mycotoxins, appropriate measures should be taken to ensure that the products to not contain such contaminants."

NOSB Proc Criteria #7: Essential & Minimum?

For many foods, they are essential to the identity of the food. Even where they are not required to be used to make an identifiable food, they are needed to produce a food of the quality consumers expect. In determining the standard of identity of natural juice, juice extracted using pectinase is usually considered minimally processed (Haight and Gump, 1995). Because they are effective in small amounts very little of any enzyme is needed to process a given food.

Discussion

Most of the organisms that produce commercial enzymes are considered fungi of some sort. These organisms include the molds Aspergillus Niger, Rhizopus oryzae, Rhizomucor meihei, blights such as Endothia parasitica and yeasts such as Candida spp and Saccharomyces spp. A considerable amount of research has been conducted on genetically modifying fungi and other organisms to increase the yields and consistencies of enzymes. Many of the prospective donor organisms are pathogenic and are being screened for genetic sequences to be inserted into non-pathogenic hosts (see, for example, Surgey, Robert-Budouy, and Condemine, 1996). Continuous improvement of production methods is possible without the use of recombinant DNA techniques. For example, classical methods of hybridization can also be used to improve enyzme-producing organisms (see Solís, S., M. E. Flores, C. Huitrón, 1997).

Although there are some important differences between enzymes used for human food and those used for livestock feed, the same guidelines should be followed for the use of enzymes as an organic livestock feed additive as are used for enyzmes used as direct food additives in organic food processing, unless otherwise specified.

32.

otherwise nontoxic proteins, and, indeed, it should still be considered speculative. However, a recent report (*53*) indicates that peptides with neuropharmacological properties are generated by the action of the natural animal digestive enzyme, pepsin, on wheat gluten or casein, i.e., the major protein of milk. The peptides are called "exorphins" because they mimic in vitro the action of opioid-like peptides, the endorphins, which are produced naturally by animals. It is suggested that such peptides may form during digestion of some food proteins in the human gastrointestinal tract, and could have physiological significance (*53*). The possibility of such peptides forming in processed foods treated with proteases was not considered.

This example illustrates the difficulty that arises when one attempts to establish absolute safety. Such a goal would be extremely difficult for a static system, and is clearly impossible when dynamic forces, such as basic scientific inquiry, continually expand our understanding and knowledge. However, there is also no reason, on the basis of available information, to fear that processed foods treated with proteases might pose a hazard, especially one that is greater than that posed by our own digestive systems. This is clearly a research area which deserves further support, especially as it relates to human physiological significance and development of specific and relevant assays.

Interactions between enzymes and other food components. It is well-known that certain drugs are not compatible with one another and that combinations of such incompatible drugs can result in interactions which are toxic (*28*). It has been suggested that such interactions might also occur between enzymes and other components of beverages or food products (*6*). However, there is no scientific basis for such speculation. It is extremely unlikely that enzymes, which are used at very low concentrations and are almost always inactivated or removed before the finished food or beverage is marketed, could produce a toxic effect due to interaction with another substance. Given the high specificity of enzyme action, it is difficult to imagine such an occurrence. The highly improbable possibility of toxic interactions involving food enzymes should not be afforded serious consideration unless supporting data appear in respected and well-refereed scientific journals.

Direct effects of food enzymes on consumers. Under the usual conditions of use in foods, enzymes do not pose a hazard for consumers. For example, ingesting an active protease at relatively low levels could hardly affect the human gastrointestinal tract, where many potent proteases, such as trypsin and pepsin, already are present at levels sufficient to digest food. This view is supported by the report of an expert committee (*11*). Proteases may adversely affect the skin, mucous membranes of the nose and throat, and lungs, and such effects are sometimes seen workers who handle large quantities of proteases. However, such occurrences are extremely rare in consumers who use much lower levels of active enzyme (*11, 15*), and it is not possible for heated foods containing inactive proteolytic enzymes to pose such a threat. Active proteases are, of course, widely distributed in fresh fruits, vegetables, cheeses and other uncooked foods which may be consumed.

We know of no reported adverse effects on humans from lipase/esterases or carbohydrases in foods. Moreover, many enzymes are inactivated in the gastrointestinal tract and digested as protein.

Concept of relative safety

The terms *nontoxigenic* and *nonpathogenic* should not be considered in an absolute sense. In the real world they are relative concepts which convey certain probabilities. A nontoxigenic organism is one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure. In the same vein, a nonpathogenic organism is one that is very unlikely to produce disease under ordinary circumstances. Thus, *Aspergillus oryzae* should be considered nontoxigenic because it does not produce detectable levels of aflatoxin (*23, 50*) and is not listed with molds known to produce other mycotoxins (*42*). Strains in commercial use did not produce detectable levels of beta-nitropropionic acid (*36*) and there are no reports of this organism producing adverse effects in animals. Likewise, *S. cerevisiae* should be considered nontoxigenic even though low levels of the carcinogen urethan are produced during fermentation (*37*) because, as far as we can tell, the amount of urethan is too low to be significant. Applying an absolute definition in this case would result in the banning of bread, wine and beer. There is no reason to believe that such an extreme measure would make our lives safer! As long as the levels of urethan in fermentative yeast enzyme preparations do not exceed those found in fermented foods and beverages, they should not be a cause of concern.

Aspergillus niger produces low levels of toxic substances (*22*), but it is only after such substances are extracted and concentrated that toxicity can be demonstrated. This example points up the important distinction between *toxin*, a chemical entity, and *toxic effect*, a biological phenomenon produced by toxins only at effective doses. Synthesizing low levels of toxins per se should not be sufficient to support redefining *A. niger* as a toxigenic organism, and it should remain classified as nontoxigenic. In the same way *B. subtilis* should be considered nonpathogenic even though one could imagine an individual with an extremely compromised immunological system succombing to a *B. subtilis* infection. Under more ordinary circumstances, *B. subtilis* does not cause disease.

These concepts are important in considering safety assessment. Absolute safety is not achievable and cannot be our goal. Rather, we should think in terms of probabilities tempered with common sense.

Animal testing for toxins

The purpose of animal testing is to assure that toxic effects are not produced by non-enzyme substances in enzyme preparations under realistic projections of use. There

Braddock, R. J. 1981. Pectinase treatment of raw orange juice an subsquent quality changes in 60°Brix concentrate. *Proc. Fla. State Hort. Soc.* 94: 270-273.

Chang, T.S., M. Siddiq, N.K. Sinha, and J.N. Cash. 1994. Plum juice quality affected by enzyme treatment and fining. *J. Food Sci.* 59: 1065-1069.

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Pariza, M.W. and E.M. Foster. 1983. Determining the safety of enzymes used in food processing. *J. Food Protection* 46: 453-468.

Servili, M., A. L. Begliomini, and G. Montedoro. 1992. Utilization of a yeast pectinase in olive oil extraction and red wine making processes. *J. Sci. Food Agric.* 58: 253-260.

Solís, S., M. E. Flores, C. Huitrón. 1997. Improvement of pectinase production by interspecific hybrids of *Aspergillus* strains. *Letters in Appl. Microbiol.* 24: 77-81.

Surgey, N., J. Robert-Baudouy, and G. Condemine. 1996. The *Erwinia chrysanthami* pecT gene regulates pectinase gene transfer. *J. Bacteriology* 178: 1593-1599.

Tucker, G.A. and L.F.J. Woods. 1995. *Enzymes in Food Processing.* London: Blackie Academic and Professional.

White, J.S. and D.C. White. 1997 *Source Book of Enzymes*. Boca Raton: CRC Press.

or any other classes of carcinogenic or mutagenic chemicals, should not be considered either a real or potential problem area in enzyme manufacture from bacteria (other than certain *Actinomycetales*).

In contrast, some antitumor agents and antibiotics produced by *Actinomycetales*, particularly certain *Streptomyces*, are weakly carcinogenic, e.g., azaserine (*34*). Moreover, some mycotoxins have carcinogenic and mutagenic activities (*33, 34, 42*). If there is reason to believe that such substances might be produced by a new culture under test, then specific chemical, biochemical or biological tests for the substances should be conducted.

Teratogens and reproductive effects. Various dietary deficiencies and excesses, hormones, drugs, agricultural and industrial chemicals, naturally-occurring toxins, and physical and biological agents produce, under some circumstances, teratogenic effects or reproductive deficiencies in experimental animals (*20, 27*). Some of these agents or conditions, such as German measles, alcohol abuse, and certain drugs and antibiotics, produce similar effects in humans. However, enzymes are not among the substances which have been shown to cause teratogenesis or reproductive deficiency. In fact, in a four-generation study in rats, a rennet preparation from *Mucor pusillus* produced no evidence of teratogenicity or toxicity towards the reproductive system (*12*), and similar negative data have been obtained for various enzymes from other microbial (*43*) and nonmicrobial (*11*) sources. Those microbial metabolites which could pose such a risk should be detected either as certain specific antibiotics (*20, 27*) or as acute/subchronic toxins (*42*).

Antibiotics. Antibiotics are chemicals produced by various species of microorganisms which kill or inhibit the growth of other microorganisms. They are really a special class of toxic agents which are useful to man in the control of disease. It is well-documented that a sensitive microorganism can acquire plasmids which confer antibiotic resistance on the host (*40*). For this and other reasons enzyme preparations intended for use in food processing should not contain antibiotics. There are methods for assessing enzyme preparations for antibiotic activity (*4*).

Allergies and primary irritations. Industrial enzymes are foreign (nonhuman) proteins, and as such, may be allergenic for humans under certain conditions. The group most likely to be affected are plant workers (*11, 15, 47, 49*). There are methods and procedures for protecting workers from this potential hazard and it is considered to be a manageable problem (*15, 47, 49*).

There are no confirmed cases of allergies or primary irritations in consumers caused by enzymes used in food processing. This is probably due, in part, to the low levels of enzymes added to foods. Foods naturally contain a wide variety of foreign (nonhuman) proteins, many of which are present at levels far higher than the industrial enzymes added as processing aids. Allergies and primary irritations from enzymes used in food processing should be considered a low priority item of concern except in very unusual circumstances. There is no justification for requiring routine testing of enzyme preparations for allergic responses or primary irritations relative to consumer safety.

Toxins involved in food posioning. A few bacterial species produce toxic proteins or peptides which can cause food poisoning. These include both enterotoxins and neurotoxins (*41*). There are immunological assays or animal systems for detecting such toxins. Within a bacterial species known to cause food poisoning via a toxin, usually only some, but not all, strains produce the toxin. Hence, nontoxigenic strains can be isolated (*41*). Some bacterial toxins are actually coded for in bacteriophage DNA which has become integrated into the bacterial genome as a prophage. "Curing" the organims of the prophage results in loss of toxicity (*41*).

Bacterial toxins which cause food poisoning are, by definition, substances which produce acute toxic responses following introduction into the gastrointestinal tracts of sensitive annimals. The nature and severity of the toxic response may vary among animal species under test, as well as the amount of toxin required to produce a measurable effect.

Products of enzymic reactions. Enzymes are used in food processing because they produce desirable changes in the natural food constituents. They are usually inactivated or removed before the final food product is marketed. As such, enzymes should be classified as *processing aids* or *secondary direct additives*. Declaring their presence on the label of a food product, in most cases, would be incorrect, since only rarely is the active enzyme present in the final product. This unique status of enzymes can lead to a new question, however. Are the products of the enzymic reaction safe? Developing an answer to this question requires an understanding of what the enzyme is doing in producing an apparently favorable transformation in the food.

Most of the enzymes used in food processing are degradative enzymes which split macromolecules, i.e., proteins, complex carbohydrates and lipids, into smaller subunits. Another important example is glucose isomerase, which catalyzes the conversion of glucose into its isomer fructose. Both glucose and fructose are nutritive and nontoxic. Only one enzymic reaction used in food processing is known to yield a potentially toxic product. Pectic enzymes increase the methanol content of treated fruit products, but the amount produced is far below the hazard level (*47*). There are reliable and rapid assays for methanol in food.

The question of hypothetical, potentially hazardous enzyme reaction products is difficult to evaluate, but probably its importance is marginal. For example, proteases from all sources degrade proteins into peptide fragments and amino acids. However, different proteases attack proteins at different sites and may produce different sets of peptide fragments from the same protein substrate (*52*). There are many biologically active peptides in nature which serve in various metabolic regulatory capacities. One may wonder if the peptides produced by proteases have any biological properties of their own. Until recently, most biochemists would have considered as highly remote the possibility that toxic peptides might be generated from

Index to Enzymes, Processing Background Information for TAP Review from OMRI, September 20, 1999

A. Letter and Table from the Enzyme Technical Association. July 26, 1999.

B. Pectin-decomposing enzymes and their use in the fruit juice, wine and jelly industries. Tauber, 1949, Chapter XXVII. Pages 458-461.

C. Enzyme Preparations. Food Chemicals Codex. 1981. Pages 107-110, 480-481.

D. Determining the safety of enzymes used in food processing. Pariza and Foster. 1983. J of Food Protection. V 46. Pages 21-36 handwritten, pages 453-458.

E. Plum juice quality affected by enzyme treatment and fining. Chang, Siddiq, Sinha, and Cash. 1994. J of Food Sci V 59. Pages 1065-1069.

F. Enzyme formulations for optimizing juice yields. Faigh. 1995. Food Technology. September. Pages 79-83.

G. Pectinase treatment of raw orange juice and subsequent quality changes in 60 degree brix concentrate. Braddock. 1981. Proc. Fla. State. Hort. Soc. 94. Pages 270-273.

H. Utilization of a yeast pectinase in olive oil extraction and red wine making processes. Servili, Begliomini, Montedoro, Petrucciloli and Federici. 1992. J. Sci. Food Agric. 58. Pages 253-260

I. Genetic Engineering. Tucker and Woods. 1995. Enzymes in food processing. Pages 22-23, 34-37.

J. Extraction and purification of enzymes using aqueous two-phase systems. Kula. 1979. Pages 71-95.

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where toxic contaminants are present, they may be removed during manufacture.

