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Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing: Update for a New Century[1]

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Microbial enzymes used in food processing are typically sold as *enzyme preparations* that contain not only a desired enzyme activity but also other metabolites of the production strain, as well as added materials such as preservatives and stabilizers. The added materials must be food grade and meet applicable regulatory standards. The purpose of this report is to present guidelines that can be used to evaluate the safety of the metabolites of the production strain that are also present in the enzyme preparation, including of course, but not limited to, the desired enzyme activity itself. This discussion builds on previously published decision tree mechanisms and includes consideration of new genetic modification technologies, for example, modifying the primary structure of enzymes to enhance specific properties that are commercially useful. The safety of the production strain remains the primary consideration in evaluating enzyme safety, in particular, the *toxigenic potential* of the production strain. Thoroughly characterized nonpathogenic, nontoxigenic microbial strains, particularly those with a history of safe use in food enzyme manufacture, are logical candidates for generating a *safe strain lineage*, through which improved strains may be derived via genetic modification by using either traditional/classical or rDNA strain improvement strategies. The elements needed to establish a safe strain lineage include thoroughly characterizing the host organism, determining the safety of all new DNA that has been introduced into the host organism, and ensuring that the procedure(s) that have been used to modify the host organism are appropriate for food use. Enzyme function may be changed by intentionally altering the amino acid sequence (e.g., protein engineering). It may be asked if such modifications might also affect the safety of an otherwise safe enzyme. We consider this question in light of what is known about the natural variation in enzyme structure and function and conclude that it is unlikely that changes which improve upon desired enzyme function will result in the creation of a toxic protein. It is prudent to assess such very small theoretical risks by conducting limited toxicological tests on engineered enzymes. The centerpiece of this report is a decision tree mechanism that updates previous enzyme safety evaluation mechanisms to accommodate advances in enzymology. We have concluded that separate mutagenicity testing is not needed if this decision tree is used to evaluate enzyme safety. Under the criteria of the decision tree, no new food enzyme can enter the market without critical evaluation of its safety. © 2001 Academic Press

INTRODUCTION

Microbial enzymes used in food processing are typically sold as *enzyme preparations* that contain not only a desired enzyme activity but also other metabolites of the production strain, as well as added materials such as preservatives and stabilizers. The added materials must be food grade and meet the standards of regulatory policy where the enzyme is used. The purpose of this report is to present guidelines that can be used to evaluate the safety of the metabolites of the production strain that are also present in the enzyme preparation, including of course, but not limited to, the desired enzyme activity itself. This discussion builds on previous reports (Pariza and Foster, 1983; IFBC, 1990; Kessler *et al.*, 1992) and includes consideration of new genetic modification technologies, for example, modifying the primary structure of enzymes to enhance specific properties that are commercially useful.

Many of the enzymes that were used or are currently used in food processing are listed in Table 1; enzymes that were listed in the Pariza and Foster (1983) publication are marked. Over time, new enzymes will be added to this list and some now on the list may be deleted. The Enzyme Technical Association (ETA) periodically updates this list and maintains it on their web site, http://www.enzymetechnicalassoc.org/.

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0273-2300/01 $35.00

TABLE 1
Enzymes Used in Food Processing Today

Trivial name	Classification	Source	Systematic Names (IUB)[a]	IUB No.[a]	CAS No.[b]
α-Amylase	Carbohydrase	(1) *Aspergillus niger* var.[c] (2) *Aspergillus oryzae* var.[c] (3) *Rhizopus oryzae* var.[c] (4) *Bacillus subtilis* var.[c] (5) *Bacillus amyloliquefaciens*[d] d-*Bacillus* (6) Barley malt[c] (7) *Bacillus licheniformis*[d] d-*Bacillus licheniformis* (8) *Bacillus stearothermophilus* (9) *Bacillus subtilis*[d] d-*Bacillus megaterium* (10) *Bacillus subtilis*[d] d-*Bacillus stearothermophilus* (11) *Microbacterium imperiale* (12) *Bacillus amyloliquefaciens* (13) *Bacillus licheniformis* var.[c] (14) *Bacillus licheniformis*[d] d-*Bacillus stearothermophilus* (15) *Aspergillus niger*[d] d-*Aspergillus niger*	1,4-α-D-Glucan glucanohydrolase	3.2.1.1	9000-90-2
Aminopeptidase	Protease	(1) *Aspergillus niger* (2) *Rhizopus oryzae* (3) *Aspergillus oryzae*	α-Aminoacyl-peptide hydrolase	3.4.11.11	
AMP deaminase	Adenosine deaminase	*Aspergillus melleus*	AMP aminohydrolase	3.5.4.6	9025-10-9
Arabinofuranosidase	Carbohydrase	(1) *Aspergillus niger* (2) *Aspergillus niger*[d] d-*Aspergillus niger*		3.2.1.55	9067-74-7
β-Amylase	Carbohydrase	(1) Barley malt[c] (2) Barley (ungerminated)	1,4-α-D-Glucan maltohydrolase	3.2.1.2	9000-91-3
Bromelain	Protease	Pineapples: *Ananas comosus* *Ananas bracteatus* (L)[c]	None	3.4.22.32 3.4.22.33	37189-34-7 9001-00-7
Catalase	Oxidoreductase	(1) *Aspergillus niger*[d] d-*Aspergillus niger* (2) bovine liver[c] (3) *Micrococcus luteus*[c] (4) *Aspergillus niger* var.[c]	Hydrogen peroxide: hydrogen peroxide oxidoreductase	1.11.1.6	9001-05-2
Cellulase	Carbohydrase	(1) *Aspergillus niger* var.[c] (2) *Trichoderma reesei*[c] (formerly *longibrachiatum*) (3) *Trichoderma reesei*[d] 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] d-calf prochymosin gene (2) *Escherichia coli* K-12[d] d-calf prochymosin gene (3) *Kluyveromyces marxianus*[d] d-calf prochymosin gene	Cleaves a single bond in κ-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	(1) *Chaetomium erraticum* (2) *Chaetomium gracile*	1,6-α-D-Glucan 6-glucanohydrolase	3.2.1.11	9025-70-1
Esterase	Lipase	*Rhizomucor miehei*		3.1.1.3	9001-62-1
Ficin	Protease	Figs: *Ficus* sp.[c]	None	3.4.22.3	9001-33-6
α-Galactosidase	Carbohydrase	(1) *Mortierella vinacea* var. *raffinoseutilizer* (2) *Aspergillus niger* (3) *Saccharomyces cerevisiae*[d] d-Guar seed	α-D-Galactoside galactohydrolase	3.2.1.22	90025-35-8

