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Food-processing enzymes from recombinant microorganisms—a review ☆

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Abstract

Enzymes are commonly used in food processing and in the production of food ingredients. Enzymes traditionally isolated from culturable microorganisms, plants, and mammalian tissues are often not well-adapted to the conditions used in modern food production methods. The use of recombinant DNA technology has made it possible to manufacture novel enzymes suitable for specific food-processing conditions. Such enzymes may be discovered by screening microorganisms sampled from diverse environments or developed by modification of known enzymes using modern methods of protein engineering or molecular evolution. As a result, several important food-processing enzymes such as amylases and lipases with properties tailored to particular food applications have become available. Another important achievement is improvement of microbial production strains. For example, several microbial strains recently developed for enzyme production have been engineered to increase enzyme yield by deleting native genes encoding extracellular proteases. Moreover, certain fungal production strains have been modified to reduce or eliminate their potential for production of toxic secondary metabolites. In this article, we discuss the safety of microorganisms used as hosts for enzyme-encoding genes, the construction of recombinant production strains, and methods of improving enzyme properties. We also briefly describe the manufacture and safety assessment of enzyme preparations and summarize options for submitting information on enzyme preparations to the US Food and Drug Administration.
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1. Introduction

Enzymes occur in all living organisms and catalyze biochemical reactions necessary to support life. Enzymes are ubiquitous in fresh and processed food and are consumed every day. Like other dietary proteins, enzymes are degraded and metabolized after ingestion. Enzymes naturally present in the human diet have not been associated with toxicity and are considered intrinsically safe.

The industrial production of enzymes for use in food processing dates back to 1874, when Danish scientist Christian Hansen extracted rennin (chymosin) from calves' stomachs for use in cheese manufacturing (Nielsen et al., 1994). Chymosin is now produced from microorganisms that contain the bovine prochymosin gene introduced through recombinant deoxyribonucleic acid (rDNA) techniques. Bovine chymosin expressed in *Escherichia coli* K-12 became the first recombinant enzyme approved for use in food by the US Food and Drug Administration (FDA) (Flamm, 1991).

Many enzymes currently used in food processing are derived from recombinant microorganisms. Enzyme manufacturers take advantage of new genetic techniques to develop and manufacture enzymes with improved properties. Such enzymes often originate from microorganisms that cannot be easily cultured under laboratory or

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industrial conditions. By judicious selection of host microorganisms, recombinant production strains can be constructed to allow efficient production of enzymes that are substantially free of undesirable enzymes or other microbial metabolites.

The increasing sophistication of food processing created a demand for a broad variety of food processing enzymes with characteristics compatible with food processing conditions. For example, commonly used sweeteners such as glucose or fructose syrups are typically produced from corn starch using hydrolytic enzymes. In the first step of starch hydrolysis, starch is liquefied with α-amylase by heating at 105 °C for 2–5 min followed by 1–2 h at 90–100 °C. With the advent of rDNA technology, it became possible to engineer α-amylases with increased heat stability and improved compatibility with other parameters of the liquefaction process. These improvements were accomplished by introducing changes in the α-amylase amino acid sequences through DNA sequence modifications of the α-amylase genes. Other enzymes currently used in food processing have also been improved using rDNA techniques.

Enzymes used in food processing are sold as enzyme preparations. An enzyme preparation typically contains the enzyme of interest and several added substances such as diluents, preservatives, and stabilizers. The added materials are usually well-known substances suitable for use in food. Enzyme preparations may also contain other enzymes and metabolites from the production organism and the residues of raw materials used in fermentation media and during isolation and purification of the enzyme. All these materials are expected to be of appropriate purity consistent with current good manufacturing practice (cGMP).

The safety evaluation of food processing enzymes from recombinant microorganisms has been extensively discussed in the literature (IFBC, 1990; Pariza and Johnson, 2001; Jonas et al., 1996) and in guidance documents issued by regulatory authorities and international organizations, for example, by the Scientific Committee for Food (SCF, 1992). In principle, the same safety considerations apply to enzymes derived from native and recombinant microorganisms. The key component in evaluating enzyme safety is the safety assessment of the production strain, in particular, its pathogenic and toxigenic potential (Pariza and Johnson, 2001). Although neither pathogenic nor toxigenic microorganisms are intentionally used in the production of food-processing enzymes, certain fungi traditionally used as sources of enzymes have been found to produce low levels of toxic secondary metabolites under fermentation conditions conducive to the synthesis of these compounds. Some of these microorganisms are now used as sources of recombinant enzymes.

In this article, we will review information on food-processing enzymes from recombinant microorganisms. We will discuss safety-related characteristics of the host microorganisms, construction of recombinant production strains, and methods of improving enzyme properties. We will also briefly describe the manufacture and safety assessment of enzyme preparations and summarize options for submitting information on enzyme preparations to FDA. We will rely on published sources, nonconfidential documents submitted to the agency, which may be obtained by the public through the Freedom of Information Act, and FDA documents published in the Federal Register or the agency's web site.

2. FDA review of submissions on enzyme preparations

Enzyme preparations can be regulated as secondary direct food additives in Title 21 of the Code of Federal Regulations (21 CFR), section 173. To establish a regulation for an enzyme preparation, a food additive petition must be submitted. In the past, FDA also reviewed generally recognized as safe (GRAS) affirmation petitions for enzyme preparations. A successful review of a GRAS affirmation petition resulted in a regulation in 21 CFR, section 184. The GRAS affirmation process is being replaced with a voluntary notification program under the agency's proposed regulation (Proposed 21 CFR 170.36 (62 FR 18938; April 17, 1997; substances generally recognized as safe (GRAS))). The successful notification process results in an FDA letter to the notifier stating that FDA has no questions regarding the notifier's conclusion that the use of the enzyme is GRAS. The GRAS notification program is described in a recent regulatory review (Gaynor, 2006).

Since 1997, FDA has reviewed more than 35 GRAS notices for enzyme preparations derived from native and recombinant microorganisms. The list of all GRAS notices submitted to FDA, including those for enzyme preparations, can be viewed at the agency's web site (http://www.cfsan.fda.gov/~rdb/opa-gras.html). The site provides links to FDA letters issued at the completion of the notification process.

3. Submissions on enzymes from recombinant microorganisms

In the last two decades, FDA has received a number of petitions and GRAS notices on food-processing enzymes derived from recombinant microorganisms. These enzymes are listed in Table 1 with their source microorganisms and references to FDA regulations, GRAS affirmation petitions, and GRAS notices. Lists of commercial enzymes used in food processing can be found at the web sites of the Enzyme Technical Association (http://www.enzymetechnicalassoc.org) and the Association of Manufacturers and Formulators of Enzyme Products (http://www.amfep.org). The scope of this review is limited to enzymes and source microorganisms listed in Table 1.