With regard to microorganisms used in enzyme manufacture, we have discussed previously our contention that mutagenesis in the laboratory does not result in the acquisition of new genes, so it is not possible for an isolate to acquire a new toxin gene by mutation. It may be theoretically possible for a mutation to alter the structure of an otherwise nontoxic enzyme in such a way that the enzyme becomes toxic (*10*), but there is no experimental basis for this notion and we consider it to be remote. Advances in DNA sequencing may ultimately be useful in providing definitive proof of nontoxicity.

Proving that a new microbial isolate does not produce a toxin elaborated by other strains in the same species is complicated by the fact that toxin production may be affected by growth conditions. Under some conditions, toxin synthesis may be high, whereas under other conditions, it may be low or undetectable. Hence, to establish that an isolate is nontoxigenic in an absolute sense may not be possible strictly from data on toxin expression. By assaying toxin production under a variety of growth conditions, the probability of demonstrating toxigenic potential is increased. Moreover, if an isolate is grown under conditions where other closely related organisms elaborate a toxin, the reliability of a negative result is strengthened even further.

In practice, enzyme preparations will not contain all of the substances that a source organisms is able to produce. For example, enzymes which are concentrated by ultrafiltration or precipitation will contain far fewer low molecular weight components than are present in crude enzyme extracts. For this reason, even if an organism produces low levels of a potentially hazardous substance, the amount of a finished enzyme preparation needed to produce a deleterious effect in animals likely will be far above the low concentrations at which enzyme preparations are employed in food processing. Published animal feeding studies and summaries of unpublished experiments reviewed by expert committees (*12, 43, 44*) fully support this conclusion.

Pathogenicity. If an isolate is known to be or suspected of being a human pathogen, it will almost certainly not be further considered for commercial enzyme production unless it is the singular source of a unique and useful enzyme. The problems inherent in maintaining and handling cultures of pathogenic organisms on an industrial scale make it unlikely that they will ever be used in the manufacture of enzymes for food processing, and there are federal regulations concerning this issue (*7*). However, high purified enzymes from pathogenic bacteria are produced commercially and used with medical supervision in the treatment of disease (*19*).

Carcinogens and mutagens. No one has ever reported an enzyme which when fed was mutagenic or intitated carcinogenesis.[3,4] Given our current understanding of the processes of carcinogenesis and mutagenesis (*34, 51*), it is implausible to expect that the protein component of an enzyme or protein with such activity will ever be discovered[4]. Rather, attention should be directed towards the relatively small organic molecules (in general, MW <500 daltons) that possess carcinogenic or mutagenic activity and which might reasonably be expected to contaminate a given enzyme preparation.

Enzymes from mammals commonly used as food in the United States will not contain mutagens or substances which can initiate [3] carcinogenesis as long as CGMPs are followed. Some plants are known to produce carcinogens (*13, 34*), but the pineapple, fig, barley and papaya are no among them. The fungal and bacterial enzyme sources listed in Table 1 also are not known to produce carcinogens or mutagens. However, fermentative yeasts, such as *Saccharomyces cerevisiae*, may produce low levels of urethan (*37*), a carcinogen which is not mutagenic in the Ames test (*1*), as a natural by-product of fermentation. For this reason bread, wine and beer often contain low levels of urethan (*37*). There are no reports of urethan in yeast enzyme preparations. Moreover, where yeast enzyme preparations are concentrated by ultrafiltration or precipitation, small molecular weight compounds, such as urethan, will be removed or greatly decreased in concentration. For this reason it is unlikely that urethan levels in yeast enzyme preparations would exceed the levels found naturally in bread, wine and beer.

Several long-term animal studies (>90 days) have been conducted with enzyme preparations from microorganisms, and none showed evidence of carcinogenicity or chronic toxicity (*12, 43*). It is necessary to conduct such long-term tests for each new microbial culture, or for each new enzyme? We think not. For example, we have been unable to locate a single confirmed report of a carcinogen or mutagen produced by bacteria, other than certain *Actinomycetales*, particularly *Streptomyces*, when grown in ordinary culture media. When nitrite and secondary amines are added to culture media, a few bacterial species appear capable of generating nitrosamines through unknow mechanisms (*35*). However, there is no reason for nitrite and secondary amines to be added to culture media intended for use in food enzyme manufacture. Nitrosamines.

[3]It is important to recognize that the process of carcinogenesis as now understood consists of two stages. The first stage is called *initiation*, the second *promotion (39)*. Some animal products, e.g., certain fats and hormones, may at high doses and in certain well-defined experimental systems promote specific types of cancers. However, it has not been shown that these substances can initiate cancer, and it is commonly accepted among experts in this field that they are not complete carcinogens. Animals exposed to carcinogens may metabolize them to other forms which retain carcinogenic activity, e.g., aflatoxin M_1 in the milk from cows exposed to aflatoxin B_1 in their diets; (*42*). Animals may also generate nitrosamines from nitrite and secondary amines in their gastrointestinal tracts (*35*). However, mammals are not known to produce substances as normal body constituents which experts would classify as carcinogens.

[4]It is possible for certain enzymes that act on nucleic acids, such as DNA-dependent DNA ploymerase, to be altered by mutation in such a way as to become error-prone, thus resulting in further mutation in the organism containing the error-prone polymerase (*48*). However, such enzymes would not be produced for use in food processing. Moreover, should such enzymes be present in food enzyme preparations, they would almost certainly not enter human cells and produce an adverse effect. They are also produced by some *Streptomyces* sp. antibiotic proteins with mutagenic and DNA-damaging activities due to the presence of nonprotein prosthetic chromophores, i.e., the apoproteins themselves are without such activity (*25a, 39a*).

A:

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July 26, 1999

AUG 02 1999

Dr. Brian Baker
Technical Director
Organic Materials Review Institute
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Eugene, OR 97440-3758

Dear Dr. Baker:

As we agreed upon during our recent telephone conversation, the Enzyme Technical Association (ETA) is sending you an updated list of enzymes marketed for use in food by ETA members. As you can see in the Table, ETA is aware of sources of enzymes which are modified using genetic engineering techniques and they have been marked with an asterisk, with "d" designating the donor strain. In instances where the donor and source are the same, this indicates the organism is a "self-cloned" or "multicopy" strain.

In our past communications with the Organic Trade Association and the National Organic Standards Board, ETA has attempted to clarify why we are unable to apply the seven Criteria for the Acceptance of Materials Used in Processing to each of the enzyme products on the ETA list. The first criterion requires that "an equivalent substance cannot be produced from a natural source and has no substitutes that are organic ingredients". Using alpha-amylase as an example, one could argue that a strong acid can be produced from a natural source and, like alpha-amylase, is capable of hydrolyzing starch. But acid hydrolysis of starch is a harsh process that produces undesirable byproducts and is not highly efficient when compared to enzymatic hydrolysis. Even among the alpha-amylases, the enzymes from different sources have their own distinct action pattern on starch. Further, each starch processor has its own unique manufacturing system which may behave differently with the various enzymes. For these reasons, it is impossible to say whether "there is no other way to produce a similar product without its use".

We also discussed whether or not the ETA had a definition for "GMO". As Dr. Lynn Hartweck of the University of Wisconsin so aptly described in the *Genetic Engineering Organic Trade Association Information Background Kit,* an exact

TABLE 2. *Selected enzymes and their maximum use in various foods based on TOS (Total Organic Solids) (5).*

Enzyme	Food category	Maximum use[a]
Papain	Baked goods	0.0078%
	Meats/meat products	0.0044%
	Beer/ale/malt beverages	0.0045%
Rennet (and other milk clotting enzymes)	Cheese	0.036%
	Gelatins/puddings/custards	0.0040%
Bromelain	Candy	0.000016%
	Fats and oils	0.000084%
	Snack foods	0.00056%
Pectinase	Baked goods	0.00000026%
	Fruits/juices	0.0035%
	Non-creamed soups	0.060%
Invertase	Candy	0.0078%
α-Amylase	Breakfast cereals	0.0030%
	Sugars/frostings	0.052%
	Gelatins/puddings/custards	0.0000020%
	Corn syrup	0.052%

[a]Percent of food based on TOS.

Life Sciences has undertaken several surveys of industrial use of food additives. In 1977, the FNB's Committee on GRAS List Survey — Phase III was asked by the FDA to organize an extensive survey of enzyme use in food processing. The Committee worked closely with AHETC and the FDA in developing questionnaires; then the AHETC distributed the survey forms to users and manufacturers of enzymes on a confidential basis. The FNB Committee received the completed forms directly for the respondents, reviewed and analyzed the data, and submitted a report to the FDA. The document is entitled *The 1978 Enzyme Survey (5)*.

The survey report contains extensive information on 23 enzymes and an analysis of their use in a detailed list of specific food items. Average and maximum use levels are estimated by TOS. Removal and inactivation of the enzymes by further processing is also tabulated. Table 2 contains some examples from this survey demonstrating the low levels at which enzymes are added to foods.

ENZYME SAFETY

Current status

Exhaustive literature reviews commissioned by the FDA for food enzymes from microbial (*43*) and nonmicrobial (*11, 44*) sources support the proposition that enzyme preparations from nontoxigenic, nonpathogenic organisms are safe to consume. This conclusion is strengthened by the report of the Joint FAO/WHO Expert Committee on Food Additives, which evaluated both published and unpublished data (*12*). There are numerous GRAS affirmation petitions currently before the FDA which also contain safety data on enzyme preparations (*46*).

It is not surprising that the enzymes used in food processing have proven to be nontoxic when tested in animals. In fact, very few toxic agents have enzymatic properties and those that do, e.g., diphtheria toxin and certain enzymes in the venoms of poisonous snakes catalyze unusual reactions which are completely unrelated to the kinds of catalytic transformations that are desirable in foods. Hence, the only relevant issue is whether enzyme preparations contain toxic contaminants. It follows that, if the source organisms do not produce toxins and if CGMPs are followed during manufacture, then the resulting enzyme preparations will not contain hazardous materials.

In practice, industrial enzymes have a strong record of safe use in food processing. However, as with all food components, it is important that scientific data be provided to show that enzyme preparations, particularly those lacking a long history of safe use, are safe to consume. To develop a logical approach to this issue, we shall first consider the factors which bear on the safety of enzymes and then present guidelines for assessing enzyme safety.

Safety considerations

Safety of source organism. The safety of the source organism should be the prime consideration in assessing the probable degree of safety of an enzyme preparation intended for use in food. For example, if the source organism is a food animal, an edible and nontoxic plant, or a nontoxigenic and nonpathogenic microorganism which does not produce antibiotics, then it follows that enzyme preparations obtained from that source organism using CGMPs (*8*) will be safe to consume at the low levels encountered in processed foods. Moreover, in other instances

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definition for GMO or genetic engineering is hard to come by. Because the various government agencies regulate GMOs differently, ETA does not currently support a particular definition at this time.

The ETA appreciates your efforts to aid the NOSB in the listing of certain enzymes as organic substances. Please let us know if we can help you further.

Sincerely,

Nancy W. Zeman

Nancy W. Zeman
ETA Chair

Enclosure(s)

28.

centration is accomplished simply by removing water through evaporation. Preservatives are almost always added during processing, and optionally in the final preparation, to prevent microbial growth and to stabilize and maintain the desired enzymic activity. Proper and appropriate use of preservatives and stabilizers serve to protect the consumer from unsafe or ineffective enzyme products (*7, 8, 16, 47*). When the enzyme is intended for addition to food, all such additives and diluents must be acceptable to the FDA for use in food. They must be of food grade quality and the levels used must not exceed specified limits.

Most industrial enzymes are not purified to any significant extent because purification is not necessary to achieve safe and useful products (*3, 4, 16, 47*). However, it is sometimes desirable to remove or destroy unwanted enzyme activities which would otherwise interfere with effective use of the desired enzyme preparation. For example, rennet produced by some microorganisms contains lipase activity which will make the finished cheese rancid. By carefully exposing the crude rennet to heat or low pH, the lipase can be inactivated without affecting the protease activity. In this example, the unwanted lipase is not physically removed (as in purification); the protein remains but is no longer catalytically active (*47*). Because of expense, physical separation normally is accomplished only when there is a market for the individual separated enzymes, although some manufacturers do highly purify certain enzymes of particular economic importance. For example, one company produces a very pure, crystalline glucose isomerase preparation for its own use (*47*).

Following extraction, concentration and stabilization, enzyme preparations are standardized (*3,4,47*). Because enzymes are catalysts, they are marketed in terms of units of catalytic activity rather than by weight or volume. A unit of catalytic activity for an enzyme preparation is defined in terms of the transformation of a given amount of substrate during a specified period of time under stated reaction conditions. Biochemists often use a unit defined by international convention, which is the amount of enzyme required to transform one micromole of substrate per minute under specific reaction conditions. However, this definition is not applicable to many commercial uses where the substrate is part of food (e.g., Swift's hamburger test for papain; *47*). Hence, many assays for industrial enzymes are based on specific application rather than uniform convention.

The standarization procedure consists of using a specific quantitative assay to determine the level of enzyme activity per milliliter or gram of the final enzyme preparation and then adjusting the activity (usually by dilution of the enzyme preparation) to conform with a desired level of activity which is convenient to use. Unstandardized enzyme preparations may also be sold, and, in this case, total activity is stated and will vary between lots.

Given that enzymes are marketed on the basis of activity rather than weight or volume per se, it follows that the activities and amounts of other enzymes, as well as the levels of nonenzymic catalytically inert materials, may vary from lot to lot and almost certainly from source to source (*47*). Moreover, since enzyme preparations are almost always relatively crude mixtures, it is apparent that anything produced by the source organims, and anything purposely or inadvertently introduced into the system during enzyme manufacture, may end up in the final enzyme preparation. For this reason, it is important that the source organism not produce or contain toxins. To avoid inadvertent contamination with unsafe substances, it is necessary that CGMPs be followed during enzyme manufacture. There are strict limits on the levels of heavy metals which will be tolerated, and there are requirements for demonstrating microbiological safety (absence of salmonellae, etc.) (*3, 4, 16, 45, 47*).