TABLE 1—*Continued*

Trivial name	Classification	Source	Systematic Names IUB[a]	IUB No[a]	CAS No[b]
β-Glucanase	Carbohydrase	(1) *Aspergillus niger* var.[c] (2) *Bacillus subtilis* var.[c] (3) *Trichoderma reesei* (formerly *longibrachiatum*) (4) *Talaromyces emersonii* (formerly *Penicillium emersonii*) (5) *Bacillus amyloliquefaciens* (6) *Aspergillus aculeatus* (7) *Bacillus amyloliquefaciens*[d] d-*Bacillus amyloliquefaciens* (8) *Disporotrichum dimorphosporum*	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.[c] (2) *Aspergillus oryzae* var.[c] (3) *Rhizopus oryzae* var.[c] (4) *Rhizopus niveus* (5) *Rhizopus delemar* (6) *Aspergillus niger*[d] d-*Aspergillus niger*	1-4-α-D-Glucan glucohydrolase	3.2.1.3	9032-08-0
Glucose isomerase	Isomerase	(1) *Actinoplanes missouriensis*[c] (2) *Bacillus coagulans*[c] (3) *Streptomyces olivaceus*[c] (4) *Streptomyces olivochromogenes*[c] (5) *Streptomyces rubiginosus*[d] d-*Streptomyces rubiginosus* (6) *Streptomyces murinus* (7) *Microbacterium arborescens* (8) *Streptomyces rubiginosus*[c]	D-Xylose ketoisomerase	5.3.1.5	9055-00-9
Glucose oxidase	Oxidoreductase	(1) *Aspergillus niger*[d] d-*Aspergillus niger* (2) *Aspergillus niger* var.[c]	β-D-Glucose: oxygen 1-oxidoreductase	1.1.3.4	9001-37-0
Glutaminase	Glutaminase	*Bacillus subtilis*	L-Glutamate aminohydrolase	3.5.1.2	9001-47-2
β-D-Glucosidase	Carbohydrase	(1) *Aspergillus niger* var. (2) *Trichoderma reesei* (formerly *longibrachiatum*)	β-D-Glucoside glucohydrolase	3.2.1.21	9001-22-3
Hemicellulase[e]	Carbohydrase	(1) *Aspergillus niger* var.[c] (2) *Aspergillus aculeatus* (3) *Aspergillus foetidus*	(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 (7) Endo-1,4-β-D-xylanase	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 9025-57-4
Hesperidinase	Carbohydrase	*Penicillium decumbens*	α-L-Rhamnoside rhamnohydrolase	3.2.1.40	37288-35-0
Invertase	Carbohydrase	*Saccharomyces* sp. (*Kluyveromyces*)[c]	β-D-Fructofuranoside fructohydrolase	3.2.1.26	9001-57-4
Lactase	Carbohydrase	(1) *Aspergillus niger* var. [c] (2) *Aspergillus oryzae* var.[c] (3) *Saccharomyces* sp.[c] (4) *Candida pseudotropicalis* (5) *Kluyveromyces marxianus* var. *lactis* (6) *Kluyveromyces marxianus* var. *lactis*[d] d-*Kluyveromyces marxianus* var. *lactis* (7) *Aspergillus oryzae*[d] d-*Aspergillus oryzae*	β-D-Galactoside galactohydrolase	3.2.1.23	9031-11-2

TABLE 1—*Continued*

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[c] (2) Animal pancreatic tissues[c] (3) *Aspergillus oryzae* var.[c] (4) *Aspergillus niger* var.[c] (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] d-*Rhizomucor miehei* (15) *Aspergillus oryzae*[d] d-*Thermomyces lanuginosus*		3.1.1.3	9001-62-1
Maltogenic amylase	Carbohydrase	*Bacillus subtilis*[d] 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	3.2.1.1	9000-90-2
			(2) Triacylglycerol acylhydrolase	3.1.1.3	9001-62-1
			(3) Protease	3.4.21.4	9002-07-7
Papain	Protease	Papaya: *Carica papaya (L)*[c]	None	3.4.22.2	9001-73-4
Pectin esterase	Carbohydrase	(1) *Aspergillus oryzae*[d] d-*Aspergillus aculeatus* (2) *Aspergillus niger* var. (3) *Aspergillus niger*[d] d-*Aspergillus niger*	Pectin pectylhydrolase	3.1.1.11	9025-98-3
Pectin lyase	Carbohydrase	*Aspergillus niger*		4.2.2.10	90025-98-3
Pectinase[e]	Carbohydrase	(1) *Aspergillus niger* var.[c] (2) *Rhizopus oryzae* var.[c] (3) *Aspergillus aculeatus*	(1) Poly(1,4-α-D-galacturonide) glycanohydrolase	3.2.1.15	9032-75-1
			(2) Pectin pectylhydrolase	3.1.1.11	9025-98-3
			(3) Poly(1,4-α-D-galacturonide) lyase	4.2.2.2	9015-75-2
			(4) Pectin lyase	4.2.2.10	9033-35-6
			(5) L-Arabinofuranoside arabinofuranohydrolase	3.2.1.55	9067-74-7
			(6) 1,5-L-Arabinan arabinofuranohydrolase	3.2.1.99	37325-54-5
			(7) Exo-polygalacturonase	3.2.1.67	
			(8) Endo-1,4-β-galactanase	3.2.1.89	
			(9) Pectin acetylesterase	3.1.1.6	
			(10) Exopolygalacturonase lyase	4.2.2.9	
Pepsin	Protease	Porcine or other animal stomach tissue[c]	None	3.4.23.1	9001-75-6
				3.4.23.2	9025-48-3
Phosphodiesterase	Nuclease	(1) *Penicillium citrinum* (2) *Leptographium procerum* (formerly *Verticicladiella procera)*	Oligonucleate 5′-nucleotidohydrolase	3.1.4.1	9025-82-5
Phospholipase A_2	Lipase	(1) *Animal pancreatic tissue* (2) *Streptomyces violaceoruber* (3) *Aspergillus niger*[d] d-porcine *pancreas*	(1) Phosphatidylcholine 2-acylhydrolase	3.1.1.4	9001-84-7
Phytase	Phosphatase	*Aspergillus niger*[d] d-*Aspergillus niger*	(1) Myo-inositol-hexakisphosphate--3-phosphohydrolase	3.1.3.8	37288-11-2 9001-77-8
			(2) Orthophosphoric monoester phosphohydrolase	3.1.3.2	