4. Steps in the development of recombinant production strains

Industrial production of recombinant enzymes is preceded by an extensive research and development phase that

Table 1
Enzymes from recombinant microorganisms (based on FDA regulations, GRAS affirmation petitions, and GRAS notices)

Source microorganism	Enzyme	Reference[a]
Aspergillus niger	Phytase	GRASP 2G0381
	Chymosin	21 CFR 184.1685
	Lipase	GRN 158
Aspergillus oryzae	Esterase–lipase	GRASP 7G0323
	Aspartic proteinase	GRN 34
	Glucose oxidase	GRN 106
	Laccase	GRN 122
	Lipase	GRN 43; GRN 75; GRN 103
	Pectin esterase	GRN 8
	Phospholipase A1	GRN 142
Bacillus licheniformis	α-amylase	GRASP 0G0363; GRN 22; GRN 24; GRN 79
	Pullulanase	GRN 72
Bacillus subtilis	α-acetolactate decarboxylase	21 CFR 173.115
	α-amylase	GRASP 4G0293; GRASP 7G0328
	Maltogenic amylase	GRASP 7G0326
	Pullulanase	GRN 20
Escherichia coli K-12	Chymosin	21 CFR 184.1685
Fusarium venenatum	Xylanase	GRN 54
Kluyveromyces marxianus var. *lactis*	Chymosin	21 CFR 184.1685
Pseudomonas fluorescens Biovar I	α-amylase	GRN 126
Trichoderma reesei	Pectin lyase	GRN 32

[a] GRASP is an acronym for a GRAS affirmation petition. The GRAS affirmation petitions listed in this Table have not resulted in regulations because the agency initiated the GRAS notification program. Some GRAS affirmation petitions were converted to GRAS notices (GRNs). A list of GRAS notices can be viewed at http://www.cfsan.fda.gov/~rdb/opa-gras.html. 21 CFR means Title 21 of the Code of Federal Regulations. Each reference to 21 CFR includes the regulation number.

culminates in the construction of a successful production strain. This process typically involves the following stages: (1) development of the host (recipient) strain; (2) construction of the expression vector; (3) transformation of the host strain; (4) identification of the best recombinant strain; (5) additional improvements; and (6) characterization of the production strain. Each of these steps is dictated by specific circumstances related to the identity and properties of the host organism and availability of genetic methods suitable for its modification and transformation. These steps will be described below and illustrated with examples.

5. Characteristics of host strains

As shown in Table 1, most host strains used to develop production strains for food-processing enzymes have been derived from a relatively small number of bacterial and fungal species primarily *B. subtilis*, *B. licheniformis*, *A. niger*, or *A. oryzae*. These microorganisms have a long history of use as safe sources of native enzymes and a proven record of efficient growth under industrial production conditions. They are also amenable to genetic manipulations and known for their ability to secrete ample quantities of enzymes into fermentation media. These characteristics make these microorganisms particularly desirable for use as hosts for a variety of heterologous enzymes. Several microorganisms with no history of use in the industrial production of native enzymes, such as *E. coli* K-12, *F. venenatum*, and *P. fluorescens*, have also been successfully utilized as hosts for expression of food-processing enzymes.

Although the majority of host microorganisms used to-date secrete enzymes to the fermentation media, the Gram-negative bacteria *E. coli* K-12 (source of chymosin) and *P. fluorescens* Biovar I (source of α-amylase) accumulate rather than secrete heterologous enzymes. Isolation and purification of the accumulated enzymes generally involve more steps during the production process than purification of the secreted enzymes.

The wild-type strains of several host microorganisms produce a variety of extracellular enzymes. Such enzymes may be carried over to the final enzyme preparation and catalyze undesirable reactions in food. Extracellular proteases are particularly troublesome because they tend to degrade heterologous enzymes. To increase enzyme yield, protease-deficient host strains have been constructed. Other modifications include developing sporulation-deficient mutant strains.

Microorganisms used as hosts for recombinant enzymes listed in Table 1 are recognized as nonpathogenic (i.e., they do not cause disease in healthy humans and animals). It is less clear whether all of these microorganisms should also be described as nontoxigenic. Some of these microorganisms, most notably *A. niger*, *A. oryzae*, and *F. venenatum*, may produce low levels of toxic secondary metabolites under certain cultivation conditions. Pariza and Foster (1983) defined the nontoxigenic organism as one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure. Under this definition, the *A. niger*, *A. oryzae*, and *F. venenatum* production strains are sometimes described as nontoxigenic in submissions to FDA. Most literature sources cited in this review do not refer to these microorganisms as nontoxigenic.

6. Bacterial host strains

6.1. Bacillus subtilis and its relatives

As shown in Table 1, several enzymes important for food processing have been recently derived from recombinant strains of the Gram-positive bacteria *B. subtilis* and *B. licheniformis*. *B. subtilis* has been used for several decades as a source of food-processing and industrial enzymes, mainly α-amylases and proteases. Of particular importance is *B. subtilis* strain 168, a well-known wild-type strain from which numerous strains widely used in research

and industrial applications were developed. Its genome has been recently sequenced (Kunst et al., 1997). Strain 168 is the progenitor of many *B. subtilis* strains that have been used as sources of food-processing enzymes. The safety of recombinant enzymes derived from *B. subtilis* is documented in petitions and GRAS notices submitted to FDA (see Table 1) and in relevant publications (for example, see Zeman and McCrea, 1985; Andersen et al., 1987; MacKenzie et al., 1989; de Boer et al., 1993). Other *Bacillus* species, including *B. licheniformis*, *B. amyloliquefaciens*, and *B. stearothermophilus* (recently reclassified as *Geobacillus stearothermophilus*; Nazina et al., 2001), have also emerged as safe sources of food-processing enzymes, primarily α-amylases. In the last decade, *B. licheniformis* and *B. amyloliquefaciens* have been successfully adapted for use as hosts for expression of recombinant enzymes. Complete genome sequences of two industrial *B. licheniformis* strains were determined and found largely homologous to the sequence of *B. subtilis* but distinct from the sequences of *B. cereus* and *B. anthracis*, which are human pathogens (Ray et al., 2004; Veith et al., 2004). *B. cereus* produces several toxins that cause food poisoning and *B. anthracis* causes anthrax.

The safety of *B. amyloliqueafaciens*, *B. licheniformis*, and *B. subtilis* was discussed in several recent reviews (de Boer and Diderichsen, 1991; de Boer et al., 1994; Pedersen et al., 2002). Pedersen et al. (2002) evaluated the cytotoxic potential of several industrial strains of these species used by Novozymes A/S in the production of enzymes. The strains were tested for cytotoxicity against Chinese hamster ovary K1 (CHO-K1) cells and for production of *B. cereus* hemolytic and nonhemolytic enterotoxins using immunological assays. The tested strains were nontoxic to CHO-K1 cells and did not react with antibodies against *B. cereus* enterotoxins. In addition, the database consisting of the DNA sequences from *B. subtilis* (full sequence) and *B. licheniformis* (96% of all genes), was searched for genes homologous to those encoding known *B. cereus* protein toxins. The search revealed no genes with homology to genes encoding the *B. cereus* protein toxins. Based on these studies, Pedersen et al. (2002) concluded that the tested strains do not produce *B. cereus*-like toxins or any other secondary metabolites with cytotoxic potential and are safe to use.

The safety of *B. subtilis* and *B. licheniformis* was evaluated by the U.S. Environmental Protection Agency. Both microorganisms were exempted from EPA review under the Toxic Substances Control Act (TSCA) (EPA, 1997). *B. subtilis* and related species are also used in the production of industrial enzymes, insecticidal and pharmaceutical proteins, antibiotics, purine nucleotides used as flavor enhancers, and other compounds with food, medical, and industrial applications (Harwood, 1992; Schallmey et al., 2004; Westers et al., 2004).

The wild-type strains of *Bacillus* species sporulate in response to nutrient limitations. While some commercially important compounds, such as *B. thuringiensis* pesticidal proteins, are produced concomitantly with sporulation (Harwood, 1992), the production of food-processing enzymes is hampered by sporulation. Consequently, nonsporulating mutants have been developed for use as host strains for recombinant enzymes.