Immobilized enzymes

Some enzymes are sold in an immobilized form, i.e., products containing enzymes that have been immobilized by adsorbtion, entrapment, reaction with cross-linking agents or covalent attachment to insoluble supports (*29*). The safety evaluation of products such as these may require consideration of factors other than the safety of the enzyme, its source and the by-products of the production methods. For this reason, safety evaluation of immobilized enzymes will not be included in this paper.

Consumption levels

Total Organic Solids (TOS). Enzymes are marketed by units of activity rather than by weight or volume, and enzyme preparations always contain other substances (salts, preservatives, stabilizers, carriers, nonenzymic organic material, etc.) (*16, 45, 47*). Further, some enzymes are added to food and remain there, although they may be inactivated by heat or other treatment in the finished food product. On the other hand, some enzymes only come in contact with the food (immobilized enzymes) but do not stay there. For these reasons, it is not an easy matter to estimate total enzyme use and consumption.

The most logical means currently available for arriving at a reliable estimate of enzyme use and consumption was developed by the Ad Hoc Enzyme Technical Committtee (AHETC), a trade group representing companies that produce or distribute enzymes for food use. AHETC set forth the concept of Total Organic Solids (TOS; *5*) as a means of determining the toxicological significance of material derived from the enzyme source. TOS is defined as the sum of the organic compounds, excluding diluents, contained in the final enzyme preparation. It is derived experimentally as follows:

$$\text{TOS}\,(\%) = 100 - A - W - D$$

where A = % ash contained in the extract or isolated enzyme, W = % water in the extract or isolated enzyme, D = % diluents (if any, or carrier if enzyme is immobilized).

The 1978 Enzyme Survey. The Food and Nutrition Board (FNB) of the National Research Council's Assembly of

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ENZYME PREPARATIONS USED IN FOOD PROCESSING

TRIVIAL NAME	CLASSIFICATION	SOURCE	SYSTEMATIC NAMES (IUB)[a]	IUB NO.[a]	CAS NO.[b]
α-Amylase	Carbohydrase	(1) *Aspergillus niger* var. (2) *Aspergillus oryzae* var. (3) *Rhizopus oryzae* var. (4) *Bacillus subtilis* (5) *Bacillus subtilis** d-*Bacillus subtilis* (6) *Bacillus amyloliquefaciens** d-*Bacillus* (7) barley malt (8) *Bacillus licheniformis** d-*Bacillus licheniformis* (9) *Bacillus stearothermophilus* (10) *Bacillus subtilis** d-*Bacillus megaterium* (11) *Bacillus subtilis** d-*Bacillus stearothermophilus* (12) *Microbacterium imperiale* (13) *Bacillus amyloliquefacians* (14) *Bacillus licheniformis* (15) *Bacillus licheniformis** *d-Bacillus stearothermophilus*	1,4-α-D-glucan glucanohydrolase	3.2.1.1	9000-90-2
Aminopeptidase	protease	*Rhizopus oryzae*	α-Aminoacyl-peptide hydrolase	3.4.11.11	
AMP deaminase	adenosine deaminase	*Aspergillus melleus*	AMP aminohydrolase	3.5.4.6	9025-10-9
β-Amylase	carbohydrase	(1) barley malt (2) barley	1,4-α-D-glucan maltohydrolase	3.2.1.2	9000-91-3
Bromelain	protease	pineapples: *Ananas comosus* *Ananas bracteatus* (L)	none	3.4.22.32 3.4.22.33	37189-34-7 9001-00-7

only relatively low rates. Moreover, the culture may also produce other undesired enzymes. For example, microbial rennet preparations often contain unwanted enzymes which can produce off-flavors in cheese on prolonged aging (*47*). Hence, it is common practice to attempt to improve the desirable qualities of the isolate by altering growth conditions, usually in conjunction with strain selection by mutation or other types of genetic manipulation. The result can be a special strain that will not survive in nature but is very useful from a commercial standpoint.

Laboratory-generated mutant strains characteristically lack certain functional or regulatory properties.[2] While the primary structures of proteins can be altered within limited ranges by mutagenesis, mutants possessing enzymes with improved catalytic activity for their normal substrates have not been reported (*30*). Moreover, no one has ever reported a mutation which transformed an otherwise nontoxic enzyme or protein into a toxin. It is now possible to introduce foreign genes into microorganisms by using DNA cloning techniques so that entirely new proteins are produced, but this should not be confused with mutagenesis where the intrinsic DNA of an organism is altered.

A useful mutant strain might be one which has lost a regulatory function that limits the synthesis of a desirable enzyme so that the mutant cannot stop synthesizing the enzyme and continues to produce it in great excess of biological need. The mutant may also have lost the ability to synthesize one or more unwanted enzymes. Additionally, it may have been manipulated genetically so that more than one copy of the gene coding for the desired enzyme is present, hence, there are more "blue-prints" available (*47*). Such organisms are really genetically impaired and are maintained in the laboratory or industrial setting by using specific, well-controlled growth conditions. These microorganisms have not been found in nature probably because they cannot compete successfully with the wild-type (nonmutant) parent or other microorganisms. It is also important to note that when the parental isolates are pathogenic, the derived mutant strains are characteristically less hazardous. Of course cultures used for food enzyme manufacture are not pathogenic, but by way of example, mutant strains of *Salmonella typhimurium* developed for routine mutagenesis testing are far less virulent than *S. typhirmurium* found in nature (*1*). Therefore, in choosing innocuous isolates for enzyme production, the process of enzyme manufacture from microorganisms becomes inherently safer.

The nonpathogenic, nontoxigenic microbial cultures traditionally used in enzyme manufacture are also ideal candidates for cloned DNA. For example, the gene for a useful enzyme that is not synthesized by *Bacillus subtilis* could be introduced into the organism. The new "strain" would then produce the new enzyme product and would not present a pathogenic or toxigenic risk greater than that of its "parents," the nonpathogenic *B. subtilis* and the gene for the useful enzyme.

Large-scale growth. There are two ways to grow microorganisms on an industrial scale. One way is to use liquid medium which is agitated and aerated, and the other way is to use solid or semi-solid medium held in large trays or drums (*16, 47*). In both cases, it is necessary to control environmental factors such as temperature, pH and degree of aeration. Equipment must be designed for easy cleaning and sterilization. Conditions must be employed which minimize the growth of contaminating microorganisms that will ruin the fermentation. During growth, cultures are routinely sampled and tested for possible contamination (*16, 47*).

All ingredients used to formulate the growth medium should be free of toxic contaminants (*7, 8, 16, 45, 47*). It is important that any "carry-over" of growth medium into the final enzyme preparation not bring with it possible toxic substances, especially when the enzyme being manufactured is intended for food processing.

Enzyme extraction, concentration and standardization. The desired enzyme may be present in the medium or inside the cells. Enzymes secreted into solid or semi-solid medium, and most intracellular enzymes, are extracted before further processing. In this context, extraction means to "wash out" and solubilize the enzyme in an aqueous solution (*16, 47*). Where the enzyme is secreted into a liquid growth medium, an extraction step is not necessary.

Enzymes secreted into solid or semi-solid media may be extracted directly into water solutions using a counter current system which filters as well as extracts (*16, 47*). Alternatively, solid or semi-solid media containing the microorganisms may be dried, ground and treated with water solutions to solubilize the desired enzyme. This method can be used to recover both intra- and extracellular enzymes. In the case of intracellular enzymes from microorganisms grown in liquid media, the cells are first collected by centrifugation or filtration and then ruptured by any of a number of physical and/or chemical procedures (*16, 47*). The enzymes are then extracted from ruptured cells with aqueous solutions.

After extraction, enzyzme solutions are usually concentrated to reduce volume. It is common to use ultrafiltration to reduce the amount of water and substances below specified molecular weights (e.g., salts, small organic molecules and peptides). Sometimes enzymes are concentrated by precipitation with salts or organic solvents, but because of organic solvent cost this method is not as common today as it was 10 years ago (*47*). In other cases, con-

[2]Under certain conditions an inducible enzyme can be made constitutive by mutation in the regulator, operator or (more rarely) the promoter region of the genetic operon. The enzyme will then be expressed in the absence of the inducer. Thus, under fermentation conditions used to produce an enzyme, production of "new" enzymes or proteins can be made to occur. These proteins or enzymes were originally present in the genetic material of the parent and would be normally synthesized under the right fermentation conditions without mutation. In addition, mutation induces minor changes in base sequence of DNA encoding for proteins and enzymes (base change, deletion, etc.). Thus, minor changes in protein structure are possible as a result of mutations affecting the structural gene. These changes can lead to increased enzymic activity or they may decrease or destroy enzymic activity (*18*).

TRIVIAL NAME	CLASSIFICATION	SOURCE	SYSTEMATIC NAMES (IUB)[a]	IUB NO.[a]	CAS NO.[b]
Catalase	oxidoreductase	(1) *Aspergillus niger** d-*Aspergillus niger* (2) bovine liver (3) *Micrococcus lysodeikticus* (4) *Aspergillus niger*	hydrogen peroxide: hydrogen peroxide oxidoreductase	1.11.1.6	9001-05-2
Cellulase	carbohydrase	(1) *Aspergillus niger* var. (2) *Trichoderma reesei* (formerly *longibrachiatum*) (3) *Trichoderma reesei** d-*Trichoderma reesei* (4) *Trichoderma viride* (5) *Aspergillus aculeatus*	Endo-1,4-(1,3;1,4)-β-D-glucan 4-glucanohydrolase	3.2.1.4	9012-54-8
Chymosin	protease	(1) *Aspergillus niger* var. *awamori** d-calf prochymosin gene (2) *Escherichia coli* K-12* d-calf prochymosin gene (3) *Kluyveromyces marxianus** d-calf prochymosin gene	cleaves a single bond in *kappa* casein	3.4.23.4	9001-98-3
Chymotrypsin	protease	bovine or porcine pancreatic extract	none	3.4.21.1	9004-07-3
Dextranase	carbohydrase	*Chaetomium erraticum* *Chaetomium gracile*	1,6-α-D-glucan 6-glucanohydrolase	3.2.1.11	9025-70-1
Ficin	protease	figs: *Ficus* sp.	none	3.4.22.3	9001-33-6
α-Galactosidase	carbohydrase	(1) *Mortierella vinacea* var. *raffinoseutilizer* (2) *Aspergillus niger* (3) *Saccharomyces cerevisiae** d-Guar seed	α-D-galactoside galactohydrolase	3.2.1.22	90025-35-8

harnessing of the enzymic process of nitrogen fixation for industrial-scale production of ammonia. Such a development would go far towards alleviating global food shortages.

As the example given above illustrates, enzymes in the broadest sense are really inexpensive alternatives to energy-requiring physical processes, such as the application of heat and/or high pressure. This is because enzymes accelerate reactions which would proceed only very slowly, or not at all, under ordinary conditions. Moreover, because enzymes are so specific in the reactions which they catalyze, many important and highly useful chemical transformations could not be accomplished without them. For these reasons, the future of enzyme technology seems exceedingly important and bright.

MANUFACTURE, COMPOSITION AND CONSUMPTION OF ENZYME PREPARATIONS

Enzymes are manufactured because we need highly specific catalysts which are safe to use. Two considerations are of primary importance: (a) catalytic activity must be preserved during production and (b) the intended and proper use of enzyme preparations must pose no health risk for plant workers or consumers. These two central principles underlie enzyme manufacture and use.

Like all biological materials, enzymes are affected by the conditions under which they are produced and handled. Economically important enzymes are obtained from animals, plants and microorganisms. In the manufacture of enzymes there must be strict adherence to current Good Manufacturing Practices (CGMPs). (*8*).

Enzymes from animals

One of the first intentional developments by man of what could be called an "enzyme preparation" was rennet, a crude extract of the lining of the fourth stomach of ruminants. This extract contains various proteolytic enzymes which cause milk to curdle, a step essential for cheese production. Rennet is still obtained from this traditional source except that modern methods of enzyme manufacture and quality control are applied to ensure a product of consistent activity which is free of pathogenic bacteria and toxic substances (*3*, *4*, *8*).

Other crude enzyme mixtures are also obtained from animals at slaughter, such as pancreatin from the pancreas (contains several proteolytic, amylolytic and lipolytic enzymes), pepsin from hog stomachs, lipase from the throat glands of young ruminants and hyaluronidase from bovine seminal vesicles (used medically to facilitate the diffusion and adsorption of local anesthetics). An important perspective of enzyme production from animals is evident from the fact that in 1975, in the Federal Republic of Germany alone, pancreas glands from 13.3 million animals were required for the production of just 100 kg of pancreatin (*44*). As in the manufacture of calf rennet, high standards of quality are maintained throughout the production process to ensure the safety and efficacy of the final enzyme preparations.

Enzymes from plants

Enzymes of commercial importance are also obtained from edible nontoxic plants. The terms *edible* and *nontoxic* are both important, since some edible plants can contain toxic substances (e.g., potatoes and rhubarb) (*13*). However, the plants used for food enzyme manufacture are not known to produce or contain such toxins. Three plant proteases (bromelin, papain and ficin) are obtained, respectively, from the stalks of pineapple plants, the fruit of papaya and the sap of fig trees. Additionally, horseradish roots serve as the source of horseradish peroxidase (an important analytical and research enzyme), and barley seeds are the source of malt which contains amylase activity and is used in brewing (*47*).

Imported raw materials are surveyed for possible insect-derived contamination. If found, the product is processed to remove the contaminant. Another consideration common to all agricultural products is possible pesticide residues or mycotoxins in plant-derived enzyme preparations. Enzymes often are separated from other plant constituents by precipitation with organic solvents such as ethanol, acetone or isopropanol (*47*). Any organic toxins initially present are likely to be separated from the enzyme-containing protein fraction which precipitates.