TABLE 1—*Continued*

Trivial name	Classification	Source	Systematic Names IUB[a]	IUB No[a]	CAS No[b]
Protease (general)	Protease	(1) *Aspergillus niger* var.[c]	None	3.4.23.18	9025-49-4
		(2) *Aspergillus oryzae* var.[c]			
		(3) *Aspergillus melleus*		3.4.21.14	9014-01-1
		(4) *Bacillus subtilis*		3.4.21.62	9014-01-1
		(5) *Bacillus subtilis*[d] d-*Bacillus amyloliquefaciens*		3.4.24.28	76774-43-1
		(6) *Bacilus amyloliquefaciens*[d] d-*Bacillus amyloliquefaciens*			9068-59-1 9073-79-4
		(7) *Bacillus licheniformis* var.		3.4.24.4	9001-61-0
		(8) *Bacillus stearothermophilus*		3.4.23.6	9080-56-2
		(9) *Rhizopus niveus*		3.4.11.1	
		(10) *Rhizopus oryzae*			
		(11) *Bacillus amyloliquefaciens*			
		(12) *Aspergillus oryzae*[d] d-*Rhizomucor miehei*			
Pullulanase	Carbohydrase	(1) *Bacillus acidopullulyticus*	α-Dextrin 6-glucanohydrolase	3.2.1.41	9075-68-7
		(2) *Bacillus licheniformis*[d] d-*Bacillus deramificans*			
		(3) *Bacillus naganoensis*[d]			
		(4) *Bacillus subtilis*[d] d-*Bacillus naganoensis*			
		(5) *Bacillus circulans*	Pullulan 6-glucanohydrolase		
Rennet	Protease	(1) Fourth stomach of ruminant animals	None	3.4.23.4 3.4.23.22	9001-98-3 37205-60-0
		(2) *Endothia parasitica*		3.4.23.23	148465-73-0
		(3) *Rhizomucor miehei*			
		(4) *Rhizomucor pusillus* (Lindt)			
		(5) *Aspergillus oryzae*[d] d-*Rhizomucor miehei*			
Transglucosidase	Glucanotransferase	*Aspergillus niger*	1,4-α-D-Glucan 4-α-D-glycosyltransferase	2.4.1.25	9032-09-1
Transglutaminase	Acyltransferase or aminotransferase	*Streptoverticillium mobaraense* var.	*R*-Glutaminly-peptide: amine γ-glutamyltransferase	2.3.2.13	80146-85-6
Trypsin	Protease	Animal pancreas	None	3.4.21.4	9002-07-7
Urease	Protease	*Lactobacillus fermentium*	None	3.5.1.5	9002-13-5
Xylanase	Carbohydrase	(1) *Trichoderma longibrachiatum*[d] d-*Trichoderma longibrachiatum*	(1) 1,4-β-D-Xylan xylanohydrolase (2) 1,3 β-D-Xylan xylanohydrolase (3) Endo-1,4(3)-β-D-hemicellulase	3.2.1.8 3.2.1.32	9025-57-4 9025-55-2
		(2) *Aspergillus niger var. awamori*[d] d-*Aspergillus* var.			
		(3) *Bacillus licheniformis*[d] d-*Bacillus licheniformis*			
		(4) *Aspergillus oryzae*[d] d-*Thermomyces lanuginosus*			
		(5) *Disporotrichum dimorphosporum*			
		(6) *Aspergillus niger*[d] d-*Aspergillus niger*			
		(7) *Trichoderma reesei* (formerly *longibrachiatum*)			
		(8) *Bacillus subtilis*[d] d-*Bacillus subtilis*			

[a] Enzyme nomenclature primarily based on the recommendations of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB). See also http://www.expasy.ch/enzyme/.
[b] *Chemical Abstract Service Registry Number.*
[c] Originally listed in the Pariza and Foster (1983) publication.
[d] A genetically modified organism. The donor organism is listed after "d-."
[e] Usually a mixture of the activities listed under the systematic name.

It is instructive to compare the number of enzymes from the 1983 list and today because the number of enzymes, and the microbial species from which production strains are derived, have greatly expanded in the past 20 years. This has occurred in response to the constantly evolving requirements of a very diverse international food processing industry.

For example, no enzyme listed in the 1983 publication is a product of recombinant DNA (rDNA) technology, because these methods were not yet in commercial use in

1983. Of course this changed in the ensuing years, and many of the production strains listed in Table 1 have been improved using rDNA technology. However, and most importantly, each of the enzymes and production strains listed in Table 1 has been evaluated for safety using criteria that are comparable to those described by Pariza and Foster (1983) and IFBC (1990).

CONSIDERATIONS PERTAINING TO FOOD ENZYME SAFETY EVALUATION

Safety of the Production Strain

Pariza and Foster (1983) discussed safety considerations for food-processing enzymes derived from production strains that were improved via traditional (nonrecombinant) methodologies. The following issues were considered: the safety of the production strain (referred to as the "source organism") with particular regard to toxigenic and pathogenic potential; allergies and primary irritations; carcinogens and mutagens; teratogens and reproductive effects; antibiotics; products of enzymatic reactions; interactions between enzymes and other food components; and direct effects of food enzymes on consumers.