One advantage of using *Bacillus* species as hosts for large scale production of heterologous enzymes and other proteins is their ability to secrete proteins directly into the fermentation medium (Simonen and Palva, 1993). However, the production of several native extracellular proteases, which tend to degrade secreted heterologous proteins, has been a major problem in using *Bacillus* species in the production of recombinant enzymes. To avoid enzyme degradation by extracellular proteases and to increase enzyme yield, enzyme manufacturers developed protease-deficient mutant strains.

Bacillus strains that are nonsporulating and extracellular protease-deficient are now routinely used as hosts for recombinant enzymes. In some instances, these strains are modified by introducing additional mutations. For example, to avoid the use of an antibiotic resistance marker for selection of cells transformed with rDNA, an auxotrophic mutant can be isolated that requires a specific amino acid (or other nutrient) for growth. The auxotrophic mutation is then complemented by providing an active copy of the mutated chromosomal gene on the transformation vector. The cells that acquire the vector attain prototrophy and regain the ability to grow in a medium without the required nutrient.

6.2. *Escherichia coli K-12*

In 1990, FDA affirmed as GRAS the chymosin enzyme preparation derived from *E. coli* K-12 (21 CFR 184.1685; Flamm, 1991). Chymosin is a milk-clotting enzyme also known as rennin. The FDA action was a milestone in the history of food-processing enzymes, because chymosin was the first recombinant enzyme regulated by the U.S. government for use in cheese and other dairy products. The chymosin production strain contains the bovine prochymosin gene. Prochymosin accumulates within the *E. coli* K-12 cells in the form of inclusion bodies. The cells are subsequently lysed and the inclusion bodies are isolated and solubilized. The prochymosin present in the solution is purified and converted to chymosin by acid treatment.

The safety of chymosin preparation was primarily based on published evidence that *E. coli* K-12 has been used as a laboratory organism for over 30 years without reported incidents of infection and that it does not produce toxins that cause illness by ingestion, such as Shiga-like toxin produced by certain toxigenic strains of *E. coli*. *E. coli* K-12 is one of the most extensively studied bacteria. Its genome was sequenced in 1997 (Blattner et al., 1997). *E. coli* K-12 has a history of safe use in the production of specialty chemicals and human drugs and was exempted from EPA review under TSCA (EPA, 1997).

6.3. *Pseudomonas fluorescens*

FDA recently reviewed a GRAS notice on the thermostable α-amylase from the recombinant *P. fluorescens*

Biovar I strain (GRN 126). Since *P. fluorescens* had no previous history of use as a source of enzymes or other compounds intended for use in food, the sponsor of the GRAS notice, Innovase, provided extensive information on the safety of *P. fluorescens*. The organism is a Gram-negative soil bacterium not known to cause disease in healthy humans. It is ubiquitous in the environment and is likely to have been consumed along with raw fruits and vegetables. The genome of *P. fluorescens* (strain Pf-5) was sequenced (Paulsen et al., 2005).

The wild-type strain MB101 that was used as a host for the recombinant α-amylase, was originally isolated from lettuce grown on a California farm. The strain has been utilized since 1989 for the large scale production of *Bacillus thuringiensis* insecticidal protein (B.t. toxin) for agricultural applications (Landry et al., 2003). Innovase characterized the MB101 strain at the genetic and phenotypic levels, and conducted studies in which mice were exposed to high oral doses of live bacteria. Innovase concluded that strain MB101 is nonpathogenic and is considered safe (Landry et al., 2003). Like *E. coli* K-12, *P. fluorescens* accumulates heterologous proteins intracellularly as inclusion bodies.

7. Fungal host strains

7.1. Aspergillus oryzae and Aspergillus niger

A. oryzae and *A. niger* are filamentous fungi well-known for their use in food production. *A. oryzae* has been used for millennia as a source of *koji* mold used in the production of fermented foods including soy sauce, soybean paste miso, and rice wine sake. *A. niger* is widely used for production of citric acid, which was affirmed by FDA as GRAS (21 CFR 184.1033). Both *A. oryzae* and *A. niger* have a long history of use as sources of enzymes used in baking, brewing, and other food applications. Several enzyme preparations derived from these microorganisms were recognized by FDA as GRAS in opinion letters issued in the early 1960s (http://www.cfsan.fda.gov/~dms/opa-enzy.html). Subsequently, a number of enzyme preparations from both natural and rDNA strains of *A. oryzae* and *A. niger* were regulated in 21 CFR either as secondary direct food additives or GRAS food ingredients, or evaluated under the proposed GRAS notification program. The *A. oryzae* genome sequence was recently determined by the Japanese *A. oryzae* genome consortium (Machida et al., 2005).

Based on the historical uses of *A. oryzae* and *A. niger* in the production of fermented foods, both organisms have traditionally been regarded as nonpathogenic and nontoxigenic, while recognizing that certain strains of both species may produce low levels of toxic secondary metabolites such as mycotoxins. In recent literature, these microorganisms continue to be described as nonpathogenic, but are rarely referred to as nontoxigenic.

The techniques for transformation of *A. niger* and *A. oryzae* with rDNA were pioneered in the late 1980s and early 1990s. Since then, both microorganisms have been successfully used as hosts for expression of heterologous enzymes. The industrial success of *A. oryzae* and *A. niger* prompted new studies and discussions on their capability to produce mycotoxins. We will summarize below recent literature on the toxigenic potential of *A. oryzae* and *A. niger* including methods of avoiding mycotoxin synthesis during industrial fermentations, and genetic modifications aimed at creating mutant strains that can no longer produce mycotoxins.

7.1.1. Safety of A. oryzae

Barbesgaard et al. (1992) reviewed information on the taxonomy, ecology, industrial use, and safety of *A. oryzae*. The authors concluded that *A. oryzae* is not pathogenic in healthy humans and described the microorganism as being a member of the *A. flavus* group that lost the ability to produce certain metabolites, e.g., aflatoxins, through thousands of years of cultivation. Several recent studies showed that certain strains of *A. oryzae*, including some strains used in the production of *koji* molds, contain structural and regulatory genes necessary for aflatoxin biosynthesis. Nevertheless, these strains appeared to be incapable of producing aflatoxins even in aflatoxin-inducing media (Kusumoto et al., 1998a; Kusumoto et al., 1998b; Watson et al., 1999; van den Broek et al., 2001). The molecular analysis of the inactive aflatoxin biosynthesis gene cluster in *A. oryzae* industrial strains revealed the presence of mutations that prevent the expression of certain genes necessary for aflatoxin production (Tominaga et al., 2006).

Certain strains of *A. oryzae* can produce low levels of mycotoxins with low-to-moderate toxicity: 3-β-nitropropionic acid, kojic acid, and cyclopiazonic acid (Barbesgaard et al., 1992; Blumenthal, 2004). Information on the toxicology and occurrence in food of these mycotoxins can be found in review articles by Burdock and Flamm (2000) and Burdock et al. (2001a,b). According to publications dating back to the 1950s and 1960s (references in Blumenthal, 2004), *A. oryzae* may also produce several other secondary metabolites including maltoryzine and violacetin. These metabolites have not been reported to be produced by the *A. oryzae* strains used as sources of enzymes.