Enzymes from microorganisms

Microorganisms are the most important source of commercial enzymes. Virtually any enzymic activity of industrial importance may be produced by one or more species of microorganism. This does not mean that microorganisms naturally synthesize animal or plant enzymes, but rather that microorganisms may produce their own enzymes to catalyze reactions that are also catalyzed by structurally different enzymes from animals or plants. Microorganisms are readily grown and manipulated on an industrial scale, and the synthesis of specific products, including enzymes by these organisms, can be regulated by using selected or genetically-engineered strains and/or varying growth conditions. Hence, the uniformity of composition of microbial enzyme preparations can be maintained.

Organism selection. Manufacturing a microbial enzyme begins with well-characterized pure cultures isolated from various sources. There are many cultures currently in use (Table 1). Microbial cultures used in food enzyme manufacture should have been tested to establish that they are nonpathogenic, nontoxigenic and do not produce antibiotics (*3*, *4*, *7*, *45*, *47*). Specific cultures often will have been subjected to many tests, and there should be little doubt that the microorganisms listed in Table 1, when handled under CGMPs, are safe for food enzyme manufacture. Cultures of the same or different species isolated anew from natural sources may also be of potential importance in food enzyme manufacture. The guidelines and procedures which we present below can be used to assess the safety of new isolates.

A culture (currently in use or isolated anew) will have been selected on the basis of its ability to synthesize a desired enzyme. However, the enzyme may be produced at

TRIVIAL NAME	CLASSIFICATION	SOURCE	SYSTEMATIC NAMES (IUB)[a]	IUB NO.[a]	CAS NO.[b]
β-Glucanase	carbohydrase	(1) *Aspergillus niger* var. (2) *Bacillus subtilis* var. (3) *Trichoderma reesei* (formerly *longibrachiatum)* (4) *Penicillium emersonii* (5) *Bacillus amyloliquefaciens* (6) *Aspergillus aculeatus*	1,3-(1,3;1,4)-β-D-glucan 3(4)-glucanohydrolase	3.2.1.6	62213-14-3
Glucoamylase (Amyloglucosidase)	carbohydrase	(1) *Aspergillus niger* var. (2) *Aspergillus oryzae* var. (3) *Rhizopus oryzae* var. (4) *Rhizopus niveus* (5) *Rhizopus delemar*	1-4-α-D-glucan -glucohydrolase	3.2.1.3	9032-08-0
Glucose Isomerase	isomerase	(1) *Actinoplanes missouriensis* (2) *Bacillus coagulans* (3) *Streptomyces olivaceus* (4) *Streptomyces olivochromogenes* (5) *Streptomyces rubiginosus** d-*Streptomyces rubiginosus* (6) *Streptomyces murinus* (7) *Microbacterium arborescens* (8) *Streptomyces rubiginosus*	D-xylose ketoisomerase	5.3.1.5	9055-00-9
Glucose Oxidase	oxidoreductase	*Aspergillus niger** d-*Aspergillus niger* *Aspergillus niger*	β-D-glucose: oxygen 1-oxidoreductase	1.1.3.4	9001-37-0
Glutaminase	glutaminase	*Bacillus subtilis*	L-Glutaamate aminohydrolase	3.5.1.2	9001-47-2
β-D-Glucosidase	carbohydrase	(1) *Aspergillus niger* var. (2) *Trichoderma longibrachiatum*	β-D-glucoside glucohydrolase	3.2.1.21	9001-22-3

preserve natural pectin content. Another variation is used in jelly manufacture. Here, the native pectin is hydrolyzed by pectic enzymes, and then, after heating to denature the enzymes, commercial pectin possessing certain desirable properties is added to produce jelly of consistent quality.

Pharmaceutical/medical applications. Because of the great versatility of enzymes, their use is not restricted to food processing. Enzymes also have gained importance in the pharmaceutical/medical industry. For example, they are used in rapid and highly reliable clinical diagnostic tests. In one such test, the enzymes glucose oxidase and peroxidase (IUB 1.11.1.7) have been combined in a specific and sensitive assay for glucose in urine (a symptom of diabetes). The glucose oxidase/peroxidase test is superior to urine-glucose tests based on chemical reduction of glucose (*9, 25*). It has also recently been applied to the detection and quantitation of glucose in blood. Other enzymes which catalyze different reactions with glucose also are used in glucose determinations. Moreover, many physiologically important substances, such as blood urea nitrogen (BUN), triglycerides and glycerol, cholesterol, uric acid, and several physiologically important enzymes, can be rapidly and specifically assayed with commercially available enzyme-based tests.

Enzymes also are employed in antibiotic manufacture to alter the chemical structure of antibiotics and thereby increase the range of microorganisms which the antibiotics can control. A related and particularly interesting example is the therapeutic application of beta-lactamase (formerly penicillinase) (IUB 3.5.2.6), an enzyme which destroys penicillin. The gene which codes for penicillinase is found on certain plasmids (extrachromosomal DNA) and the acquisition of such plasmids by pathogenic bacteria confers penicillin resistance. However, the purified enzyme can also be used to treat people who are hypersensitive to penicillin but were inadvertently exposed to the drug (*47*). Thus, imaginative application has resulted in health benefit from an enzyme which functions in nature to the detriment of human health.

There are many other similar examples of the therapeutic uses of purified enzymes from pathogenic microorganisms, from the venom of poisonous snakes, from human urine and from a variety of other plant, animal and microbial sources (*19*). Enzymes may be used in the treatment of human maladies ranging from cancer and thrombosis to prevention of tooth decay (*19, 47*).

Enzyme detergents. The addition of enzymes to laundry products to aid in stain removal was developed by Rohm, who patented the idea in 1913. Various improvements were made on the original concept, and, by 1969, enzyme detergents claimed 50% of the market in Europe and almost 45% in the United States (*49*). Then, following widely circulated, unfavorable publicity concerning the possible development of allergies to enzymes inhaled as a result of dust formation, the use of enzymes in laundry products in the United States declined dramatically. However, an expert committee, with support from the United States Food and Drug Administration (FDA), has concluded that irritation from enzyme detergents does not exceed that of detergents which do not contain enzymes (*15*). In addition, methods have been developed to encapsulate enzymes in polymeric matrices which are too large to be dispersed in air as dust particles, yet retain enzyme catalytic activity in the laundry product. Hence, it is now possible to produce an essentially dust-free enzyme detergent (*49*).

The use of enzymes in laundry products offers prospects for decreasing energy (heating) costs as well as minimizing water pollution (diminishing the need for other chemical additives). Enzymes are being used widely and successfully in laundry products without evidence of adverse health effects in consumers (*49*).

Other uses. There are many other practical applications of enzymes. For example, enzymes are used widely in the textile and leather industries to remove undesirable substances from products during manufacture. Additionally, commercial enzyme preparations are available for use in septic tanks. Such preparations often contain many enzymes for decomposing complex carbohydrates, proteins and lipids, as well as viable microorganisms which use the enzyme-liberated products as nutrients and produce additional degradative enzymes to continue the cycle. Microorganisms producing appropriate enzymes are also used to detoxify pesticides, and other bacteria can remove nitrate and nitrite from water supplies (*47*). Certain microorganisms and their enzymes are gaining particular attention in the production of alcohol as fuel as well as in the production of food from inedible materials or by-products (*47*).

Future applications of enzymes

It is now apparent that additional useful and important applications of enzymes to societal improvement are limited only by the depth of our imagination and our resolve as a nation to encourage experimentation and innovation. Technological application of enzymology is a direct outgrowth of our scientific preeminence, and once reasonable safety has been established, new developments should be allowed to proceed unfettered. Many problems which disturb us and plague much of the rest of the world, such as unavailability of food, fuel, adequate medical and pharmaceutical supplies, clean water and pollution control, are amenable to enzyme technology. Enzymes are an immensely valuable renewable natural resource, and their imaginative use in improving human welfare should be nurtured.

By way of specific example, one area of great potential is enzymic nitrogen fixation. Nitrogen is an essential element for life [indeed, all enzymes contain about 16% nitrogen (*52*)], yet atmospheric nitrogen cannot be utilized by animals, plants and most microorganisms. Nitrogen can be "fixed" as ammonia (a biologically usable form of the element) by industrial processes which consume much energy (*31*). In contrast, blue-green algae and certain species of bacteria can produce ammonia from nitrogen and hydrogen in a much more efficient manner, although energy is still required (*52*). Hence, an important challenge is the

TRIVIAL NAME	CLASSIFICATION	SOURCE	SYSTEMATIC NAMES (IUB)[a]	IUB NO.[a]	CAS NO.[b]
Hemicelluase[c]	carbohydrase	(1) *Aspergillus niger* var. (2) *Trichoderma longibrachiatum* (3) *Aspergillus aculeatus*	(1) α-L-arabinofuranoside arabinofuranohydrolase (2) 1,4-β-D-mannan mannanohydrolase (3) 1,3-β-D-xylan xylanohydrolase (4) 1,5-α-L-arabinan 1,5-α-L arabinanohydrolase (5) 1,4,β-D-Xylan xylanohydrolase (6) 1,4,β-D-Xylan xylohydrolase	3.2.1.55 3.2.1.78 3.2.1.32 3.2.1.99 3.2.1.8 3.2.1.37	9025-56-3
Hesperiginase	carbohydrase	*Penicillium decumbens*	α-L-Rhamnoside rhamnohydrolase	3.2.1.40	37288-35-0
Invertase	carbohydrase	*Saccharomyces* sp., (*Kluyveromyces)*	β-D-fractofuranoside fructohydrolase	3.2.1.26	9001-57-4
Lactase	carbohydrase	(1) *Aspergillus niger* var. (2) *Aspergillus oryzae* var. (3) *Saccharomyces* sp. (4) *Candida pseudotropicalis* (5) *Kluyveromyces marxianus* var. *lactis*	β-D-galactoside galactohydrolase	3.2.1.23	9031-11-2

24.

Pepsin	Protease	Porcine or other animal stomachs	None	3.4.23.1
Protease (general)	Protease	(1) *Aspergillus niger*, var. (2) *Aspergillus oryzae*, var. (3) *Bacillus subtilis*, var. (4) *Bacillus licheniformis*, var.	None	3.4.21.14 3.4.24.4
Rennet	Protease	(1) Fourth stomach of ruminant animals (2) *Endothia parasitica* (3) *Mucor miehei*, *M. pusillus*	None	3.4.23.4 3.4.23.6 3.4.23.6
Trypsin	Protese	Animal pancreas	None	3.4.21.4

[a]*Enzyme Nomenclature: Recommendations (1978) of the Nomenclature Committee of the International Union of Biochemistry*. Academic Press, New York. 1979.
[b]Usually a mixture of polygalacturonase, pectin methylesterase and pectate lyase.

ganisms are in wide use today, although it is now known that enzymes produced by these organisms are the actual agents responsible for the conversion of grapes to wine, milk to buttermilk or yogurt, etc. In addition to modern applications of ancient discoveries, enzymes extracted from living organisms also are widely employed in the food industry.

Enzymes used by food manufacturers are derived from edible and nontoxic plants, animals, and nonpathogenic, nontoxigenic microorganisms (*47*). Some of the enzymes used in food processing are given in Table 1 along with the sources of each. Because enzymes are catalysts, the amounts added to food (usually at an early or intermediate step in processing) represent only a minute fraction of the total food mass (*5*). Even this small amount may be reduced by further processing. For example, heating to produce desired organoleptic properties enhance shelf-life and ensure the absence of pathogenic microorganisms will denature or destroy the activity of most enzymes. The protein molecules which comprised the enzymes will still be present, but their physical shape will have been irreversibly altered by heating so that they no longer possess catalytic activity. There are also other methods of enzyme removal and/or inactivation such as raising or lowering the pH beyond limits which the enzyme can tolerate (*47*). Every enzyme exhibits a range of pH stability above or below which inactivation occurs. Many enzymes are inactivated by the acidity of the stomach.

The main organic constituents of foods are carbohydrates, proteins and lipids. It is often desirable to alter one or more of these constituents with enzymes during the conversion of raw to finished product. An important example of this involves the use of carbohydrases and isomerase to produce corn syrups from starch (*29, 32, 47*).

In one example of this conversion, alpha-amylase (IUB 3.2.1.1) first breaks long-chain starch molecules into shorter chains. Then glucoamylase (IUB 3.2.1.3) cleaves the individual glucose molecules from the chains. The resulting corn syrup has many commercial applications, but it is not as sweet as sucrose, the common table sugar obtained from sugar cane and sugar beets.

This deficiency of corn syrups has been overcome in recent years by the discovery of glucose isomerase (IUB 5.3.1.5), which converts glucose into fructose. The resulting high fructose corn syrup (HFCS) approaches the sweetness of sucrose and is less expensive. It is replacing the disaccharide in many applications.

There are many other novel and important applications of enzymes. For example, some foods and beverages do not store well in the presence of oxygen. By use of the enzyme glucose oxidase (IUB 1.1.3.4), which adds molecular oxygen to glucose to produce gluconic acid, it is possible to remove atmospheric oxygen safely and effectively from foods or beverages that are susceptible to oxygen.

Another interesting example is the production of juices from certain fruits and vegetables, where pectin content may become an important consideration (*47*). Pectin and pectic substances occur in plants. They are complex carbohydrates which are insoluble in water but nonetheless absorb water and, when dispersed, greatly increase viscosity. This is a desirable property for certain juices, such as those made from tomatoes, apricots and oranges, but the resulting lack of clarity is undesirable in apple and grape juices. Unfortunately, nature does not necessarily accommodate human taste. Raw apple and grape juice can contain considerable amounts of pectin even though most of us may not like them that way. For this reason, it is usually necessary to add pectic enzymes to raw apple and grape juices during processing to hydrolyze the pectin. Additionally, considerable amounts of juice can remain trapped in masses of pectic material. Through the use of pectic enzymes, such trapped juice can be freed. This makes juice extraction more efficient and economical, hence it lowers the price for consumers.