It was concluded that the safety of the production strain should be the primary consideration in evaluating enzyme safety. The primary issue in evaluating the safety of a production strain is its *toxigenic potential*, specifically the possible synthesis by the production strain of toxins that are active via the oral route. *Pathogenic potential* is not usually an area of concern for consumer safety because enzyme preparations rarely contain viable organisms. Pathogenicity is, however, important to worker safety.

Toxigenic potential. Microbial toxins that are active via the oral route may be produced by certain bacteria or certain filamentous fungi (molds). Yeasts, by contrast, are not known to produce such toxins.

The oral toxins produced by bacteria cause food poisoning. They are proteinaceous in nature and elicit a rapid response. *In vivo* and/or *in vitro* tests are available for the detection of these toxins. The principal bacterial food poisoning toxins have been purified and many of their corresponding genes have been sequenced. Most of the toxins have been well characterized and exhaustively studied (Aktories and Just, 2000; Alouf and Freer, 1999; Rappuoli and Montecucco, 1997). The bacteria and fungi that produce these toxins have also been extensively characterized (Doyle *et al.*, 1997; Fischetti, 2000). This information provides the basis for testing new bacterial isolates for toxigenic potential.

The oral toxins produced by filamentous fungi are small molecular weight organic molecules, usually less than 1000 Da in size (Chu, 2000). These are referred to as mycotoxins. Most mycotoxins are acutely toxic, and many of them may also induce chronic toxicity (e.g., cancer) and developmental toxic effects when repeatedly administered to test animals. Chemical tests have been developed for the more important known mycotoxins (Chu, 2000). These tests can measure low levels of mycotoxins that would not elicit an acute response.

Pathogenic potential. It is extremely unlikely that a frank human pathogen would ever be used in food enzyme manufacture. Moreover, food enzyme preparations rarely contain viable production organisms. Hence the issue of pathogenicity is largely moot as regards food enzyme production strains. Nonetheless it is common industrial practice to evaluate previously untested host organisms for potential pathogenicity, using animal models.

It is important to distinguish between pathogenicity and opportunistic infection. Many microorganisms will produce opportunistic infections if they gain access to tissue sites that are normally protected by host barriers. Examples are infections in deep wounds produced by otherwise harmless microbes or infections by normally harmless microorganisms in individuals with a compromised immune system. By contrast, a true pathogen will produce disease or infection in an individual who would otherwise be considered healthy. Hence, a pathogen must be able to cross or evade noncompromised host barriers (Falkow, 1997; Mims, 1991). Accordingly, one cannot assess potential pathogenicity in compromised hosts.

It is important not to confuse the effect of the microbe itself with the host response to the microbe. For example, injecting dead bacteria into animals may lead to a catabolic cascade that may end in death by septic shock. This is not the result of pathogenicity, since the bacteria are dead and cannot produce an infection. Rather the reaction is caused by the release of hormone-like substances (*cytokines*) from the host immune cells in response to the presence of the dead bacteria (Beutler and Cerami, 1997). Accordingly, simply injecting microorganisms into animals is not an appropriate way to assess potential pathogenicity.

Information on the human pathogenic potential of microorganisms is readily available, for example, at http://www.cdc.gov/ncidod/dvbid/Biosafety_manual_rev_1994.pdf, or the NIH Guidelines for Research Involving Recombinant DNA Molecules at http://www4.od.nih.gov/oba/guidelines.html.

Safe Strain Lineage

Thoroughly characterized nonpathogenic, nontoxigenic microbial strains, particularly those with a history of safe use in food enzyme manufacture, are logical candidates for generating a *safe strain lineage*, through which improved strains may be derived via genetic modification either by using traditional/classical or rDNA strain improvement strategies (IFBC, 1990). The

elements needed to establish a safe strain lineage include thoroughly characterizing the host organism, determining the safety of all new DNA that has been introduced into the host organism, and ensuring that the procedure(s) that have been used to modify the host organism are appropriate for food use.

Historically the original isolates, from which contemporary microbial enzyme production strains were derived, were screened for vigorous growth under industrial scale fermentation conditions and selected for adequate yield of the desired enzyme product. The isolates were then subjected to mutagenesis (chemically induced or UV induced) and screened for randomly induced changes that would further increase yields. More recently rDNA technology has provided a directed means of enhancing strain improvement programs as well as for isolating and manipulating the genes coding for desired enzyme proteins.

Microbial taxonomy has advanced greatly during the past decade, particularly for bacteria and filamentous fungi (Balows and Duerden, 1998; Claridge *et al.*, 1997; Fink, 1999; Pitt and Hocking, 1997; Ward, 1998). The ability to amplify genomic DNA and obtain the sequences of microbial genomes has revolutionized our understanding of microbial taxonomy, phylogeny, and pathogenicity (Fink, 1999; Strauss and Falkow, 1997). DNA sequence data coupled with phenotypic analyses permit an accurate assessment of the taxonomy of donor and production organisms used for industrial enzyme development and manufacture. The safety evaluations of several microorganisms used in the food industry and for enzyme manufacture have been published including *Aspergillus oryzae* (Barbesgaard *et al.*, 1992), *Bacillus licheniformis* (de Boer *et al.*, 1994), *Bacillus subtilis* and *Bacillus amyloliquefaciens* (de Boer and Diderichsen, 1991), *Kluyveromyces lactis* (Bonekamp and Oosterom, 1994), and *Trichoderma reesei* (Nevalainen *et al.*, 1994). Recent technical and taxonomic treatises have provided considerable insight into the safety of enzyme production organisms based on detailed studies of phylogenetics and systematics of bacteria, yeasts, and fungi (Balows and Duerden, 1998; Kurtzman and Fell, 1999; Pitt and Hocking, 1997; Wolf, 1995). Hence, using traditional and modern molecular techniques, it is now possible to precisely determine the degree of relationships of microorganisms used in food enzyme production (e.g., Geiser *et al.*, 1998; Kuhls *et al.*, 1996). Further, according to the United States Food and Drug Administration (FDA, 1993), "... if internationally accepted rules of nomenclature are followed, changes in the taxonomic placement of an organism should not affect the ability to identify scientific references to the organism of interest, including scientific references to its toxigenicity, pathogenicity, or use in the production of food or enzymes."