EPA evaluated the safety of *A. oryzae* under TSCA for use as a recipient microorganism in the production of industrial compounds such as enzymes used in detergent formulations (EPA, 1997). EPA concluded that "Risks from use of the recipient microorganism *A. oryzae* are low. *A. oryzae* has a long history of commercial use. While some strains of *A. oryzae* are known to produce mycotoxins, these mycotoxins are not highly toxic to humans and their production under usual commercial conditions does not appear to pose a significant risk to human health." Based on this assessment, EPA exempted *A. oryzae* from its review.

7.1.2. A. oryzae host strains with reduced toxigenic potential

Table 1 includes several recombinant enzymes derived from *A. oryzae*. Most of these enzymes are derived from the

A. oryzae production strains that are descendants of the wild type strain IFO 4177, also known as strain A1560. Enzyme preparations derived from strain A1560 or its rDNA derivatives were tested and shown to be safe for their intended uses in food. Strain A1560 is capable of producing low levels of 3-β-nitropropionic acid, kojic acid, and cyclopiazonic acid, especially when cultivated in media conducive to the synthesis of these compounds. Strain A1560 is also known to contain nonfunctional genes related to aflatoxin synthesis (GRN 142). Strain A1560 has been recently modified by site-directed disruption of three endogenous TAKA-amylase genes, one alkaline protease gene, and one neutral metalloprotease gene. The modified strain was subsequently subjected to classical mutagenesis and selection to reduce its toxigenic potential. As a result, a mutant strain was isolated (designated BECh2) from which the aflatoxin gene cluster and the genes involved in the synthesis of cyclopiazonic acid were deleted. The strain also contains a mutation that results in reduced kojic acid production under inducing conditions. The BECh2 strain has been used as a host for expression of triacylglycerol lipase (GRN 103), glucose oxidase (GRN 106), and phospholipase A1 (GRN 142).

7.1.3. Safety of A. niger

A. niger has been used for several decades in the production of citric acid and food-processing enzymes. Citric acid derived from several sources including *A. niger* is regulated as a GRAS food ingredient (21 CFR 184.1033). FDA recognized as GRAS several enzymes from *A. niger* in opinion letters issued in the early 1960s (http://www.cfsan.fda.gov/~dms/opa-enzy.html). Carbohydrase and cellulase from *A. niger* are regulated as secondary direct food additives (21 CFR 173.120) and recombinant chymosin derived from *A. niger* var. *awamori* is regulated as a GRAS food ingredient (21 CFR 184.1685).

The safety of *A. niger* has been re-visited in several recent reviews (Schuster et al., 2002; van Dijck et al., 2003; Blumenthal, 2004). According to these reviews, a relatively small number (3%–10%) of known *A. niger* strains produce the nephrotoxic and carcinogenic mycotoxin ochratoxin A under certain fermentation conditions. Schuster et al. (2002) recommend testing *A. niger* isolates for their potential to produce ochratoxin A at the start of the development of the production process for enzymes used in food processing. Some strains of *A. niger* can also produce other secondary metabolites that are not considered to be mycotoxins, including nigragillin, nigerazine B, malformins (cyclic peptides), naphtho-γ-pyrones, and oxalic acid. The chemical nature and toxicity of these metabolites are discussed by Schuster et al. (2002) and Blumenthal (2004). Certain strains of *A. niger* produce a proteinaceous hemolysin, nigerlysin, when incubated on sheep's blood agar. Purified nigerlysin was toxic to mouse neuronal cells in culture (Donohue et al., 2006). Early reports of aflatoxin production by *A. niger* have not been confirmed by later studies (Schuster et al., 2002).

EPA exempted *A. niger* from its review under TSCA (EPA, 1997). EPA concluded that despite the widespread human exposure to *A. niger*, there are only several reports of aspergillosis with *A. niger* and that *A. niger* is not a significant human pathogen. EPA also concluded that, although *A. niger* is capable of producing several mycotoxins, mycotoxin production appears to be controlled by the conditions of fermentation.

7.1.4. A. niger host strains developed for targeted DNA integration

van Dijck et al. (2003) describe the development of *A. niger* host strains that allow targeted introduction of heterologous genes into the host genome. The intention of these modifications was to address concerns that random DNA integration may perturb metabolic pathways and increase the production of toxic metabolites. The novel host strains were developed from strain DS03043, which was derived from the ancestral *A. niger* strain NRRL3122 using classical genetic methods of mutagenesis and selection. The DS03043 strain was used as a host for expression of heterologous enzymes such as phytase and xylanase derived from other *A. niger* strains. The strain contains seven copies of the glucoamylase-encoding gene *gla*A. All seven copies of the *gla*A gene were deleted from the DS03043 strain and the resulting strain was used to construct several production strains that contain genes encoding desired enzymes integrated at the deleted loci. At each locus, several copies of the intended gene were integrated to achieve high expression of the target enzyme. All these strains were tested under conditions optimal for mycotoxin production and showed a pattern of secondary metabolites similar to that of strain DS03043 and the ancestral strain NRRL3122, which can produce secondary metabolites, such as nigragillin and naphtho-γ-pyrones, under stress conditions. The strains were also tested under conditions representative of large-scale production conditions. None of the secondary metabolites normally produced by the strains under stress conditions were detected either in broth samples or the final enzyme products.

7.1.5. General recommendations for safe use of A. oryzae and A. niger

As discussed above, certain strains of *A. oryzae* and *A. niger* used for enzyme production have the potential for producing mycotoxins or other secondary metabolites with varying degrees of toxicity. The formation of these substances usually occurs under stress conditions and can be avoided by controlling the fermentation process. Several precautionary measures have been discussed in the literature (Schuster et al., 2002; Blumenthal, 2004), which may be summarized as follows: (1) whenever possible, the production strains or host strains should be chosen or developed from strains that have a history of safe use and were examined for their ability to produce mycotoxins under industrial fermentation conditions; (2) new isolates or uncharacterized strains should be thoroughly examined

and tested for mycotoxin production capability before they are developed as production or host organisms; and (3) if a strain has the potential to produce mycotoxins, a control system should be implemented to assure that the mycotoxins do not end up in the enzyme preparation at toxicologically-significant levels; the manufacturing process should be carefully designed, operated, and monitored, and the enzyme preparation should be routinely tested for relevant mycotoxins.

7.2. Fusarium venenatum

In 2001, FDA reviewed a GRAS notice for the xylanase enzyme preparation derived from a recombinant strain of the filamentous fungus *F. venenatum* containing the xylanase gene from *Thermomyces lanuginosus* (GRN 54). The *F. venenatum* strain that was used as a host for expression of xylanase is a descendant of the wild-type strain A3/5. The A3/5 strain was isolated in 1968 from a soil sample in the United Kingdom. It was initially identified as *Fusarium graminearum* Schwabe and deposited in several international culture depositories including the American Type Culture Collection (Manassas, VA, USA) where the strain was designated as ATCC 20334. The A3/5 strain was subsequently reclassified as *F. venenatum* based on morphological, molecular, and mycotoxin data (O'Donnell et al., 1998; Yoder and Christianson, 1998).

F. venenatum has been used as a source of mycoprotein, a protein-rich product sold for use in food in the U.K. since 1985 under the trade name "Quorn." In recent years, mycoprotein has also been commercialized in several European countries (Wiebe, 2002) and the US. In 2001, FDA reviewed a GRAS notice submitted by the manufacturer of mycoprotein, Marlow Foods, Inc. (UK) notifying the agency that, based on the opinion of qualified experts, mycoprotein is generally recognized as safe for use in food as a meat replacer (GRN 91). Mycoprotein is currently manufactured from the *F. venenatum* strain deposited in ATCC under the Accession No. PTA-2684. The mycoprotein production strain PTA-2684 and the previously deposited strain ATCC 20334 are both derived from the original isolate A3/5.