It is important to recognize that pectic enzymes (a mixture of three enzymes — see Table 1), as well as pectin, are naturally present in fruit juices, and where more enzyme activity is required, additional pectic enzymes may be added as indicated above. However, where high pectin content is preferred (e.g., apricot nectar, tomato and orange juices) the juice may be heated at an early stage in processing to denature native pectic enzymes and thereby

TRIVIAL NAME	CLASSIFICATION	SOURCE	SYSTEMATIC NAMES (IUB)[a]	IUB NO.[a]	CAS NO.[b]
Lipase	lipase	(1) edible forestomach tissue of calves, kids, and lambs (2) animal pancreatic tissues (3) *Aspergillus oryzae* var. (4) *Aspergillus niger* var. (5) *Rhizomucor miehei* (6) *Candida rugosa* (7) *Candida lipolytica* (8) *Rhizopus delemar* (9) *Rhizopus oryzae* (10) *Rhizopus niveus* (11) *Penicillium roqueforti* (12) *Penicillium camembertii* (13) *Mucor javanicus* (14) *Aspergillus oryzae** *d-Rhizomucor miehei* *(15) Aspergillus oryzae** *d-Thermomyces lanuginosus*		3.1.1.3	9001-62-1
Maltogenic Amylase	carbohydrase	*Bacillus subtilis** d-*Bacillus stearothermophilus*	1,4-α-D-glucan α-maltohydrolase	3.2.1.133	160611-47-2
Naringinase	carbohydrase	*Penicillium decumbens*	α-L-Rhamnoside rhamnohydrolase	3.2.1.40	37288-35-0
Pancreatin	mixed; carbohydrase, lipase, and protease	bovine and porcine pancreatic tissue	(1) 1,4-α-D-glucan glucanohydrolase (2) triacylglycerol acylhydrolase (3) protease	3.2.1.1 3.1.1.3 3.4.21.4	9000-90-2 9001-62-1 9002-07-7
Papain	protease	papaya: *Carica papaya (L)*	none	3.4.22.2	9001-73-4

23.

TABLE 1. *Enzyme preparations use* *d processing (3).*

Trivial name	Classification	Source	Systematic name (IUB)[a]	IUB No.[a]
α-Amylase	Carbohydrase	(1) *Aspergillus niger*, var. (2) *Aspergillus oryzae*, var. (3) *Rhizopus oryzae*, var. (4) *Bacillus subtilis*, var. (5) Barley malt (6) *Bacillus licheniformis*, var.	1,4-α-D-Glucan glucanohydrolase	3.2.1.1
β-Amylase	Carbohydrase	Barley malt	1,4-α-D-Glucan maltohydrolase	3.2.1.2
Bromelain	Protease	Pineapples: *Ananas comosus*, *Ananas bracteatus* (L)	None	3.4.22.4
Catalase	Oxidoreductase	(1) *Aspergillus niger*, var. (2) Bovine liver (3) *Micrococcus lysodeikticus*	Hydrogen peroxide: hydrogen peroxide oxidoreductase	1.11.1.6
Cellulase	Carbohydrase	(1) *Aspergillus niger*, var. (2) *Trichoderma reesei*	1,4-(1,3;1,4)-β-D-Glucan 3(4)-glucanohydrolase	3.2.1.4
Ficin	Protease	Figs: *Ficus* sp.	None	3.4.22.3
β-Glucanase	Carbohydrase	(1) *Aspergillus niger*, var. (2) *Bacillus subtilis*, var.	1,3-(1,3;1,4)-β-D-Glucan 3(4)-glucanohydrolase	3.2.1.6
Glucoamylase (Amyloglucosidase)	Carbohydrase	(1) *Aspergillus niger*, var. (2) *Aspergillus oryzae*, var. (3) *Rhizopus oryzae*, var.	1,4-α-D-Glucan glucohydrolase	3.2.1.3
Glucose isomerase	Isomerase	(1) *Actinoplanes missouriensis* (2) *Bacillus coagulans* (3) *Streptomyces olivaceus* (4) *Streptomyces olivochromogenes* (5) *Streptomyces rubiginosus*	D-Xylose ketolisomerase	5.3.1.5
Glucose oxidase	Oxidoreductase	*Aspergillus niger*, var.	β-D-Glucose: oxygen oxidoreductase	1.1.3.4
Hemicellulase	Carbohydrase	*Aspergillus niger*, var.	None	None
Invertase	Carbohydrase	*Saccharomyces* sp. (*Kluyveromyces*)	β-D-Fructofuranoside fructohydrolase	3.2.1.26
Lactase	Carbohydrase	(1) *Aspergillus niger*, var. (2) *Aspergillus oryzae*, var. (3) *Saccharomyces sp.*	β-D-Galactoside galactohydrolase	3.2.1.23
Lipase	Lipase	(1) Edible forestomach tissue of calves, kids, and lambs (2) Animal pancreatic tissues (3) *Aspergillus oryzae*, var. (4) *Aspergillus niger*, var.	Carboxylic-ester hydrolase Triacylglycerol acylhydrolase	3.1.1.1 3.1.1.3
Papain	Protease	Papaya: *Carica papaya* (L)	None	3.4.22.2
Pectinase[b]	Carbohydrase	(1) *Aspergillus niger*, var. (2) *Rhizopus oryzae*, var.	Poly (1,4-α-D-galacturonide) glycanohydrolase Pectin pectyylhydrolase Poly (1,4-α-D-galacturonide) lyase	3.2.1.15 3.1.1.11 4.2.2.2

TRIVIAL NAME	CLASSIFICATION	SOURCE	SYSTEMATIC NAMES (IUB)[a]	IUB NO.[a]	CAS NO.[b]
Pectin esterase	carbohydrase	*Aspergillus oryzae** d-*Aspergillus aculeatus*	pectin pectylhydrolase	3.1.1.11	9025-98-3
Pectinase[d]	carbohydrase	(1) *Aspergillus niger* var. (2) *Rhizopus oryzae* var. (3) *Aspergillus aculeatus*	(1) poly(1,4-α-D-galacturonide) glycanohydrolase (2) pectin pectylhydrolase (3) poly(1,4-α-D-galacturonide) lyase (4) pectin lyase	3.2.1.15 3.1.1.11 4.2.2.2 4.2.2.10	9032-75-1 9025-98-3 9015-75-2 9033-35-6
Pepsin	protease	porcine or other animal stomach tissue	none	3.4.23.1 3.4.23.2	9001-75-6 9025.48.3
Phosphodiesterase	nuclease	*Penicillium citrinum* *Leptographium procerum*	Oligonucleate 5'-nucleotidohydrolase	3.1.4.1	9025-82-5
Phospholipase A_2	lipase	(1) animal pancreatic tissue (2) *Streptomyces violaceoruber*	(1) phosphatidylcholine 2-acylhydrolase	3.1.1.4	9001-84-7
Phytase	phosphatase	*Aspergillus niger* var.	(1) myo-inositol-hexakisphosphate--3-phosphohydrolase (2) orthophosphoric mono ester phosphohydrolase	3.1.3.8 3.1.3.2	37288-11-2 9001-77-8

strate upon which the enzyme acts; e.g., the sugar *lactose* is acted upon by *lactase*, *proteins* are degraded by *proteases*, intramolecular rearrangements *(isomerizations)* are catalyzed by *isomerases*. Additionally, many well-known and long-used enzymes have trivial (common, historical) names, e.g., papain from papaya. To minimize confusion, each enzyme activity is assigned a four-part number (called the IUB[1] number) and a systematic name based on the reaction. However, this system does not distinguish between different enzymes from different organisms which catalyze the same reaction (*47*).

All living organisms produce and contain many enzymes, but no one organism has enzymes for all or even most possible biotransformations. Organisms may produce one specific enzyme to act on a given substrate. Organisms may also produce two or more different enzymes which catalyze the same reaction; such enzymes are called isoenzymes. The reasons for this are not known, but it is believed related to the apparent necessity of organisms to maintain precise control over enzyme synthesis, degradation and activity (*52*). Although enzymes catalyzing the same reaction but produced by different species may be similar, it is also possible that they may be entirely different (*21, 52*). Similarities and differences between enzymes and other proteins is one way of estimating evolutionary divergence among species (*21, 52*).

Catalytic activity is ultimately derived from the sequence of specific amino acids which comprise an enzyme. Amino acid sequence, in turn, determines the shape of the enzyme molecule. The shape or configuration is all-important. Disrupting the shape destroys activity.

Enzyme activity is operationally defined by kinetic parameters such as maximum catalytic rate and the affinity of the enzyme for its substrate. Virtually any environmental factor (pH, ionic strength, temperature, etc.) affects enzyme activity. Enzymes are also subject to inhibition by various means (*47, 52*). These properties permit cells to regulate the activities of enzymes which they synthesize and contain. A thorough understanding of the properties of individual enzymes also permits their optimal use in industry.

Historical examples of enzyme use

Most of what we call "food" is really tissue derived from living organisms (animals or plants); in some cases (e.g., milk), food is a secretion from living cells. Many of the enzymes in the cells of tissues remain active after cell death. For example, meat is "aged" by hanging animal carcasses in refrigerated rooms for several days after slaughter. During this time cells in the tissues break down, freeing various degradative enzymes, which then partially digest the connective tissue to give a more tender product. The tenderizing process can be accelerated by adding proteolytic enzymes derived from other sources to the meat at various stages before consumption, such as injecting proteases into the vascular system of the animal before slaughter or sprinkling papain (protease from papaya) on the meat before cooking. The tenderizing process is simply the first step in digestion which continues in the gastrointestinal tract of the consumer.

Enzymes have always been present in human food even though they have only recently been recognized as such. In addition to tissue-derived enzymes, microorganisms (because they are ubiquitous) also pervade the food supply, and the enzymes in microorganisms can alter the character of food. It was discovered early in the development of human civilization that some microbial transformations are desirable.

One of the first to be recognized was the souring of milk, a necessary step in making cheese. According to legend, cheesemaking was discovered several thousand years ago when an Arabian merchant carried milk in a pouch made of sheep's stomach. Rennet in the lining of the pouch caused the milk to curdle. We must assume that microorganisms grew at the same time and produced other enzymic changes that came to be regarded as desirable.

During the intervening centuries, man has learned how to make hundreds of kinds of cheese by controlling the environment and by adding types of microorganisms that produce enzymes which can bring about desirable changes. Lipases and proteases from various animal and microbial sources can also be added to achieve certain desired qualities.

We now use the term "fermentation" to describe milk souring and similar processes involving mass growth of microorganisms to produce useful products (*52*). Originally, however, the term described the transformation of grape juice into wine. Production of wine from grapes through fermentation also has its origin in antiquity. Among the treasures placed in the tombs of Egyptian pharaohs were casks of wine. The ancient Greeks attributed to the god Bacchus the discovery of fermentation (*52*). We now know that it is not yeast per se, but rather a system comprised of several enzymes contained in yeast that is ultimately responsible for the production of ethanol and carbon dioxide from the sugar in grape juice. This enzyme system was one of the first to be extensively studied and characterized. In fact, the word "enzyme", introduced by Kuehne, means "in yeast," although it has been expanded and now applies to all proteinaceous catalysts from any biological source (*52*).

Other ancient processes of food alteration and/or preservation involving enzymic action include breadmaking (yeast) and the production of vinegar from wine (*Acetobacter*). Only within the past 100 years has it been recognized that enzymes exist as discrete entities, and can, in fact, function in isolated systems outside living cells (*52*). This realization has led to remarkable advances through technological application of enzymes to many areas of human need.

Modern uses of enzymes

Food processing. Fermentations involving living or-

[1]The enumeration system of the Enzyme Commission of the Third International Congress of the International Union of Biochemistry (*47*).

TRIVIAL NAME	CLASSIFICATION	SOURCE	SYSTEMATIC NAMES (IUB)[a]	IUB NO.[a]	CAS NO.[b]
Protease (general)	protease	(1) *Aspergillus niger* var. (2) *Aspergillus oryzae* var. (3) *Aspergillus melleus* (4) *Bacillus subtilis* (5) *Bacillus subtilis** d-*Bacillus amyloliquefaciens* (6) *Bacillus licheniformis* var. (7) *Bacillus stearothermophilus* (8) *Rhizopus niveus* (9) *Rhizopus oryzae* (10) *Bacillus amyloliquefaciens*	none	3.4.23.18 3.4.21.14 3.4.21.62 3.4.24.28 3.4.24.4 3.4.23.6 3.4.11.1	9025-49-4 9014-01-1 9014-01-1 76774-43-1 9068-59-1 9073-79-4 9001-61-0 9080-56-2
Pullulanase	carbohydrase	(1) *Bacillus acidopullulyticus* (2) *Bacillus licheniformis** d-*Bacillus deramificans* (3) *Bacillus naganoensis* (4) *Bacillus subtilis** d-*Bacillus naganoensis* (5) *Bacillus circulans*	α-Dextrin 6-glucanohydrolase Pullulan 6-glucanohydrolase	3.2.1.41	9075-68-7
Rennet	protease	(1) fourth stomach of ruminant animals (2) *Endothia parasitica* (3) *Rhizomucor miehei* (4) *Rhizomucor pusillus* (Lindt)	none	3.4.23.4 3.4.23.22 3.4.23.23	9001-98-3 37205-60-0 148465-73-0
Transglucosidase	glucanotransferase	*Aspergillus niger*	1,4-α-D-glucan 4-α-D-glycosyltransferase	2.4.1.25	9032-09-1
Trypsin	protease	animal pancreas	none	3.4.21.4	9002-07-7
Urease	protease	*Lactobacillus fermentium*	none	3.5.1.5	9002-13-5

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Determining the Safety of Enzymes Used in Food Processing

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ABSTRACT

Enzymes are proteins that catalyze chemical reactions. They are highly specific and needed in only minute quantities. Certain enzymes have long been used to produce specific foods (e.g., cheese). Today they have numerous applications and are increasing in commercial importance. There has never been a health problem traced to the use of an enzyme per se in food processing. However, it is important that scientific data be provided to show that enzyme preparations, particularly those lacking a long history of safe use, are in fact safe to consume. The purpose of this report is to propose guidelines for assessing enzyme safety. We conclude that the enzymes per se now used or likely to be used in the future in food processing are inherently nontoxic. Safety evaluation should focus on possible contaminants which could be present. Assuming that current Good Manufacturing Practices (CGMPs) are followed, toxic contaminants could only come from the enzyme source itself (animal, plant or microbial). Hence, the safety of the source organism should be the prime consideration. Enzymes from animals or plants commonly regarded as food need not be subjected to animal feeding studies. Some food plants produce toxins and chemical assays may be used in these cases to assess safety. For enzymes from bacteria, it should be shown that antibiotics and acute toxins active via the oral route (enterotoxins and certain neurotoxins) are absent. Small molecular weight toxins (< 500 daltons) may be produced by certain fungi and actinomycetes. It should be shown that enzymes from such organisms are free of these materials. If it is established that a microbial culture does not produce antibiotics or toxins active via the oral route, then enzymes manufactured from that culture using CGMPs may be regarded as safe for use in food processing.