Improvements in the efficiency of enzyme production may be achieved by developing microbial strains that directly or indirectly increase the amount of enzyme protein that is synthesized and available for harvest and purification (Archer and Peberdy, 1997; Demain and Davies, 1999). Genetic modifications are utilized to increase growth rate, expand the number of gene copies, enhance gene expression, and elevate enzyme secretion.

Traditional and molecular genetic techniques are also used to reduce or eliminate specific undesired endogenous enzyme activities or other characteristics. In some cases, these side activities may cause unwanted reactions in particular applications. For example, many microbial species secrete copious amounts of various proteases. Although this characteristic is desirable for certain applications, it can also lead to unwanted degradation of other secreted enzymes or have undesirable effects in certain food applications. Specific production strains have been developed in which one or several protease genes have been deleted or inactivated.

In some cases potentially useful enzyme activities have been discovered in microorganisms that are not suitable for use as production organisms in industrial fermentation. In these cases the genetic sequence encoding the desired enzyme protein can be cloned from the unsuitable microorganism and then transferred to and subsequently expressed in a well-characterized production strain with a history of safe use. This heterologous expression of enzymes is commonplace in the industry today and will remain standard practice in the commercialization of many enzymes.

Further improvements of enzyme producing microorganisms through genetic modification will continue as more knowledge is accumulated on the molecular basis of gene expression in the commonly used yeast, fungal, and bacterial enzyme production strains.

Engineered Enzymes

Protein engineering is the intentional alteration of the amino acid sequence of a protein to affect function (Arnold and Volkov, 1999; Atwell and Wells, 1999; Cleland and Craik, 1996, 1999; Kuchner and Arnold; 1997, Shaw *et al.*, 1999). This can be accomplished by inducing or introducing random mutations by chemical mutagenesis, UV irradiation, mutator strains, error-prone PCR, and other related techniques. Alternatively, site-directed mutagenesis techniques can be used to target changes to specific sites in the gene that are thought to be responsible for a particular protein characteristic. The more recent approach of directed molecular evolution employs other techniques, such as gene shuffling, to recombine gene fragments or functional blocks of gene sequence to generate variants of the protein gene sequence. In all cases, an effective and efficient selection or screening method is required to identify the altered protein having the desired functional characteristic.

When applied to enzymes, protein engineering can modify specific properties and improve the enzyme for

1. Is the production strain[a] genetically modified?[b]
   If yes,[c] go to 2. If no, go to 6.
2. Is the production strain modified using rDNA techniques?
   If yes, go to 3. If no, go to 5.
3. Issues relating to the introduced DNA[d,e] are addressed in 3a–3e.

3a. Do the expressed enzyme product(s) which are encoded by the introduced DNA have a history of safe use in food?[f]
   If yes, go to 3c. If no, go to 3b

3b. Is the NOAEL[g] for the test article[h] in appropriate short-term oral studies[i] sufficiently high[j] to ensure safety?
   If yes, go to 3c. If no, go to 12.

3c. Is the test article free of transferable antibiotic resistance gene DNA?[k]
   If yes, go to 3e. If no, go to 3d.

3d. Does the resistance gene(s) code for resistance to a drug substance used in treatment of disease agents in man or animal?
   If yes, go to 12. If no, go to 3e.

3e. Is all other introduced DNA well characterized and free of attributes that would render it unsafe for constructing microorganisms to be used to produce food-grade products?
   If yes, go to 4. If no, go to 12.

4. Is the introduced DNA randomly integrated into the chromosome?
   If yes, go to 5. If no, go to 6.
5. Is the production strain sufficiently well characterized so that one may reasonably conclude that unintended pleiotropic effects which may result in the synthesis of toxins or other unsafe metabolites will not arise due to the genetic modification method that was employed?
   If yes, go to 6. If no, go to 7.
6. Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure?[l]
   If yes, the test article is ACCEPTED. If no, go to 7.
7. Is the organism nonpathogenic?[m]
   If yes, go to 8. If no, go to 12.
8. Is the test article free of antibiotics?[n]
   If yes, go to 9. If no, go to 12.
9. Is the test article free of oral toxins[o] known to be produced by other members of the same species?
   If yes, go to 11. If no, go to 10.
10. Are the amounts of such toxins in the test article below levels of concern?[p]
   If yes, go to 11. If no, go to 12.
11. Is the NOAEL[q] for the test article in appropriate oral studies sufficiently high to ensure safety?
   If yes, the test article is ACCEPTED.[r]
   If no, go to 12.
12. An undesirable trait or substance may be present and the test article is not acceptable for food use. If the genetic potential for producing the undesirable trait or substance can be permanently inactivated or deleted, the test article may be passed through the decision tree again.

[a] Production strain refers to the microbial strain that will be used in enzyme manufacture. It is assumed that the production strain is nonpathogenic, nontoxigenic, and thoroughly characterized; steps 6–11 are intended to ensure this.

[b] The term "genetically modified" refers to any modification of the strain's DNA, including the use of traditional methods (e.g., UV or chemically-induced mutagenesis) or rDNA technologies.

[c] If the answer to this or any other question in the decision tree is unknown, or not determined, the answer is then considered to be NO.

[d] Introduced DNA refers to all DNA sequences introduced into the production organism, including vector and other sequences incorporated during genetic construction, DNA encoding any antibiotic resistance gene, and DNA encoding the desired enzyme product. The vector and other sequences may include selectable marker genes other than antibiotic resistance, noncoding regulatory sequences for the controlled expression of the desired enzyme product, restriction enzyme sites and/or linker sequences, intermediate host sequences, and sequences required for vector maintenance, integration, replication, and/or manipulation. These sequences may be derived wholly from naturally occurring organisms or incorporate specific nucleotide changes introduced by *in vitro* techniques, or they may be entirely synthetic.

[e] If the genetic modification served only to delete host DNA, and if no heterologous DNA remains within the organism, then proceed to step 5.

[f] Engineered enzymes are considered *not* to have a history of safe use in food, unless they are derived from a safe lineage of previously tested engineered enzymes expressed in the same host using the same modification system.

FIG. 1. Decision tree for evaluating the safety of microbially derived food enzymes.