F. venenatum is not known to be pathogenic. However, studies on secondary metabolite profiles conducted with strain A3/5 and its derivatives revealed that *F. venenatum* is capable of producing the mycotoxins, trichothecenes, culmorins, and fusarins, and a cyclic peptide enniatin B under conditions optimized for the production of these compounds (O'Donnell et al., 1998; Miller and MacKenzie, 2000; Song et al., 2004; GRN 54). Mycotoxin production by *F. venenatum* can be avoided by controlling the fermentation conditions. For example, mycoprotein is manufactured under conditions that are not conducive to mycotoxin synthesis (Johnstone, 1998). To confirm the absence of mycotoxin production, mycoprotein is regularly tested for representative trichothecenes and fusarins (GRN 91).

The *F. venenatum* strain used for production of *T. lanuginosus* xylanase was derived from strain MLY3 (GRN 54). The MLY3 strain is a spontaneous mutant of *F. venenatum* strain CC1-3 which, in turn, is a morphological mutant that arose spontaneously during mycoprotein production. Thus, the MLY3 strain is a descendant of the original strain A3/5. Although the MLY3 strain has been shown to produce a trichothecene diacetoxyscirpenol, it was modified during the construction of the xylanase production strain to delete the *tri5* gene encoding trichodiene synthase, the enzyme that catalyzes the first step in the trichothecene biosynthetic pathway (GRN 54; Royer et al., 1999). The resulting xylanase production strain LyMC4.B was tested for mycotoxin production and confirmed to have lost the capacity of producing trichothecenes. However, the strain produced low levels (μg/l) of culmorins and trace levels of enniatin B under conditions inducing the synthesis of these compounds (Miller and MacKenzie, 2000; GRN 54). Although fusarin C may also be produced by strain LyMC4.B under inducing conditions, there was no indication of its synthesis under industrial fermentation conditions used in xylanase production (GRN 54).

Culmorins are structurally related sesquiterpenes found in grains contaminated by *Fusarium graminearum* and related fungi. They are not mutagenic in the Ames test and show either no or low toxicity in other studies (Pedersen and Miller, 1999). Fusarin C has not been thoroughly studied, because it is labile upon exposure to light and heat. Published toxicological studies show that fusarin C is a mutagen but its carcinogenic potential is unclear (Gelderblom et al., 1984, 1986; Lu and Jeffrey, 1993; IARC, 1993).

Enniatin B is one of several enniatins produced by certain species of the genus *Fusarium*. Enniatins are cyclic peptides that exhibit antibacterial and insecticidal activities. Very few studies on mammalian toxicity of enniatins have been performed. The existing data indicate that the toxicity of enniatins to higher animals is low (GRN 54 and references therein).

Based on its low toxigenic potential, lack of toxin production under industrial fermentation conditions, and well-characterized inserted DNA, strain LyMC4.B was considered to be a safe source of xylanase (GRN 54). The safety of the xylanase preparation was evaluated and confirmed (Pedersen and Broadmeadow, 2000). *F. venenatum* has also been used for expression of other enzymes including serine carboxypeptidase from *A. oryzae* (Blinkovsky et al., 1999), aminopeptidase from *A. oryzae* (Blinkovsky et al., 2000), glucoamylase from *A. niger* (Gordon et al., 2001), and lactose oxidase from *Microdochium nivale* (Ahmad et al., 2004). Of all these enzymes, only xylanase and lactose oxidase were derived from the production strains that do not contain the trichodiene synthase gene.

7.3. Kluyveromyces marxianus var.lactis

In 1992, FDA affirmed as GRAS the chymosin enzyme preparation derived from a genetically modified strain of

yeast *K. marxianus* var. *lactis* (21 CFR 184.1685). *K. marxianus* var. *lactis* strain SL56 was used as a host for the bovine prochymosin gene because of its well-characterized fermentation properties and its ability to secrete high levels of prochymosin to the fermentation medium (van den Berg et al., 1990). Prochymosin is converted to active chymosin by pH adjustment.

K. marxianus var. *lactis* was once known as *Saccharomyces lactis* and subsequently as *Kluyveromyces lactis*. The current classification, *K. marxianus* var. *lactis*, was established in 1984 (van der Walt and Johannsen, 1984). The safety of *K. marxianus* var. *lactis* is well-documented. The organism has been used for many years as a source of lactase used for conversion of lactose to galactose and glucose in milk and milk products. The lactase enzyme preparation from *K. lactis* was affirmed by FDA as GRAS (21 CFR 184.1388) in 1984. The agency reviewed the safety of *K. marxianus* var. *lactis* in relation to the regulations for lactase and chymosin enzyme preparations and concluded that the organism is nonpathogenic and nontoxigenic and is a safe source of both enzyme preparations.

7.4. Trichoderma reesei

T. reesei is a filamentous fungus well-known as a source of enzymes that hydrolyze cellulose and hemicellulose. *T. reesei* has also emerged as a host organism for expression of heterologous enzymes. FDA has reviewed a GRAS notice on the pectin lyase preparation from a *T. reesei* strain containing the pectin lyase gene from *A. niger* var. *awamori* (GRN 34).

T. reesei was first isolated from cotton canvas in the Solomon Islands in 1944 (Kuhls et al., 1996). The original isolate, QM6a, is the parent of practically all *T. reesei* industrial production strains (Nevalainen et al., 1994). During the 1980s, *T. reesei* was considered to be identical to *Trichoderma longibrachiatum*. More recent studies suggest that *T. reesei* is the asexual form of a tropical fungus, *Hypocrea jecorina* (Kuhls et al., 1996).

In 1999, FDA affirmed as GRAS the cellulase enzyme preparation derived from a nonpathogenic and nontoxicogenic strain of *T. longibrachiatum* (now known as *T. reesei*) (21 CFR 184.1250). Cellulases from *T. reesei* have been used safely in food, animal feed, and pharmaceuticals since the 1960s (reviewed in Nevalainen et al., 1994). Major food applications of *Trichoderma* cellulases include baking, malting, and grain alcohol production. A comprehensive review of *Trichoderma* enzymes used in food and feed can be found in a review by Gallante et al. (1998). Penttilä et al. (2004) reviewed the molecular biology of *Trichoderma* and various aspects of its use as a host for production of heterologous enzymes. Watts et al. (1988) reported that a strain of *T. reesei* produced two antifungal metabolites, one of which was identified as trichodermin, a trichothecene mycotoxin. However, Blumenthal (2004) cites another opinion (van Dijck, personal communication) that among *Trichoderma* species, the only producer of trichodermin is a strain from *T. harzianum*.

8. Construction of recombinant production strains

8.1. Expression vectors

Genes encoding recombinant enzymes are typically introduced into host strains using expression vectors. An expression vector is a DNA plasmid that carries the expression cassette. Essential components of the expression cassette include a promoter, the gene encoding the desired enzyme, and a terminator. The promoter and terminator are regulatory sequences that control the transcription of the enzyme-encoding gene. Expression vectors also contain DNA derived from bacterial plasmids. Generally, well characterized, commercially-available plasmids are used to construct specific expression vectors. Most commonly used plasmids are pUB110, pUC18, and pUC19.