BACKGROUND

To understand and apply the proposed guidelines for determining safety of enzymes used in food processing, it is necessary to consider what enzymes are, how they act, how they are prepared and how they are used. That is the purpose of this section.

General considerations

Enzymes are proteins which catalyze chemical reactions. Like all catalysts enzymes increase the rates at which reactions achieve equilibrium. For example, there are instances where certain enzymes increase the rates of specific reactions by 10 million times (*47*). Enzymes act by lowering activation energy. Since they cannot create energy, enzymes will only affect reactions which, because of a "downhill" net energy flow, could occur spontaneously. Like other catalysts, enzymes are not consumed by the reactions which they catalyze. Hence, one enzyme molecule can, through time, catalyze the transformation of many molecules of substrate (*47, 52*).

Most complex chemical reactions not controlled by catalysts produce a variety of products. However, in general, enzymes accelerate specific reactions which result in the generation of specific products. High degrees of specificity and strong catalytic activities are the most important functional properties of enzymes. Clearly, without enzymes DNA could not be replicated nor could RNA and proteins be synthesized and degraded. The controlled and orderly array of metabolic processes of living cells, which in fact define life, would not be possible. Life on earth is absolutely dependent upon enzymes. Every cell comprising every organism alive at this moment contains enzymes which are functioning in highly ordered and specific ways to transform one chemical into another as dictated by biological necessity.

Like all proteins, enzymes are synthesized inside cells by a complex process involving DNA, RNA, cellular structures called ribosomes, various small molecules such as amino acids, energy-rich phosphorus compounds and certain cations, and enzymes to catalyze specific reactions (*52*). The fact that enzymes are a necessary component in the biological mechanism which produces new enzymes underscores the fundamental importance of these remarkable biological catalysts.

After synthesis, enzymes may remain inside cells or they may be secreted into the extracellular milieu. Secreted enzymes are hydrolytic and their purpose is to decompose macromolecules into small units which then can be taken up by cells and used (under enzymic direction) as needed in metabolic processes. Enzymes which remain inside cells (intracellular) are of all classes and may be involved in synthesis or degradation of various substances. Economically important enzymes are found among both the intracellular and extracellular groups (*47*).

The name given to an enzyme is determined according to the reactions which is catalyzed. It is customary to attach the suffix "-ase" to the name of the principal sub-

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TRIVIAL NAME	CLASSIFICATION	SOURCE	SYSTEMATIC NAMES (IUB)[a]	IUB NO.[a]	CAS NO.[b]
Xylanase	carbohydrase	(1) *Trichoderma longibrachiatum** d-*Trichoderma longibrachiatum* (2) *Aspergillus niger var. awamori** d-*Aspergillus var.* (3) *Bacillus licheniformis* d-*Bacillus licheniformis* *4) Aspergillus oryzae** *d-Thermomyces lanuginosus*	1) 1,4 β-D-xylan xylanohydrolase 2) 1,3 β-D-xylan xylanohydrolase	3.2.1.8 3.2.1.32	9025-57-4 9025-55-2

[a]*Enzyme Nomenclature: recommendations (1992) of the Nomenclature Committee of the International Union of Biochemistry*, Academic Press, New York, 1992.

[b]*Chemical Abstract Service Registry Number*

[c]Usually a mixture of the activities listed under the systematic name.

[d]Usually a mixture of the activities listed under the systematic name.

*The asterisk indicates a genetically modified organism. The donor organism is listed after "d-."

Enzyme Preparations Used in Food Processing (*continued*)

Trivial Name	Classification	Source	Systematic Name (IUB)[a]	IUB No.[a]
Pepsin	protease	porcine or other animal stomachs	none	3.4.23.1
Protease (general)	protease	(1) *Aspergillus niger*, var. (2) *Aspergillus oryzae*, var. (3) *Bacillus subtilis*, var. (4) *Bacillus licheniformis*, var.	none	3.4.21.14 3.4.24.4
Rennet	protease	(1) fourth stomach of ruminant animals (2) *Endothia parasitica* (3) *Mucor miehei*, *M. pusillus*	none	3.4.23.4 3.4.23.6 3.4.23.6
Trypsin	protease	animal pancreas	none	3.4.21.4

[a] *Enzyme Nomenclature: Recommendations (1978) of the Nomenclature Committee of the International Union of Biochemistry*. Academic Press, New York, 1979.
[b] Usually a mixture of polygalacturonase, pectin methylesterase, and pectate lyase.

Comparator Use either the standard Hellige comparator (Catalog No. 607) or the pocket comparator (Catalog No. 605) with prism attachment (Catalog No. 605A). The comparator should be illuminated with a 100-W frosted lamp placed 6 in. from the rear opal glass of the comparator and mounted so that direct rays from the lamp do not shine into the operator's eyes.

Comparator Tubes Use the precision-bored square tubes with a 13-mm viewing depth that are supplied with the Hellige comparator. Suitable tubes are also available from other apparatus suppliers (e.g., Coleman Universal Distributors).

Reagents and Solutions

Buffer Solution (pH 4.8) Dissolve 164 g of anhydrous sodium acetate in about 500 ml of water, add 120 ml of glacial acetic acid, and adjust the pH to 4.8 with glacial acetic acid. Dilute to 1000.0 ml with water, and mix.

β-Amylase Solution .Dissolve 250 mg of β-amylase, free from α-amylase, in 5 ml of water. The enzyme, which has been standardized to 2000° diastatic power, is distributed by Sturge Enzymes, Div. of Henley and Co., Inc., 750 Third Ave., New York, N.Y. 10017. (NOTE: The enzyme should be stored in a refrigerator, and it should be allowed to warm to room temperature before opening, in order to prevent condensation of moisture.)

Special Starch Use starch designated as "Starch (Lintner) Soluble," Baker Analyzed Reagent Catalog No. 4010. Before using new batches, test them in parallel with previous lots known to be satisfactory. Variations of more than ±3° diastatic power in the averages of a series of parallel tests indicate an unsuitable batch.

Buffered Substrate Solution Disperse 10.0 g (dry-weight basis) of *Special Starch* in 100 ml of cold water, and pour slowly into 300 ml of boiling water. Boil with stirring for 1 to 2 min, then cool, and add 25 ml of *Buffer Solution*, followed by all of the *β-Amylase Solution*. Quantitatively transfer the mixture into a 500-ml volumetric flask with the aid of water saturated with toluene, dilute to volume with the same solvent, and mix. Store the solution at 30° ± 2° for not less than 18 or more than 72 h before use. (This solution is also known as "buffered limit dextrin substrate.")

Stock Iodine Solution Dissolve 5.5 g of iodine and 11.0 g of potassium iodide in about 200 ml of water, dilute to 250 ml with water, and mix. Store in a dark bottle and make a fresh solution every 30 days.

Dilute Iodine Solution Dissolve 20 g of potassium iodide in 300 ml of water, and add 2.0 ml of *Stock Iodine Solution*. Quantitatively transfer into a 500-ml volumetric flask, dilute to volume with water, and mix.

Sample Preparation Prepare a solution of the sample so that 5 ml of the final dilution will give an endpoint between 10 and 30 min under the conditions of the assay.

For barley malt, finely grind 25 g of the sample in a Miag-Seck mill, available from the Schock Gusmer Division of the Pfaudler Co., 1000 West Avenue, Rochester, N.Y. 10003, or from Ludwig Baer Machinery, Inc., 270 Madison Avenue, New York, N.Y. 10016. Quantitatively transfer the powder into a 1000-ml Erlenmeyer flask, add 500 ml of a 0.5% solution of sodium chloride, and allow the infusion to stand for 2.5 h at 30° ± 0.2°, agitating the contents by gently rotating the flask at 20-min intervals. (*Caution:* The infusion must not be mixed by inverting the flask, and the quantity of the grist left adhering to the inner walls of the flask as a result of agitation must be as small as possible.) Filter the infusion through a 32-cm fluted filter of Whatman No. 1, or equivalent, paper on a 20-cm funnel, returning the first 50 ml of filtrate to the filter. Collect the filtrate until 3 h have elapsed from the time the sodium chloride solution and the sample were first mixed. Pipet 20.0 ml of the filtered infusion into a 100-ml volumetric flask, dilute to volume with the 0.5% sodium chloride solution, and mix.

Procedure Pipet 5.0 ml of *Dilute Iodine Solution* into a series of 13- × 100-mm test tubes, and place them in a water bath maintained at 30° ± 0.1°, allowing 20 tubes for each assay.

Pipet 20.0 ml of the *Buffered Substrate Solution*, previously

Enzyme Preparations Used in Food Processing

Trivial Name	Classification	Source	Systematic Name (IUB)[a]	IUB No.[a]
α-Amylase	carbohydrase	(1) *Aspergillus niger*, var. (2) *Aspergillus oryzae*, var. (3) *Rhizopus oryzae*, var. (4) *Bacillus subtilis*, var. (5) barley malt (6) *Bacillus licheniformis*, var.	1,4-α-D-glucan glucanohydrolase	3.2.1.1
β-Amylase	carbohydrase	barley malt	1,4-α-D-glucan maltohydrolase	3.2.1.2
Bromelain	protease	pineapples: *Ananas comosus*, *Ananas bracteatus* (L)	none	3.4.22.4
Catalase	oxidoreductase	(1) *Aspergillus niger*, var. (2) bovine liver (3) *Micrococcus lysodeikticus*	hydrogen peroxide: hydrogen peroxide oxidoreductase	1.11.1.6
Cellulase	carbohydrase	(1) *Aspergillus niger*, var. (2) *Trichoderma reesei*	1,4-(1,3;1,4)-β-D-glucan 3(4)-glucanohydrolase	3.2.1.4
Ficin	protease	figs: *Ficus* sp.	none	3.4.22.3
β-Glucanase	carbohydrase	(1) *Aspergillus niger*, var. (2) *Bacillus subtilis*, var.	1,3-(1,3;1,4)-β-D-glucan 3(4)-glucanohydrolase	3.2.1.6
Glucoamylase (Amyloglucosidase)	carbohydrase	(1) *Aspergillus niger*, var. (2) *Aspergillus oryzae*, var. (3) *Rhizopus oryzae*, var.	1,4-α-D-glucan glucohydrolase	3.2.1.3
Glucose Isomerase	isomerase	(1) *Actinoplanes missouriensis* (2) *Bacillus coagulans* (3) *Streptomyces olivaceus* (4) *Streptomyces olivochromogenes* (5) *Streptomyces rubiginosus*	D-xylose ketolisomerase	5.3.1.5
Glucose Oxidase	oxidoreductase	*Aspergillus niger*, var.	β-D-glucose: oxygen oxidoreductase	1.1.3.4
Hemicellulase	carbohydrase	*Aspergillus niger*, var.	none	none
Invertase	carbohydrase	*Saccharomyces* sp. *(Kluyveromyces)*	β-D-fructofuranoside fructohydrolase	3.2.1.26
Lactase	carbohydrase	(1) *Aspergillus niger*, var. (2) *Aspergillus oryzae*, var. (3) *Saccharomyces* sp.	β-D-galactoside galactohydrolase	3.2.1.23
Lipase	lipase	(1) edible forestomach tissue of calves, kids, and lambs (2) animal pancreatic tissues (3) *Aspergillus oryzae*, var. (4) *Aspergillus niger*, var.	carboxylic-ester hydrolase triacylglycerol acylhydrolase	3.1.1.1 3.1.1.3
Papain	protease	papaya: *Carica papaya* (L)	none	3.4.22.2
Pectinase[b]	carbohydrase	(1) *Aspergillus niger*, var. (2) *Rhizopus oryzae*, var.	poly(1,4-α-D-galacturonide) glycanohydrolase pectin pectylhydrolase poly(1,4-α-D-galacturonide) lyase	3.2.1.15 3.1.1.11 4.2.2.2

B: Tauber 1949

CHAPTER XXVII

PECTIN-DECOMPOSING ENZYMES AND THEIR USE IN THE FRUIT JUICE, WINE, AND JELLY INDUSTRIES

Pectins are parts of the cell walls of plants. They are polymerization compounds of galacturonic acid of an unknown constitution. The pectic compounds of the middle lamella are believed to be the cementing link between cells. Softening of fruits during ripening is probably caused by enzymic breakdown of the cementing pectins (1, 2). Pectins are soluble carbohydrates of colloidal nature. They are formed from an insoluble compound, called protopectin, by boiling in water or dilute acids or by the action of enzymes. Lemons (albedo), oranges (albedo), apples, sugar beets, flax stalks, strawberries and raspberries, and many other fruits are good sources of pectin.

The pectins, or the pectic substances as they are sometimes called, are very important industrially. They are used in the setting of jams and jellies, and as emulsifying agents in the manufacture of oil emulsions and other foods. The pectic substances and the pectin-decomposing enzymes have been extensively studied in connection with the preparation of textile fibers, the clarification of fermented and unfermented juices, and the stabilization of "clouds" in tomato and citrus juices.