[g] NOAEL is the acronym for the term *no observed adverse effect level*. It is the maximum dose of a test article (see below) that may be given to an animal in an appropriate repeated-dose oral toxicity test (see below) that does not produce an adverse effect. Ordinarily a NOAEL is derived from long-term feeding studies. However, given the established database indicating that microbial toxins that are active via the oral route are also acute toxins (see text), we conclude that in this very limited case, for the purposes of enzyme safety evaluation, a NOAEL can be determined from short-term gavage/feeding tests described in footnote *i*.

[h] Test article refers to the enzyme-containing material that is actually tested. It may differ from the commercial enzyme preparation in either form or formulation. For example, a lyophilized powder or other concentrated form of the enzyme preparation may be required so that it can be administered to test animals at higher concentrations. The test article may differ in formulation in that it is devoid of these safe and suitable preservatives, stabilizers, or other materials that may be commonly added to the enzyme that could affect palatability, nutrition, or some other aspect of a toxicity study.

The process for producing the test article should be representative of the process used for the final enzyme product. For instance, one would not produce a test article from a submerged culture growing in a yeast extract medium if the enzyme is to be produced commercially from cultures growing in a soy-based medium in open trays. The test article is often produced using the production process, stopping before the final purification and formulation steps.

A test article intended for pathogenicity testing would most likely consist of viable vegetative cells, spores, conidia, or other reproductive cells depending on the microorganism under test. These are commonly suspended in water, buffer, or other materials to minimize trauma to the test animals.

[i] We consider two animal toxicity tests to be appropriate for evaluating the safety of enzymes. Both are conducted using the oral route of administration as that is the intended route of exposure for consumers. The choice of which test to use is made on a case-by-case basis, depending largely on the species of the host organism and consideration of the nature of toxins that could theoretically be present (e.g., mycotoxin or bacterial enterotoxin).

The first test is an acute oral toxicity test in the rat following a single dose of the test article, as proposed by Pariza and Foster (1983). The dose to be used for this test should be at least 100 times the estimated mean human exposure (based on total organic solids (TOS)) or at least 2000 mg/kg body wt according to the OECD (Organization for Economic Cooperation and Development) Guideline for Testing of Chemicals, Acute Oral Toxicity, Guideline No. 401, Limit Test only (adopted on February 24, 1987) (Paris, 1983).

It should be emphasized that this test is not intended to establish a LD_{50}. Rather it is a specially designed test for determining the safety of bacterial enzymes, since with rare exception the only toxins known to be produced by bacteria are proteins or peptides (enterotoxins and certain neurotoxins) which are acute toxins that are produced by only a few bacterial species.

The second proposed toxicity test is a repeated-dose oral study (14–91 days) in one animal species, preferably the rat because of the historical data available on this species. The test article can be administered either in the feed or via gavage. The lowest dose used for this study should be at least 100 times the estimated mean human exposure (based on TOS). This test will detect toxicity that would be associated with the known microbial toxins that are active via the oral route.

In addition, all new enzymes should be analyzed for toxins that might be reasonably expected, using chemical, biochemical, or biological methods. For example, all test material from mold sources should be assayed for mycotoxins that are known to be synthesized by closely related species. Aflatoxins, zearalenone, T-2 toxin, ochratoxin A, and sterigmatocystin analyses are required by JECFA for all enzyme products produced by any mold (Patterson and Roberts, 1979).

[j] The NOAEL should provide at least a 100-fold margin of safety for human consumption, calculated using standard methods (Klaassen, 1996; Lehman and Fitzhugh, 1954; ILSI, 1997).

[k] Antibiotic resistance genes are commonly used in the genetic construction of enzyme production strains to identify, select, and stabilize cells carrying introduced DNA. Principles for the safe use of antibiotic resistance genes in the manufacture of food and feed products have been developed (IFBC, 1990; "FDA Guidance for Industry: Use of Antibiotic Resistance Marker Genes in Transgenic Plants," http://vm.cfsan.fda.gov/~dms/opa-armg.html).

[l] In determining safe strain lineage one should consider the host organism, all of the introduced DNA, and the methods used to genetically modify the host (see text). In some instances the procedures described by Pariza and Foster (1983) and IFBC (1990) may be considered comparable to this evaluation procedure in establishing a safe strain lineage.

[m] The issue of pathogenicity is addressed in the text. Unless an enzyme preparation contains live organisms, we do not consider this to be a relevant consumer safety issue.

[n] In this context the term antibiotic refers to antimicrobial substances that are positive in the JECFA test (FAO, 1981).

[o] The toxins of concern for food enzymes are those which are active via the oral route.

FIG. 1—*Continued*

[p] It is important to distinguish between a toxin and a toxic effect. A toxin is a chemical substance that produces a toxic effect when administered to an animal or a human in an amount that is high enough to induce the toxic effect. At lower levels of exposure there may be no adverse effect and no cause for concern (Klaassen, 1995).

[q] In the case of enzymes that lack a history of safe use in foods that are produced via genetically modified production strains, for example, newly isolated enzymes, this issue will have been addressed at step 3b. In these cases it is not necessary to repeat the animal tests again.

[r] In case of a new strain (new isolate) this testing will be fully comprehensive per this decision mechanism. However, with increased knowledge of strain and background (safe strain lineage with a number of products from this lineage tested) the depth of safety testing may be reduced. This is a case-by-case judgment; e.g. Scientific Committee for Foods (SCF) Guidelines, Section 10, also gives exemptions from the basic-full-toxicologic requirements (Scientific Committee for Food, 1992).

FIG. 1—*Continued*

particular applications. Examples include changing the pH optimum, increasing thermal stability, reducing the requirement for cofactors such as metal ions, and stabilizing the enzyme against chemical oxidation.

It may be asked if such modifications might also affect the safety of an otherwise safe enzyme. To address this question we should consider what is known about the natural variation in enzyme structure and function.