Plasmid pUB110 was originally isolated from *Staphylococcus aureus* (Keggins et al., 1978) and was subsequently sequenced by McKenzie et al. (1986, 1987). The plasmid replicates in *B. subtilis* and is used in the construction of expression vectors for production of enzymes in *B. subtilis* and other *Bacillus* species. The plasmid carries the *kan*^{r} (kanamycin or neomycin resistance) gene, also known as the *neo* or *nptII* gene, and the *ph1* (phleomycin resistance) gene. The plasmid also carries two other genes, one encoding the primary replication initiation protein (ORF alpha) which initiates the copying of the plasmid, and the other encoding the mobilization protein (ORF beta), which enables the mobilization of the plasmid for transfer from one *Bacillus* strain to another (Selinger et al., 1990). The mobilization gene is routinely deleted during construction of the expression vectors to avoid vector instability. The *kan*^{r} and *ph1* genes are useful as selectable markers during construction of the transformation vector. However, they are not always carried over to the final expression vector. Most expression vectors contain either the *kan*^{r} or the p*h*1 gene and some expression vectors contain neither gene. In the latter case, bacterial transformants are selected either on the basis of enzyme activity or the complementation of an auxotrophic mutation in the host strain.

The pUC18 and pUC19 plasmids were developed for cloning in *E. coli* (Yanish-Perron et al., 1985). They contain an origin of replication active in *E. coli* and the *amp*^{r} gene (also known as the *bla* gene) that confers ampicillin resistance. The pUC18 and pUC19 plasmids are used in the construction of certain bacterial and fungal expression vectors in *E. coli* using the *amp*^{r} gene as a selectable marker. Because the *amp*^{r} gene is under the control of a bacterial promoter and is not expressed in fungal hosts, fungal expression vectors must also contain a selectable marker gene suitable for selection in fungi. A commonly used selectable marker is the *amdS* gene (see Section 8.3 for more information). In recently developed fungal expression vectors the *amp*^{r} gene has been replaced with the *URA3*

gene from *Saccharomyces cerevisiae* (baker's yeast), which complements the *pyrF* mutation in the *E. coli* strain used as an intermediate host for vector construction (see Section 8.3 for more information).

There are other bacterial plasmids (described in the literature and petitions or GRAS notices submitted to FDA) that have been utilized in the construction of expression vectors for production of recombinant enzymes. In some instances, fragments derived from two or more plasmids are combined. Most expression vectors carry their own selectable marker gene. However, in some instances, two separate vectors are constructed; one carries the expression cassette, while the other carries the selectable marker gene. In such a case, the host strain is co-transformed with both vectors.

An interesting plasmid system was developed for inducible production of a thermostable α-amylase in a Gram-negative bacterium, *Pseudomonas fluorescens* (Richardson et al., 2002; GRN 126). The expression vector carries the α-amylase gene under the control of an inducible *tac* promoter. The auxiliary plasmid carries the *lacI* gene from *E. coli*. The *lacI* gene encodes the LacI repressor protein, which binds to the *tac* promoter and inhibits α-amylase expression. Only after the desired cell growth has been achieved is production of α-amylase induced by addition to the fermentation medium of the lactose analog isopropyl-thio-β-D-galactopyranoside (IPTG), which prevents binding of the LacI repressor to the *tac* promoter.

Bacterial expression vectors may be designed either for integration into the host chromosome or for extrachromosomal (autonomous) replication. Autonomously replicating bacterial expression vectors contain origins of replication compatible with the host bacterium and replicate at multiple copies per cell to assure high production of the target enzyme. Expression vectors used for enzyme production in yeasts and filamentous fungi are usually designed for integration into the host genome. Most frequently, the complete expression vector is integrated into the fungal host genome. Alternatively, the expression vector is cut with a restriction enzyme and a vector fragment containing the expression cassette and a selectable marker gene is transformed into the host.

In recent years, techniques for integration of vector DNA at multiple loci within the host genome have been developed. In such a case, the DNA intended for integration contains DNA sequences homologous to the DNA sequences of a host gene. Depending on the specific host/vector combination, vector DNA that carries either both the expression cassette and a selectable marker gene or only the expression cassette is used for transformation. The expression cassette may be directed into predetermined loci in the host genome and may either replace a dispensable host gene or integrate in the vicinity of the host gene without affecting its function. An example of targeted integration at several loci is the construction of a *B. licheniformis* strain for production of the thermostable α-amylase. Three copies of the gene encoding thermostable α-amylase flanked with appropriate DNA sequences homologous to the host DNA were introduced into the host strain by homologous recombination at the *amyL* (α-amylase), *xyl* (xylose isomerase), and *gnt* (gluconate permease) loci. The introduced DNA sequences contained only the α-amylase gene and *B. licheniformis* chromosomal DNA sequences (GRN 79).

8.2. Expression cassettes

The expression cassette is the essential genetic element that must be present in the production microorganism. The simplest expression cassette carries the gene of interest placed under the control of regulatory sequences, the promoter and terminator. Heterologous genes are usually placed under the control of promoters and terminators derived from genes native to the host microorganism or related species. For example, bacterial genes encoding enzymes expressed in Bacilli are usually placed under the control of regulatory sequences derived from *Bacillus* species. Likewise, fungal genes are placed under the control of fungal promoters and terminators derived either from the host species or closely related species.

Promoter strength is essential for achieving efficient expression of the target enzyme. Examples of strong promoters used in *Bacillus* species include promoters of the *amyL* (*B. licheniformis* α-amylase) gene and *amyM* (*B. stearothermophilus* maltogenic amylase) gene (GRN 79; GRASP 7G0326). Heterologous genes expressed in *A. oryzae* are frequently placed under the control of the TAKA amylase promoter from *A. oryzae* (Christensen et al., 1988; GRN 34; GRN 43; GRN 122). Increasing knowledge about promoter function has recently led to the development of improved promoters by introducing mutations into promoter sequences, or by fusing sequences derived from two or more promoters and creating tandem promoters or hybrid promoters. An example of a modified promoter is the Pna2/TPA promoter used for expression of several enzymes in *A. oryzae* and *A. niger* (GRNs 75, 103, 106, and 158). The Pna2/TPI promoter is the neutral amylase II promoter from *A. niger*, in which the 5′ nontranslated part has been replaced with the corresponding part of the *A. nidulans* triose phosphate isomerase (TPI) promoter. An even more complex promoter was developed for expression of phospholipase A1 in *A. oryzae* (GRN 142). It consists of a modified Pna2 promoter from which the TATA box has been removed, and the Pna2/TPI promoter described above. In some instances, it is advantageous to use inducible promoters that are activated by the addition of an inducer to the fermentation medium. An example of an inducible promoter that controls the expression of the thermostable α-amylase gene in *P. fluorescens* is described in Section 8.1.

8.3. Transformation and identification of transformed cells

The technique used to transfer vector DNA into host cells depends on the properties of the host and the nature of

the expression vector. For enzyme production, bacterial hosts have been transformed with vector DNA using conjugation (cell-to-cell contact, in which protein pili on cell surfaces mediate direct transfer of DNA), electroporation, DNA uptake by competent cells (known as bacterial transformation), or vector incubation with protoplasts, i.e., cells from which cell walls were chemically removed. Yeasts and filamentous fungi are usually transformed by incubating DNA with protoplasts.