The Agricultural and Food Chemistry Division of the American Chemical Society proposed the following nomenclature for the pectic substances (3):

Pectic Substances. "Pectic substances" is a group designation for those complex, colloidal carbohydrate derivatives which occur in or are prepared from plants and contain a large proportion of anhydrogalacturonic acid units which are thought to exist in a chainlike combination. The carboxyl groups of polygalacturonic acids may be partly esterified by methyl groups and partly or completely neutralized by one or more bases.

Protopectin. The term "protopectin" is applied to the water-insoluble parent pectic substance which occurs in plants and which upon restricted hydrolysis yields pectin or pectinic acids.

Pectinic Acids. The term "pectinic acids" is used for colloidal polygalacturonic acids containing more than a negligible proportion of methyl ester groups. Pectinic acids, under suitable conditions, are capable of forming gels with sugar and acid or, if suitably low in methoxyl content, with certain metallic ions. The salts of pectinic acids are either normal or acid pectinates.

Pectin. The general term "pectin" (or pectins) designates those wat soluble pectinic acids of varying methyl ester content and degree of neutralizati which are capable of forming gels with sugar and acid under suitable conditions

Pectic Acids. The term "pectic acids" is applied to pectic substances mos composed of colloidal polygalacturonic acids and essentially free from metl ester groups. The salts of pectic acids are either normal or acid pectates.

Many bacteria, molds, and higher plants contain active pecti destroying enzymes. For excellent reviews see references 2 and Three pectic enzyme systems are now known: *protopectinase, pectina (polygalacturonase, pectolase), and pectase (pectin-methoxylase).*

1. Protopectinase. Protopectinase is the enzyme that softens pla tissue by hydrolyzing the middle lamella of plants (protopectir The exact nature of this reaction is not known. Various pathoger microorganisms, fungi, and bacteria-infecting plants contain tl enzyme.

Good sources of protopectinase are *B. carotovorus* (5), *B. mesente cus* (6), *Botrytis cinerea* (7), *Rhizopus* (8), *Sclerotinia cinerea* (t plum-rotting organism), and *Fusarium chromiophthoron* (9). T softening of fruits such as apples, pears, peaches (10, 11), and of soi vegetables such as tomatoes is caused by protopectinase, which present in these plants. Ehrlich (12) prepared a highly active prot pectinase by extracting the mycelium of *Penicillium* with water a precipitating the enzyme with alcohol. This preparation dissolv up to 60 per cent of the sugar-beet tissue in 24 hours.

2. Pectinase (Pectolase, Polygalacturonase). This enzyme spl polygalacturonic acid into monogalacturonic acid by opening glycosi linkages. It is mostly present in fungi and bacteria and is frequent accompanied by pectase (pectin-methoxylase) (2).

Although barley and barley malt do not contain pectin, pectina may be prepared from the barley malt (13). Pectinase is present *Sclerotinia cineria* (14) and in a great variety of bacteria (1 especially those microorganisms thriving on fruits (16). Various fu such as *Rhizopus tritici* (17), *Sclerotinia cinerea, Botrytis cinerea* (1 *Penicillium ehrlichii* (19), and other penicillia (20) contain pectina Menon (21) described the pectinases of the parasite molds *B. ciner Monilia fructizena, Pythium de baryanum, Phytophthora erythros tica, Fusarium fructizenum,* and *Glocosporium fructizenum.*

Jansen and MacDonnel (22) have reported on the rate of glycosi hydrolysis of pectin and enzyme- and alkali-prepared pectic ac by the commercial enzyme mixture "Pectinol 100 D." This pa contains many interesting observations. However, the preparat of specific enzymes from molds offers no difficulties, and resu

GENERAL REQUIREMENTS

Enzyme preparations are produced in accordance with good manufacturing practices. Regardless of the source from which they are derived, they cause no increase in the total microbial count in the treated food over the level accepted for the respective food.

Animal tissues used for the production of enzymes must comply with the applicable federal meat inspection requirements and must be handled in accordance with good hygienic practices.

Plant material used in the production of enzymes, or culture media used for the growth of microorganisms, consists of components that leave no residues harmful to health in the finished food under normal conditions of use.

Preparations derived from microbial sources are produced by methods and under culture conditions that ensure a controlled fermentation, thus preventing the introduction of microorganisms that could be the source of toxic materials and other undesirable substances.

The carriers, diluents, and processing aids used in the production of the enzyme preparations shall be substances that are acceptable for general use in foods, including water and substances that are insoluble in foods but removed from the foods after processing.

Although tolerances have not been established for mycotoxins, appropriate measures should be taken to ensure that the products do not contain such contaminants.

ADDITIONAL REQUIREMENTS

Assay Not less than 85% and not more than 115% of the declared activity.
Arsenic (as As) Not more than 3 ppm.
Coliforms Not more than 30 per g.
Heavy Metals (as Pb) Not more than 0.004%.
Lead Not more than 10 ppm.
Salmonella sp. Negative by test.

TESTS

Assay The following procedures, which are included in the *General Tests* section under *Enzyme Assays*, page 479, are provided for application as necessary in determining compliance with the declared representations for enzyme activity:* Alpha-Amylase Activity (Nonbacterial); Bacterial Alpha-Amylase Activity (BAU); Catalase Activity; Cellulase Activity; Diastase Activity (Diastatic Power, DP); β-Glucanase Activity; Glucoamylase Activity (Amyloglucosidase Activity); Glucose Isomerase Activity; Glucose Oxidase Activity; Hemicellulase Activity; Invertase Activity; Lactase (β-Galactosidase) Activity; Lipase Activity; Lipase/Esterase (Forestomach) Activity; Milk-Clotting Activity; Pepsin Activity; Plant Proteolytic Activity; Proteolytic Activity, Bacterial (PC); Proteolytic Activity, Fungal (HUT); Proteolytic Activity, Fungal (SAP); and Trypsin Activity.

Arsenic A *Sample Solution* prepared as directed for organic compounds meets the requirements of the *Arsenic Test*, page 464.

Coliforms Determine as directed in Section 46.039, *Official Methods of Analysis of the AOAC*, Thirteenth Edition, 1980, page 825.

Heavy Metals Prepare and test a 500-mg sample as directed in *Method II* under the *Heavy Metals Test*, page 513, using 20 μg of lead ion (Pb) in the control (*Solution A*).

Lead A *Sample Solution* prepared as directed for organic compounds meets the requirements of the *Lead Limit Test*, page 518, using 10 μg of lead ion (Pb) in the control.

Salmonella sp. Determine as directed in Chapter VI, Procedure 7, *Bacteriological Analytical Manual*, Fifth Edition, Food and Drug Administration, 1978.

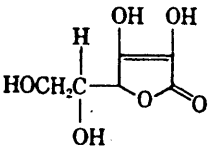
Packaging and Storage Store in tight containers in a cool, dry place.

Functional Use in Foods Enzyme (see discussion under *Classification* above).

*Because of the varied conditions under which pectinases are employed, and because laboratory hydrolysis of a purified pectin substrate does not correlate with results observed with the natural substrates under use conditions, it is recommended that pectinase suppliers and users develop their own assay procedures that would relate to the specific application under consideration.

Erythorbic Acid

D-Araboascorbic Acid



$C_6H_8O_6$ Mol wt 176.13

DESCRIPTION

White or slightly yellow crystals or powder. On exposure to light it gradually darkens. In the dry state it is reasonably stable in air, but in solution it rapidly deteriorates in the presence of air. It melts between 164° and 171° with decomposition. One g is soluble in about 2.5 ml of water and in about 20 ml of alcohol. It is slightly soluble in glycerin.

REQUIREMENTS

Identification

A. A 1 in 50 solution slowly reduces alkaline cupric tartrate TS at 25°, but more readily upon heating.
B. To 2 ml of a 1 in 50 solution add a few drops of sodium nitroferricyanide TS, followed by 1 ml of approximately 0.1 *N* sodium hydroxide. A transient blue color is produced immediately.
C. Dissolve about 15 mg in 15 ml of a trichloroacetic acid solution (1 in 20), add about 200 mg of activated charcoal,

obtained with enzymes of known origin and definite purity are of greater value to the enzymologist.

The optimum *p*H of mold pectinase is at *p*H 3.0 to 3.5, and it is influenced, as in other enzymes, by the composition of the reaction mixture, source of the enzyme, purity of the enzyme, etc.

3. Pectase (Pectin-Methoxylase, Pectinesterase). Pectase, the enzyme that splits off methoxy groups (methyl alcohol) from pectin and converts soluble pectin, in the presence of calcium salts, into a gel, was discovered by Fremy in 1840. There are some indications that pectase is a non-specific esterase identical with plant esterases (lipases).

Preparation and Properties of Pectase. The press juice of fresh-cut alfalfa converts pectin into a solid gel within a few seconds. Mehlitz (23) obtained 1682 grams of crude press juice from 5000 grams of alfalfa. He further purified this juice by preserving it with chloroform, placing it in a dark place, and allowing it to settle for 22 hours. Then the crude juice was filtered and the pectase was precipitated by the addition of 2 volumes of 90 per cent alcohol. The precipitate was collected and dissolved in 400 cc. of distilled water by allowing it to remain in the water for 15 hours with occasional shaking. Then the pectase was separated from the insoluble residue by filtration. The enzyme was precipitated again with 90 per cent alcohol. The precipitate was dried over calcium chloride in a vacuum desiccator. The dry enzyme powder kept well for 4 months, whereas its 10 per cent solution lost most of its activity in 3 to 4 days. A calcium concentration of 0.15 per cent was found to be the optimum amount for gel formation, and the *p*H optimum of the alfalfa pectase was at 4.8 to 5.0 at a calcium pectate concentration of 0.7 per cent.

Paul and Grandseigne (24) prepared active pectase by extracting sprouting legumes with water. The extract was mixed with a colloid, such as starch or tragacanth, and was precipitated with acetone. The resulting gel was dried. Tzerevitinov and Rozanova (25) examined a series of plants and grasses and found that potato plants and Swedish clover were rich sources for pectase.

It was found that this enzyme removes the methoxyl group from pectin and that gel formation is a secondary reaction influenced mainly by the state of the substrate. For this reason Kertesz (26) differentiates between the "methoxylase" reaction and the "pectase" (gelation) reaction. According to Kertesz, gel formation depends on the composition of the mixture and is influenced by *p*H changes brought about by the liberated carboxyl groups. Acid formation may increase the *p*H of the medium to such an extent that gel formation is greatly

delayed. Kertesz proposes the method applied by Knaffl-Lenz (27) for lipase for the titration of the carboxyl groups of the galacturonic acids set free during the reaction. By this method alkali is added at short intervals, the *p*H being kept constant (*p*H 6.2) during the course of the reaction. This cannot be accomplished with buffers. The pectin methoxylase shows increased activity with increasing *p*H. In an alkaline solution, however, pectin is demethoxylated, so that enzyme activity can be determined on the acid side of the *p*H scale only.

Table LIV shows the pectin methoxylase content of some plants

TABLE LIV

ENZYMATIC PECTIN DEMETHOXYLATION BY VARIOUS MATERIALS

Material	Applied in Determination (cc.)	Dry Matter (per cent)	Pectin Methoxylase Units	
			Per Cc.	Per Gram
Filtered juice of Windsor sweet cherries	5.0	11.14	0.182	1.6
	10.0		0.189	1.7
Juice of shipped Florida tomatoes	1.5	4.25	2.39	56
	2.0		2.42	57
	2.5		2.57	60
	3.0		2.48	58
				58 (Average)
Juice of ripe hothouse tomatoes, Forcing Wonder	1.0	3.59	5.02	140
	2.0		4.90	137
10 per cent extract of dried tobacco powder	2.0	3.86	0.44	11.4
	3.0		0.42	10.9
Press juice of leaves of hothouse-grown (Kentucky) green White Burley tobacco	1.0	3.82	1.69	44
	2.0		1.66	43
Press juice from alfalfa	1	6.99	1.08	15.5
	2		1.17	16.7
	3		1.13	16.1
	4		1.04	14.9
				15.8 (Average)

Steapsin "Difco" (6 years old) 5.6 units per gram per cc.

and Table LV shows that of some commercial enzyme mixtures as reported by Kertesz (26).

It had been known for some time that pectin methoxylase is an esterase similar in action to certain lipases and perhaps identical with plant lipases. Experiments supporting this view had been reported by Kertesz, who showed that castor-bean lipase and pancreatic lipase have both pectin methoxylase and pectase activity. Owing to the non-

Micrococcus lysodeikticus. Major active principle: *catalase*. Typical application: manufacture of cheese.

Glucose Isomerase (*Actinoplanes missouriensis*, *Bacillus coagulans*, *Streptomyces olivaceus*, *Streptomyces olivochromogenes*, or *Streptomyces rubiginosus*, var.) Produced by the controlled fermentation of any of the above organisms as off-white to tan or brown or pink amorphous powders, granules, or liquids. They are partially soluble in water, and are insoluble in alcohol, in chloroform, and in ether. Major active principle: *glucose* (or *xylose*) *isomerase*. Typical applications: manufacture of high-fructose corn syrup and other fructose starch syrups.

Glucose Oxidase (*Aspergillus niger* var.) Produced by the controlled fermentation of *Aspergillus niger* var. as yellow to brown solutions or as yellow to tan or off-white powders. Practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) *glucose oxidase* and (2) *catalase*. Typical applications: removal of sugar from liquid eggs; deoxygenation of citrus beverages.

Lipase (*Aspergillus niger* var.) Produced by the controlled fermentation of *Aspergillus niger* var. as off-white to tan amorphous powders. Soluble in water (the solutions usually being light yellow in color) but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *lipase*. Typical applications: hydrolysis of lipids (e.g., fish oil concentrates).

Lipase (*Aspergillus oryzae* var.) Produced by the controlled fermentation of *Aspergillus oryzae* var. as off-white to tan amorphous powders, or as liquids. Soluble in water (the solutions usually being light yellow in color) but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *lipase*. Typical application: hydrolysis of lipids (e.g., fish oil concentrates).