The enzymes in Table 1 are listed by their systematic names according to the Nomenclature Committee of the International Union of Biochemistry (i.e., their IUB or EC number) (IUB, 1992) and by their Chemical Abstract Service (CAS) registry number. However, in addition to the traditional IUB scheme which is based mainly on catalytic activity, several databases are available that describe the nucleotide sequences of the genes encoding the enzymes, the corresponding amino acid sequences, and information regarding the three-dimensional structures of enzymes (Brenner *et al.*, 1998; Doolittle, 1996). This information is valuable for determining the evolutionary relationships of enzymes and provides considerable insight into their structure/function determinants. Molecular analyses have demonstrated that enzymes within a given class are composed of characteristic folds that comprise domains within the entire enzyme molecule (Creighton, 1993; Doolittle, 1996; Henrissat and Davies, 1997; Jancek *et al.*, 1999).

Families/superfamilies of enzymes obtained from microorganisms found in diverse habitats retain their general tertiary structure and enzymatic properties (Siezen and Leunissen, 1997; Jancek *et al.*, 1999; Conrad *et al.*, 1995; Todd *et al.*, 1999) but they may differ in certain functional characteristics such as stability and substrate specificity (Creighton, 1993). We know of no instance in which such natural variation within enzyme families has resulted in the generation of a toxin active via the oral route. This also follows from the observation that toxicity is an unusual property among proteins. Pariza and Foster (1983) pointed out that whereas there are thousands of proteins in food, only a tiny fraction exhibit toxigenic potential by the oral route. These known toxigenic proteins differ greatly in structure from commercially available food enzymes.

Extensive studies on engineered enzymes have also demonstrated that enzymes within families/superfamilies (e.g., subtilases) that are altered by these techniques still retain their characteristic three-dimensional structure and catalytic activities (Bott *et al.*, 1992). Hence, engineered enzymes exhibit variation that is similar to that observed in nature.

An examination of enzyme structure and function indicates that it is unlikely that changes which improve upon desired enzyme function will result in the creation of a toxic protein. In our opinion it is prudent to assess this very small theoretical risk by conducting limited toxicological tests on engineered enzymes. We anticipate that when a manufacturer synthesizes a series of products through protein engineering, inserting the engineered gene into the same host with the same vector system and demonstrating through appropriate toxicological testing that each product is safe, there will come a point after which further testing of additional similar products should be considered redundant and unnecessary. The point at which this may occur would be established by independent experts on a case-by-case basis.

These conclusions should be reassessed on a regular basis, as the body of knowledge from such testing grows.

A Word about in Vitro Genotoxicity Testing

Despite the questions raised by Pariza and Foster (1983) regarding the scientific rationale and need for testing new food enzyme preparations for mutagenic activity, the practice continues, driven largely by regulatory requirements in some locales. Accordingly it is worth noting that, to our knowledge, the requirement that new enzyme preparations be tested for *in vitro* genotoxicity has failed to reveal the presence of a single mutagen or clastogen that would not have been detected using the more comprehensive decision tree approach described by Pariza and Foster (1983) and IFBC (1990), which involve analytical chemistry and limited animal feeding tests.

There appear to be three reasons for this, as follows. Proteins, including food-borne enterotoxins and neurotoxins produced by some bacteria, are not genotoxic. All known mycotoxins, some of which are genotoxic, also induce other toxic effects in test animals that are easily determined in short-term feeding tests. There are reliable analytical procedures available for virtually all of the known food-borne protein toxins and mycotoxins, which are used routinely in determining the safety of new production strains.

As of June 1999, members of the Enzyme Technical Association (ETA) reported conducting 102 bacterial mutagenesis tests (Ames *et al.*, 1975; OECD, 1984; EEC, 1992) and 63 chromosome aberration tests on enzyme preparations (Amacher *et al.*, 1980; OECD, 1984; EEC, 1992; Clive and Spector, 1975; Clive *et al.*, 1979, 1987). The chromosome aberration tests included *in vitro* cytogenicity tests in cultured mammalian cells (human peripheral lymphocytes, mouse lymphoma cells, or Chinese hamster ovary cells, for example) and *in vivo* tests in mice to detect damage to the chromosomes or the mitotic apparatus (OECD, 1984; EEC, 1992; Amacher *et al.*, 1980; Clive and Spector, 1975; Clive *et al.*, 1979, 1987). The enzyme preparations were from traditionally and genetically modified production organisms (i.e., 49 Ames tests and 27 chromosome aberration tests were conducted on enzyme preparations from genetically modified microorganisms). The production organisms were: *Actinoplanes missouriensis, Aspergillus melleus, A. niger, A. oryzae, Bacillus alcalophilus, B. amyloliquefaciens, B. licheniformis, B. naganoeneis, B. subtilis, Candida pseudotropicales, C. rugosa, Chaetomium erraticum, Disporotrichium dimorphosporum, Kluyveromces lactis, Leptographium procerum, Microbacterium imperial, Mucor javanicus, Penicillium camembertii, P. citrinum, P. decumbens, P. roqueforti, Pseudomonas alcaligenes, Rhizomucor miehei, Rhizopus niveus, R. oryzae (R. delemar), Streptomyces lividans, Talaromyces emersonii, Trichoderma reesei*, and *Verticidadiella procera*.

Seven of the Ames tests were false positive (6 on enzyme preparations from genetically modified microorganisms) and 6 of the chromosome aberration tests were false positive (2 on enzyme preparations from genetically modified microorganisms). The remainder (95 Ames and 57 chromosome aberration) were negative.

The false-positive results from the Ames tests were demonstrated to be due to the growth-enhancing effects of histidine in the enzyme preparations (this was verified by repeating the assay using the treat and plate method and observing no evidence of mutagenesis).

The false-positive results from the chromosome aberration assays have several explanations, as follows:

—Clastogenicity was observed in human lymphocyte cells; additional *in vitro* studies with Chinese hamster ovary cells were clearly negative (two studies/one from genetically modified microorganism);

—*In vitro* positive results were not confirmed by *in vivo* cytogenetic tests (three studies); and

—Some enzymatic reactions result in the production of hydrogen peroxide, which is known to cause clastogenic aberrations (one study/one from genetically modified microorganism).

These findings underscore the conclusion that testing enzyme preparations from traditional and genetically modified microorganisms for genotoxicity is unnecessary for safety evaluation.