As discussed in Section 8.1, most expression vectors used in enzyme production contain one or more genes that enable selection of transformed cells. Selection of bacterial transformants is usually conducted using either antibiotic resistance selectable marker genes or by complementing a chromosomal auxotrophic mutation with the functional gene provided on the expression vector. In some instances, antibiotic resistance markers are deleted in the final construction steps of bacterial expression vectors. In such cases, selection is conducted by screening microbial colonies for enzyme activity (GRASP 4G0293). Positive selection methods are used when a gene function is restored as a result of DNA integration. For example, the α-amylase activity is restored in the α-amylase-deficient host strain transformed with a recombinant α-amylase gene (see, for example, GRN 79).

Most expression vectors used to-date for production of enzymes in Bacilli contain the *kan*^{r} gene which encodes an enzyme, aminoglycoside 3′-phosphotransferase II, known either as APH(3′)II or NPTII. APH(3′)II catalyzes the phosphorylation of kanamycin or neomycin thereby inactivating these antibiotics. APH(3′)II has often been used as a selectable marker in the development of bioengineered plants. The safety of the APH(3′)II protein has been discussed in numerous publications including a recent review by Goldstein et al. (2005). FDA issued a regulation for use of the APH(3′)II protein in the development of genetically modified cotton, oilseed rape, and tomatoes (21 CFR 173.170).

Other antibiotic resistance markers used in enzyme production include the *amp*^{r} gene and the *tet* gene. The *amp*^{r} gene was used for selection of transformants during construction of the *E. coli* K-12 strain for production of bovine chymosin affirmed by FDA as GRAS (21 CFR 184.1685). The *amp*^{r} gene encodes β-lactamase, which catalyzes the hydrolysis of penicillin antibiotics including ampicillin. The *tet* (tetracycline resistance) and *kan*^{r} genes were used as selectable markers in the construction of the *P. fluorescens* strain for production of the thermostable α-amylase (GRN 126). The *tet* gene confers resistance to tetracycline and encodes a membrane-bound protein that pumps tetracycline out of the microbial cell. Antibiotic resistance genes, including those used as selectable markers in the production of enzymes, are widely spread among bacteria.

Methods of manufacturing enzymes include steps to prevent carryover of intact copies of antibiotic resistance genes to the final enzyme products. For example, enzymes secreted to the fermentation broth are separated from microbial cells. Enzymes that accumulate within bacterial cells as inclusion bodies (e.g., chymosin expressed in *E. coli* or α-amylase expressed in *P. fluorescens*) are isolated from the microbial mass and processed to hydrolyze any DNA that may have been released from the production strain (GRASP 8G0337; GRN 126). Enzyme manufacturers often test enzyme preparations for the presence of antibiotic resistance genes using either a DNA transformation assay or assessing the size of the DNA fragments. The results of these tests were provided in submissions to FDA and showed that all tested enzyme preparations contained neither transformable DNA nor full-size copies of the antibiotic resistance genes.

Several selectable markers suitable for use in filamentous fungi are described in the literature (see, for example, Howe, 1995). The selection system based on the *amdS* gene isolated from *Aspergillus nidulans* is favored by enzyme manufacturers because fungi commonly used as hosts, such as *A. niger* and *A. oryzae*, lack an endogenous *amdS* gene. The *amdS* gene encodes acetamidase, an enzyme that catalyzes the hydrolysis of acetamide to acetic acid and ammonia, which the fungus uses as carbon and nitrogen sources, respectively. Consequently, the fungal cells transformed with an expression vector containing the *amdS* gene can grow on acetamide as a sole nitrogen source. The *amdS* gene was initially shown to function as a selectable marker in *A. niger* (Kelly and Hynes, 1985) and subsequently in *A. oryzae* (Christensen et al., 1988). More recently, the *amdS*-based selection system has also been developed for *F. venenatum* (Royer et al., 1995).

Fungal expression vectors often contain the *amp*^{r} gene used for selection in *E. coli*. This marker gene is under the control of a bacterial promoter and is, therefore, not expressed in fungal production strains. As noted in Section 8.1, new fungal expression vectors have been developed without the *amp*^{r} gene (GRN 103; GRN 142; GRN 158). These vectors contain the *URA3* gene from *S. cerevisiae* for selection in *E. coli* and the *amdS* gene for selection in fungi. The *URA3* gene encodes orotidine 5′-monophosphate decarboxylase, the enzyme that catalyzes the last step in the pyrimidine biosynthetic pathway. The *URA3* gene complements the *pyrF* mutation in the *E. coli* strain used as an intermediate host for the construction of the expression vector. The *pyrF* mutant requires uridine for growth. This requirement is alleviated in the *E. coli* transformants carrying the *URA3* gene provided on the expression vector. The *URA3*-based selection system allows the construction of fungal production strains without the use of antibiotic resistance genes.

9. Sources of recombinant enzymes

Recombinant enzymes can be derived from a variety of sources including microorganisms, plants or animal tissue. They are often identical to well-known enzymes with a long history of use in food. For example, chymosin derived from recombinant strains of *E. coli* K-12, *K. marxianus* var.

lactis, and *A. niger* var. *awamori* is identical to that present in animal-derived rennet (21 CFR 184.1685).

Most recombinant enzymes currently used in food are derived from well-characterized culturable microorganisms. However, the development of modern highly-efficient screening techniques facilitated discovery of new enzymes from microorganisms sampled from the environment. In this approach, DNA is isolated directly from the environmental samples and used to create expression libraries in *E. coli* or other suitable expression hosts. The expression libraries are then screened to identify enzymes with desired characteristics (Short, 1997). Modern PCR techniques are used to limit the DNA intended for introduction into the host organism to the sequence encoding the desired enzyme. This approach precludes the transfer of any extraneous or unidentified DNA from the donor organism(s) to the production strain.

Thermophilic enzymes with optimized properties are important in baking and starch processing. Several genes encoding such enzymes, including thermostable α-amylases and xylanases, have been isolated from thermophillic microorganisms and expressed in heterologous production strains. Enzyme properties may also be adapted to specific use conditions by using modern genetic techniques. Site-specific mutagenesis can be used to introduce specific changes in the amino acid sequence of the enzyme. Site-specific mutagenesis is most effective when the three-dimensional structure of the enzyme is known and the relationships between structure and enzyme properties have been elucidated.

In recent years, a powerful approach for improving enzyme properties known as molecular or directed evolution has been developed. The process of molecular evolution consists of several steps, often performed in an iterative manner. In the first step, one or several "parent" genes are chosen. If several genes are used, they are often derived from diversified sources to provide sequence diversity. These genes are subsequently mutagenized in a random manner using techniques such as an error-prone PCR mutagenesis, sequential random mutagenesis, or gene shuffling to create a large number of gene variants. A library of altered genes is then constructed in a suitable host microorganism. The clones are screened using high-throughput methods to identify those expressing improved enzymes. Genes encoding these enzymes are isolated, sequenced and usually recycled through the process until an enzyme with the desired characteristics is identified. Extensive information on directed enzyme evolution is available in published sources such as at F. Arnold's web page (http://www.cheme.caltech.edu/groups/fha/) or in recent reviews, for example, Kirk et al. (2002), van Beilen and Li (2002), Roodveldt et al. (2005), and Yuan et al. (2005).