Protease (*Aspergillus niger* var.) Produced by the controlled fermentation of species of *Aspergillus niger* var. The purified enzyme occurs as off-white to tan amorphous powders. Soluble in water (the solution usually being light yellow in color) but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *protease*. Typical application: production of protein hydrolysates.

Protease (*Aspergillus oryzae* var.) Produced by the controlled fermentation of species of *Aspergillus oryzae* var. The purified enzyme occurs as off-white to tan amorphous powders. Soluble in water (the solutions usually being light yellow in color) but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *protease*. Typical applications: chillproofing of beer; bakery products; meat tenderizing; production of protein hydrolysates.

Rennet, Microbial (*Endothia parasitica*) Produced by the controlled fermentation of nonpathogenic species of *Endothia parasitica* as an off-white to tan amorphous powder, or as a liquid. The powders are soluble in water (the solutions usually being tan to dark brown in color) but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *protease*. Typical application: manufacture of cheese.

Rennet, Microbial (*Mucor* species) Produced by the controlled fermentation of *Mucor miehei* or *M. pusillus* as white to tan amorphous powders. The powders are soluble in water (the solutions usually being light yellow in color) but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *protease*. Typical application: manufacture of cheese.

REACTIONS CATALYZED

NOTE: The reactions catalyzed by any given active component are essentially the same, regardless of the source from which that component is derived.

α-Amylase Hydrolysis of α-1,4-glucan bonds in polysaccharides (starch, glycogen, etc.), yielding dextrins and oligo- and monosaccharides.

β-Amylase Hydrolysis of α-1,4-glucan bonds in polysaccharides (starch, glycogen, etc.), yielding *beta* limit dextrins.

Bromelain Hydrolysis of polypeptides, amides, and esters (especially at bonds involving basic amino acids, or leucine or glycine), yielding peptides of lower molecular weight.

Catalase $2H_2O_2 \rightarrow O_2 + 2H_2O$.

Cellulase Hydrolysis of β-1,4-glucan bonds in such polysaccharides as cellulose, yielding β-dextrins.

Ficin Hydrolysis of polypeptides, amides, and esters (especially at bonds involving basic amino acids, or leucine or glycine), yielding peptides of lower molecular weight.

β-Glucanase Hydrolysis of β1,3- and β1,4-linkages in β-D-glucans, yielding oligosaccharides and glucose.

Glucoamylase (*Amyloglucosidase*) Hydrolysis of α-1,4- and α-1,6-glucan bonds in polysaccharides (starch, glycogen, etc.), yielding glucose (dextrose).

Glucose Isomerase Isomerization of glucose to fructose, and xylose to xylulose.

Glucose Oxidase β-D-glucose + $O_2 \rightarrow$ D-glucono-δ-lactone + H_2O_2.

Hemicellulase Hydrolysis of β-1,4-glucan bonds in such polysaccharides as locust (carob) bean and guar gums, yielding β-dextrins.

Invertase Hydrolysis of sucrose to a mixture of glucose and fructose (invert sugar).

Lactase Hydrolysis of lactose to a mixture of glucose and galactose.

Lipase Hydrolysis of triglycerides of simple fatty acid esters, yielding mono- and diglycerides, glycerol, and free fatty acids.

Pectinase

Pectin Methylesterase Demethylation of pectin.

Polygalacturonase Hydrolysis of α-1,4-galacturonide bonds in pectin.

Pepsin Hydrolysis of polypeptides, including those with bonds adjacent to aromatic or decarboxylic L-amino acid residues, yielding peptides of lower molecular weight.

Protease (general) Hydrolysis of polypeptides, yielding peptides of lower molecular weight.

Rennin Hydrolysis of polypeptides; specificity may be similar to pepsin.

Trypsin Hydrolysis of polypeptides, amides, and esters at bonds involving the carboxyl groups of L-arginine and L-lysine, yielding peptides of lower molecular weight.

sample taken by the formula $25C \times A_U/A_S$, in which C is the exact concentration of the Reference Standard solution, in μg per ml, A_U is the absorbance of the sample solution, and A_S is the absorbance of the Reference Standard solution.

Amino Acids To 5 ml of a 1 in 1000 solution of the sample add 1 ml of ninhydrin TS. No color is produced.

Ammonium Salts Transfer about 100 mg of the sample into a small test tube, and add 50 mg of magnesium oxide and 1 ml of water. Moisten a piece of red litmus paper with water, suspend it in the tube, cover the mouth of the tube, and heat in a water bath for 5 min. The litmus paper does not change to blue.

Arsenic A *Sample Solution* prepared as directed for organic compounds meets the requirements of the *Arsenic Test*, page 464.

Barium Dissolve 1 g of the sample in 100 ml of water, filter, and add 5 ml of diluted sulfuric acid TS to the filtrate. Any turbidity is not greater than that produced in a similar solution containing 1.5 ml of *Barium Standard Solution* (150 μg Ba).

Clarity and Color of Solution A 500-mg portion of the sample dissolved in 10 ml of water is colorless and shows no more than a trace of turbidity.

Heavy Metals Prepare and test a 1-g sample as directed in *Method II* under the *Heavy Metals Test*, page 513, using 20 μg of lead ion (Pb) in the control (*Solution A*).

Lead A *Sample Solution* prepared as directed for organic compounds meets the requirements of the *Lead Limit Test*, page 518, using 10 μg of lead ion (Pb) in the control.

Other Nucleotides Prepare a strip of Whatman No. 2 or equivalent filter paper about 20 × 40 cm, and draw a line across the narrow dimension about 5 cm from one end. Using a micropipet, apply on the center of the line 10 μl of a 1 in 100 solution of the sample in water, and dry the paper in air. Fill the trough of an apparatus suitable for descending chromatography (see page 473) with a 160:3:40 mixture of saturated ammonium sulfate solution, *tert*-butyl alcohol, and 0.025 *N* ammonia, respectively, and suspend the strip in the chamber, placing the end of the strip in the trough at a distance about 1 cm from the pencil line. Seal the chamber, and allow the chromatogram to develop until the solvent front descends to a distance about 30 cm from the starting line. Remove the strip from the chamber, dry in air, and observe under shortwave (254 nm) ultraviolet light in the dark. Only one spot is visible.

pH of a 1 in 20 Solution Determine by the *Potentiometric Method*, page 531.

Water Determine by the *Karl Fischer Titrimetric Method*, page 552.

Packaging and Storage Store in well-closed containers.

Functional Use in Foods Flavor enhancer.

Enzyme Preparations

DESCRIPTION

Enzyme preparations used in food processing are derived from animal, plant, or microbial sources (see *Classification* below). They may consist of whole cells, parts of cells, or cell-free extracts of the source used, and they may contain one or more active components as well as diluents, preservatives, antioxidants, and other substances consistent with good manufacturing practice.

The individual preparations are usually named according to the substance to which they are applied, such as *Protease* or *Amylase*; such traditional names as *Malt*, *Pepsin*, and *Rennet* are also used, however.

The color of the preparations—which may be liquid, semiliquid, or dry—may vary from virtually colorless to dark brown. The active components consist of the biologically active proteins, which are sometimes conjugated with metals, carbohydrates, and/or lipids. Known molecular weights of the active components range from approximately 12,000 to several hundred thousand.

The activity of enzyme preparations is measured according to the reaction catalyzed by individual enzymes (see below) and is usually expressed in activity units per unit weight of the preparation. In commercial practice (but not for *Food Chemicals Codex* purposes), the activity of the product is sometimes also given as the quantity of the preparation to be added to a given quantity of food in order to achieve the desired effect.

Additional information relating to the nomenclature and the sources from which the active components are derived is provided in the *General Tests* section under *Enzyme Assays*, page 479.

CLASSIFICATION

Animal-Derived Preparations

Catalase (bovine liver) Partially purified liquid or powdered extracts from bovine liver. Major active principle: *catalase*. Typical application: manufacture of certain cheeses.

Lipase, Animal Obtained from two primary sources: (1) edible forestomach tissue of calves, kids, or lambs, and (2) animal pancreatic tissue. Produced as purified edible tissue preparations or as aqueous extracts. Dispersible in water; insoluble in alcohol. Major active principle: *lipase*. Typical applications: manufacture of cheese; modification of lipids.

Pepsin Obtained from the glandular layer of hog stomach. White to light tan water-soluble powders, amber pastes, or clear amber to brown aqueous liquids. Major active principle: *pepsin*. Typical applications: preparation of fish meal and other protein hydrolysates; clotting of milk in manufacture of cheese (in combination with rennet).

Rennet Aqueous extracts made from the fourth stomach of calves, kids, or lambs. Clear amber to dark brown liquid preparations, or white to tan powders. Major active principle: *protease* (rennin). Typical application: manufacture of cheese.

Rennet, Bovine Aqueous extracts made from the fourth stomach of bovine animals, sheep, and goats. Clear amber to dark brown liquids, or white to tan powders. Major active principle: *protease* (rennin). Typical application: manufacture of cheese.

Trypsin Obtained from purified extracts of porcine or bovine pancreas. White to tan amorphous powders, which are soluble in water but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *trypsin*. Typical applications: baking; meat tenderizing; production of protein hydrolysates.

Plant-Derived Preparations

Bromelain The purified proteolytic substance derived from the pineapples *Ananas comosus* and *Ananas bracteatus* L. White to light tan amorphous powder. Soluble in water (the solution being colorless to light yellow and somewhat opalescent) but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *bromelain*. Typical applications: chillproofing of beer; meat tenderizing; preparation of precooked cereals; production of protein hydrolysates.

Ficin The purified proteolytic substance derived from the latex of *Ficus* sp., which include a variety of tropical fig trees. White to off-white powders, which are completely soluble in water. (Liquid fig latex concentrates are light brown to dark brown in color.) Major active principle: *ficin*. Typical applications: chillproofing of beer; meat tenderizing; dough conditioner in baking.

Malt The product of the controlled germination of barley. Clear amber to dark brown liquid preparations, or white to tan powders. Major active principles: (1) *α-amylase* and (2) *β-amylase. Typical applications: baking; manufacture of alcoholic beverages; manufacture of syrups.*

Papain The purified proteolytic substance derived from the fruit of the papaya *Carica papaya* L. (Fam. Caricaceae). Produced as white to light tan amorphous powders, or as liquids. Soluble in water (the solution being colorless or light yellow and somewhat opalescent) but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) *papain* and (2) *chymopapain*. Typical applications: chillproofing of beer; meat tenderizing; preparation of precooked cereals; production of protein hydrolysates.

Microbially Derived Preparations

Carbohydrase (*Aspergillus niger* var.) Produced by the controlled fermentation of *Aspergillus niger* var. as off-white to tan amorphous powders, or as tan to dark brown liquids. Practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) *α-amylase*; (2) *pectinase* (usually a mixture of pectin methylesterase, polygalacturonase, and pectate lyase); (3) *cellulase*; (4) *glucoamylase* (amyloglucosidase); (5) *hemicellulase*; (6) *lactase*; and (7) *β-glucanase*. Typical applications: preparation of starch syrups, alcohol, beer, ale, fruit juices, chocolate syrup, bakery products, liquid coffee, wine, dextrose, and dairy products.

Carbohydrase (*Aspergillus oryzae* var.) Produced by the controlled fermentation of *Aspergillus oryzae* var. as off-white to tan amorphous powders, or as liquids. Soluble in water (the solutions being light yellow to dark brown in color) but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) *α-amylase*, (2) *glucoamylase* (amyloglucosidase), and (3) *lactase*. Typical applications: preparation of starch syrups, alcohol, beer, ale, bakery products, and dairy products.

Carbohydrase (*Rhizopus oryzae* var.) A group of enzyme preparations produced by the controlled fermentation of *Rhizopus oryzae* var. as powders or liquids. Major active principles: (1) *α-amylase*; (2) *pectinase*; and (3) *glucoamylase* (amyloglucosidase). Typical applications: preparation of starch syrups and fruit juices; manufacture of cheese.

Carbohydrase (*Saccharomyces* species) The purified enzyme produced by the controlled fermentation of a number of species of *Saccharomyces* traditionally used in the manufacture of food. White to tan amorphous powders. Soluble in water (the solutions usually being light yellow in color) but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) *invertase* and (2) *lactase*. Typical applications: manufacture of candy and ice cream; modifications of dairy products.

Carbohydrase (*Trichoderma reesei* var.) Produced by the controlled fermentation of *Trichoderma reesei* var. as off-white to tan amorphous powders or liquids. Soluble in water (the solutions usually being tan to brown in color) but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *cellulase*. Typical applications: preparation of fruit juices, wine, vegetable oils, and beer.

Carbohydrase and Protease, Mixed (*Bacillus licheniformis*) Produced by the controlled fermentation of *Bacillus licheniformis* var. as off-white to brown amorphous powders or as liquids. Soluble in water (the solution usually being light yellow to dark brown in color) but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) *α-amylase* and (2) *protease*. Typical applications: preparation of starch syrups, alcohol, beer, dextrose, fish meal, protein hydrolysates.

Carbohydrase and Protease, Mixed (*Bacillus subtilis*) Produced by the controlled fermentation of *Bacillus subtilis* var. as off-white to tan amorphous powders, or as liquids. Soluble in water (the solutions usually being light yellow to dark brown in color) but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) *α-amylase* and *β-glucanase*, and (2) *protease*. Typical applications: preparation of starch syrups, alcohol, beer, dextrose, bakery products, fish meal; meat tenderizing; preparation of protein hydrolysates.

Catalase (*Aspergillus niger* var.) Produced by the controlled fermentation of *Aspergillus niger* var. as off-white to tan amorphous powders, or as liquids. Soluble in water (the solutions usually being tan to brown in color) but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *catalase*. Typical applications: manufacture of cheese and egg products.

Catalase (*Micrococcus lysodeikticus*) Partially purified liquid or powdered extracts from submerged fermentations of