A FOOD ENZYME SAFETY EVALUATION STRATEGY FOR THE 21ST CENTURY

New enzymes with improved properties are now being derived in numerous ways, including genetic modification (protein engineering) (Arnold and Volkov, 1999; Atwell and Wells, 1999; Cleland and Craik, 1996; Ford, 1999; Shaw *et al.*, 1999), protein "breeding" (Minshull and Stemmer, 1999), chemical modification (DeSantis and Jones, 1999), and by isolation from newly explored environments (Adams *et al.*, 1995; Demain and Davies, 1999; Hunter-Cevera, 1998; Madigan and Marrs, 1997;

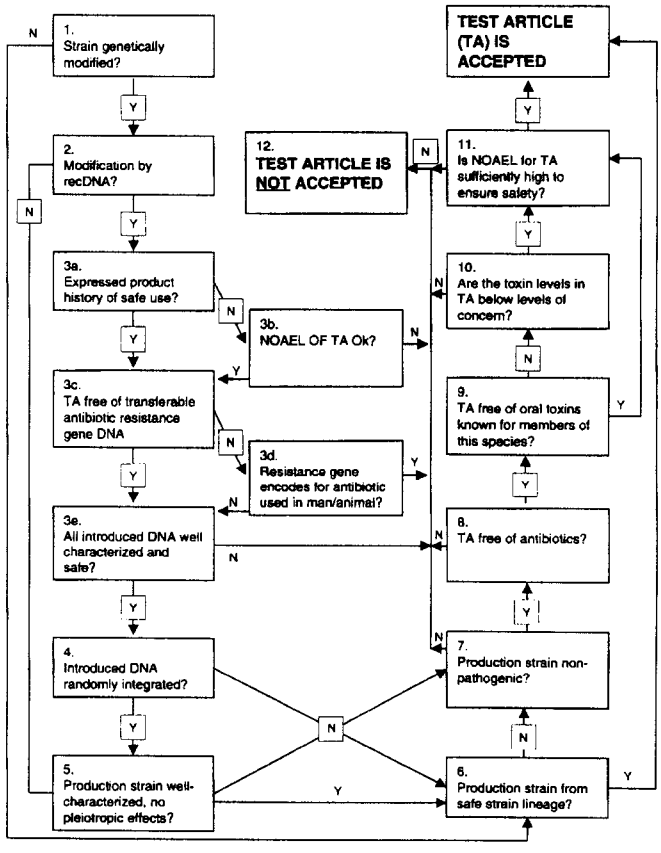

FIGURE 2

Marrs *et al.*, 1999; Pace, 1997). Accordingly, it is important to update previous enzyme safety evaluation mechanisms (Pariza and Foster, 1983; IFBC, 1990) to accommodate these advances in biology. The decision tree shown in Fig. 1 was developed to accomplish this goal. Figure 2 is a schematic representation.

The footnotes to the decision tree contain additional explanation and discussion. It is important to note that under the criteria of the decision tree, no new enzyme can enter the market without critical evaluation of its safety. It is also important to note that along with meeting the criteria in the decision tree, a safe food enzyme should also be produced under current Good Manufacturing Practices and meet or exceed the specifications for food enzymes described in the Food Chemicals Codex (FCC, 1996) and/or FAO/JECFA (JECFA, 1992).

CONCLUSION

Microbial enzymes used in food processing are typically sold as *enzyme preparations* that contain not only a desired enzyme activity but also other metabolites of the production strain, as well as added materials such as preservatives and stabilizers. The added materials must be food grade and meet applicable regulatory standards. In this report we present guidelines that can be used to evaluate the safety of the metabolites of the production strain that are also present in the enzyme preparation, including of course, but not limited to, the desired enzyme activity itself. This discussion builds on previous reports (Pariza and Foster, 1983; IFBC, 1990) and includes consideration of new genetic modification technologies, for example, modifying the primary structure of enzymes to enhance specific properties that are commercially useful.

The safety of the production strain should remain as the primary consideration in evaluating enzyme safety. The primary issue in evaluating the safety of a production strain is its *toxigenic potential*, specifically the possible synthesis by the production strain of toxins that are active via the oral route. *Pathogenic potential* is not usually an area of concern for consumer safety because enzyme preparations rarely contain viable organisms. Pathogenicity is, however, important to worker safety.

Thoroughly characterized nonpathogenic, nontoxigenic microbial strains, particularly those with a history of safe use in food enzyme manufacture, are logical candidates for generating a *safe strain lineage*, through which improved strains may be derived via genetic modification either by using traditional/classical or rDNA strain improvement strategies (IFBC, 1990). The elements needed to establish a safe strain lineage include thoroughly characterizing the host organism, determining the safety of all new DNA that has been introduced into the host organism, and ensuring that the procedure(s) that have been used to modify the host organism are appropriate for food use.

Enzyme function may be changed by intentionally altering the amino acid sequence (e.g., protein engineering). Examples include changing the pH optimum, increasing thermal stability, reducing the requirement for cofactors such as metal ions, and stabilizing the enzyme against chemical oxidation. It may be asked if such modifications might also affect the safety of an otherwise safe enzyme. To address this question we consider what is known about the natural variation in enzyme structure and function, and conclude that it is unlikely that changes which improve upon desired enzyme function will result in the creation of a toxic protein. It is prudent to assess such very small theoretical risks by conducting limited toxicological tests on engineered enzymes.

Despite the questions raised by Pariza and Foster (1983) regarding the scientific rationale and need for testing new food enzyme preparations for mutagenic activity, the practice continues, driven largely by regulatory requirements in some locales. Accordingly it is worth noting that, to our knowledge, the requirement that new enzyme preparations be tested for *in vitro* genotoxicity has failed to reveal the presence of a single mutagen or clastogen that would not have been detected using the more comprehensive decision tree approach described by Pariza and Foster (1983) and IFBC (1990).

The centerpiece of this report is a decision tree mechanism that updates previous enzyme safety evaluation mechanisms (Pariza and Foster, 1983; IFBC, 1990) to accommodate advances in enzymology. Under the criteria of this decision tree, no new food enzyme can enter the market without critical evaluation of its safety.

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