FDA reviewed several GRAS notices on enzymes improved by using either one or a combination of modern genetic techniques. For example, the α-amylase from *B. licheniformis* present in the mixed carbohydrase and protease enzyme preparation affirmed as GRAS in 1983 (21 CFR 184.1027), was modified by replacing its amino terminus with the corresponding sequence from a *B. amyloliquefaciens* α-amylase and by introducing five additional amino acid substitutions. The modified enzyme is thermostable, active at low pH, and does not require added calcium when used for starch hydrolysis in the production of high fructose corn syrup and other similar sweeteners. The enzyme is produced from a recombinant strain of *B. licheniformis* (GRN 22). Another α-amylase with similar characteristics was developed using molecular evolution from three α-amylases discovered in nature. The microorganisms that produce these α-amylases were identified as belonging to the order Thermococcales within Archaea (previously known as Archaebacteria). The hybrid α-amylase is produced from *P. fluorescens* Biovar I and is intended for use in starch processing and fermentation of ethanol for alcoholic beverages (GRN 126; Richardson et al., 2002; Landry et al., 2003) (see Section 6.3).

10. Fine tuning of the production strain

Recombinant production strains can be further improved using classical mutagenesis. Fungal expression vectors may integrate into the host genome at different loci and various copy numbers. Consequently, the transformation procedure yields a population of transformants that produce different levels of the intended enzyme. These transformants are subsequently grown under different conditions and assessed for enzyme expression and other characteristics. Once a satisfactory transformant is identified, it can be subjected to mutagenesis using either a chemical mutagen or UV or ionizing radiation. Subsequently, the population of mutants is screened for enzyme yield to identify the best performer (Novozymes, 2005). For example, the production of laccase from *Myceliophthora thermophila* expressed in *A. oryzae* was increased by chemical mutagenesis and selection (GRN 122; WHO, 2004).

11. Fermentation and processing

Microbial enzymes, whether native or recombinant, are manufactured by controlled fermentation of the production strains. In most instances, the fermentation is conducted as a batch process in large-scale aerated fermenters under strictly controlled fermentation parameters such as temperature, pH, and aeration. The culture is periodically tested to assure the absence of microbial contaminants. Fermentation media contain nutrients and compounds that facilitate the fermentation process. Commonly used media components include dextrose, corn steep liquor, starch, soybean meal, yeast extract, ammonia, urea, and minerals such as phosphates, chlorides or carbonates. Other components may include antifoaming agents and acid or alkali for pH adjustment. For optimal production, the composition of the fermentation medium must satisfy the nutritional requirements of the production strain.

Most recombinant enzymes manufactured today are secreted to the fermentation medium. After the fermentation has been completed, the fermentation broth is separated from the cellular debris using flocculation and filtration. The enzyme is subsequently concentrated by ultrafiltration or a combination of ultrafiltration and evaporation. Enzymes that accumulate within cells are isolated from the cellular mass, solubilized, and concentrated. The enzyme concentrate is then sterilized by germ filtration and formulated with compounds such as sucrose, maltose, maltodextrin, potassium sorbate, or sodium benzoate. For certain food applications, enzymes may also be formulated as granulates or tablets or immobilized using solid support materials. The final product is commonly referred to as an enzyme preparation. Enzyme preparations may contain, in addition to the formulating aids and the enzyme of interest, metabolites derived from the production microorganism or compounds used in fermentation and processing.

12. Assessment of the enzyme preparation

Enzymes are used in food processing at very low levels. Often, they are either not carried over to food as consumed or are inactivated during cooking or baking. Exposure to enzyme preparations used in food processing is typically calculated on the basis of total organic solids (TOS). TOS includes the enzyme itself as well as other organic material that originated from the production organism and enzyme processing. Enzyme preparations are tested according to generally accepted procedures discussed in several publications and guidance documents (IFBC, 1990; SCF, 1992; Pariza and Johnson, 2001). The test material is usually the concentrated enzyme before formulation. The final formulated enzyme product is assessed for compliance with specifications established for enzyme preparations by the Food Chemicals Codex (FCC, 2004) and JECFA (2001).

Some enzyme preparations derived from recombinant microorganisms were tested for the presence of transformable DNA (i.e., DNA that can be taken up by competent bacteria) encoding antibiotic resistance markers. So far, no such DNA was detected in enzyme preparations described in dossiers submitted to FDA. The number of enzyme preparations derived from recombinant strains that do not contain antibiotic resistance markers is increasing. Such enzyme preparations are not expected to be tested for transformable DNA encoding antibiotic resistance genes.

13. Conclusions

Enzymes found in nature have been used in the production of fermented foods for millennia. The production of enzyme preparations isolated from natural sources dates back to the late 19th century. The developments in molecular genetics and cell biology in the last four decades have reshaped enzyme production. It became possible to clone genes encoding enzymes and express them in host microorganisms that are well-adapted to large-scale industrial fermentation. Enzyme yield could be substantially increased by using efficient promoters and introducing multiple copies of the enzyme-encoding gene. It also became possible to tailor enzyme properties to food-processing conditions such as temperature or pH. This has been accomplished by modifying the amino acid sequence of the enzyme using either rational design or molecular evolution. As examples, we described α-amylases that were modified for increased thermostability to match the conditions of starch hydrolysis during production of sweeteners from corn.

The safety of enzyme production strains continues to be a focus of attention. The concept of developing safe strain lineages using well-characterized nonpathogenic, nontoxigenic microbial strains, particularly those with a history of safe use in food enzyme manufacture (Pariza and Johnson, 2001) has been embraced by the enzyme industry. Industrial strains of microorganisms used as sources of native enzymes are now often used as hosts for heterologous enzymes.

It has been recognized that certain fungi traditionally used as sources of food-processing enzymes and considered to be nonpathogenic and nontoxigenic, for example, *A. oryzae* or *A. niger*, are capable of producing low levels of certain mycotoxins under fermentation conditions conducive to the synthesis of these compounds. Mycotoxin production by these fungi can be minimized or avoided by controlling the fermentation conditions used in the production of enzymes. Advances in genetics and molecular biology enriched the knowledge about these microorganisms and provided new tools for reducing their toxigenic potential. Some fungal host strains described above have been altered using classical mutagenesis and/or rDNA methods to inactivate or impair their mycotoxin synthetic pathways. Most effective is the inactivation of the entire pathway by deletion or disruption of genes encoding key enzymes involved in mycotoxin synthesis. For example, the *F. venenatum* strain, from which the trichodiene synthase gene was deleted, lost the capacity to produce trichothecene mycotoxins.

Another interesting approach relies on avoiding potential unintended effects of DNA insertion, such as an increase in the levels of secondary metabolites, by targeting the cloned genes into designated chromosomal loci in the host genome. Once it has been shown that the DNA insertion does not affect secondary metabolite pathways, it is assumed that other cloned genes can be safely inserted into the same locus without triggering unintended effects. This strategy is useful for host strains that naturally produce secondary metabolites at toxicologically insignificant levels.

Microbial host strains have also been genetically modified to improve enzyme production. For example, several bacterial and fungal species used as sources of food-processing enzymes naturally produce extracellular proteases that may degrade the target enzyme. Over the years, classical mutagenesis has been effectively employed to generate protease-deficient mutants of these microorganisms. However, upon the identification of genes encoding proteases, it

became possible to generate strains deficient in specific proteases.

Current strain improvement strategies have already contributed to creating more efficient and safer enzyme production strains. This trend will undoubtedly continue as the knowledge about the genetic make-up of microorganisms used for enzyme production expands and new genetic techniques emerge.

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