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On the safety of a new generation of DSM *Aspergillus niger* enzyme production strains

Piet W.M. van Dijck,[a,*] Gerard C.M. Selten,[b] and Rixta A. Hempenius[a]

[a] *DSM Food Specialties, Department of Regulatory Affairs, P.O. Box 1, 2600 MA Delft, Netherlands*
[b] *DSM Food Specialties, Department of Genetics, P.O. Box 1, 2600 MA Delft, Netherlands*

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Abstract

Consumers safety of enzyme preparations is determined by three variables: the producing organism, the raw materials used in the production, and the production process itself. The latter one is embedded in current Good Manufacturing Practice (cGMP) and Hazard Analysis of Critical Control Points (HACCP); therefore the safety focus can be directed to raw materials and the producing organism. In this paper, we describe the use of novel genetically modified strains of *Aspergillus niger*—made by a design and build strategy—from a lineage of classically improved strains with a history of safe use in enzyme production. The specifics of the host strain allow for integration and over-expression of any gene of interest at a targeted integration site implying that the rest of the host genome is not affected by this integration. Furthermore due to the fact that the newly integrated gene copies are put under the genetic regulation of the host's own glucoamylase promoter, the recipe of the production process of any new production strain can be kept constant with respect to the raw materials composition. Consequently the safety of a new enzyme product from these novel genetically modified strains is determined by the background of the production organism. The use of a strain with a history of safe use and targeted integration according to the concept described above has consequences for the safety studies on the final product. If a known enzymatic activity is over-expressed the safety of a new enzyme preparation is covered by the results of the safety studies performed for other strains from this specific *Aspergillus niger* strain lineage. In this paper an overview is given on the available toxicity tests with these strains. We conclude that for new enzyme products produced with strains from this lineage using the design and build technology no new sub-acute/chronic oral toxicity studies are needed. This also has the benefit that no longer test animals are needed to demonstrate the safety of products produced by these strains.

Keywords: Enzyme safety; Micro-organism; *Aspergillus niger*; Genetic engineering; Secondary metabolites

1. Introduction

Microbial enzymes are since many decades used in many food processing and animal feed applications. Although they are sold for a specific enzymatic function they seldom are pure enzyme products. The same holds for enzyme preparations derived from plant or animal origin. Microbial enzymes may contain other enzymatic proteins, metabolites from the production organism, remnants of the fermentation raw materials as well as added materials such as preservatives and stabilizers.

Enzymes in general are consumed in large amounts in fresh as processed foods such as meat, eggs, nuts and grains, dairy products, fruits, and vegetables. Enzymes are proteins and upon ingestion are degraded to peptides and amino acids, which are completely metabolised by humans. Enzymes in the diet are as a rule completely non-toxic (Aunstrup et al., 1979; Reed, 1975) and can be considered intrinsically safe (Noordervliet and Toet, 1987). Therefore, the safety assessment of microbial enzymes for the food processing and animal feed industry focuses more on the "contaminants" in an enzyme preparation than on the enzyme itself (IFBC, 1990; Kessler et al., 1992; Pariza and Foster, 1983; Pariza and Johnson, 2001). Toxic contaminants may be introduced during the whole production process by the

* Corresponding author. Fax: +31-15-2793614.
E-mail address: piet.dijck-van@dsm.com (P.W.M. van Dijck).

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production organism, the fermentation process (substrates, raw materials etc.), and the isolation and purification procedures. Note that the latter ones are embedded in current Good Manufacturing Practice (cGMP) and Hazard Analysis of Critical Control Points (HACCP).

For many decades, *Aspergillus niger* has been safely used in the commercial production of various food enzymes, such as glucose oxidase, pectinase, α-amylase, and glucoamylase. Industrial production of citric acid by *A. niger* has taken place since 1919 (Röhr et al., 1983).

This long experience of industrial use has resulted in a good knowledge of the characteristics of *A. niger* and understanding of the metabolic reactions. Recently its genome has been elucidated (Groot et al., 2002). Genetically modified industrial strains of *A. niger* have been used as a host to over-express food and feed enzymes such as phytase and xylanase. Traditionally, the genes encoding these enzymes are integrated in a random fashion in the genome of the host organism. These enzymes are on the market now for almost a decade.

New technological developments have enabled us to construct a new generation of strains according to a design and build concept, in which the genes are introduced targeted to a predetermined region in the genome of the strain. The safety of this novel generation of enzymes for the consumer and the consequences with regard to safety studies are discussed in this paper.

2. The safety of *A. niger*

A. niger is known to naturally occur in foods. The fungus is commonly present in products like rice, seeds, nuts, olives, and dried fruits.

The long industrial use and wide distribution of *A. niger* in nature has never led to any pathogenic symptoms. Its non-pathogenic nature has been confirmed by several experimental studies, as reviewed by Schuster et al. (2002). *A. niger* is therefore generally accepted as a non-pathogenic organism.

Even though products from *A. niger* have been used in food for many decades, there has never been any evidence that the industrial strains used are able to produce detectable levels of toxins. The non-toxicogenicity has been confirmed by a large amount of toxicological tests, as well as batch testing of the various end products for toxins. This non-toxicogenicity does, however, not hold for all *A. niger* strains. A small percentage of natural isolates have been shown to possess the potential to produce ochratoxin A, a nephrotoxic, and carcinogenic mycotoxin (Abarca et al., 2001; Varga et al., 2000). Other secondary metabolites isolated from *A. niger* strains comprise nigragillin, the malformins, and the naphtho-γ-pyrones. These compounds are not considered to be mycotoxins.

The whole body of knowledge from the literature is carefully taken into consideration when testing industrial strains for any possible risk during the development of a fermentation process. Whenever possible the production organism is chosen from strains which have been in use for many years and which are examined for their ability to produce known toxins under the fermentation conditions. In addition, the products are regularly checked that they meet the requirements of the health authorities as given in the Food Chemical Codex (1996) or in the FAO/WHO Joint Expert Committee on Food Additives (JECFA) specifications (1992).

When a production process at our company DSM is developed with a new fungal strain the strain is routinely tested for its potential to produce toxic secondary metabolites, including mycotoxins. To this end the strains are cultured on agar-media, which are known from literature to give optimal expression of these toxic secondary metabolites (Frisvad and Filtenborg, 1989; Frisvad and Thrane, 1993). All cultures are incubated for 14 days in darkness at 24 °C. For metabolite analysis, the samples are extracted by the method described by Frisvad and Thrane (1987) and analysed by high performance liquid chromatography (HPLC) equipped with a diode array detection (DAD) system (Frisvad and Thrane, 1993). The metabolites found are compared to a spectral UV library made from authentic standards run at the same conditions, and retention indices were compared with those of the standards. An extra control run is carried out with a fluorescence monitor that detects ochratoxin.

3. The DSM *A. niger* GAM strain lineage

The DSM industrial strain used as a host for over-expression of enzymes is *A. niger* DS03043. Strain DS03043 is an over-producer of the enzyme glucoamylase, which is shortened to GAM. Consequently this lineage of strains is called the GAM strain lineage and is derived by classical strain improvement techniques (mutagenic treatment followed by selection for improved isolates) from the original strain *A. niger* NRRL3122 from the Culture Collection Unit of the Northern Utilization Research and Development Division, US Department of Agriculture, Peoria, IL, USA. The strain NRRL3122 and its classical derivatives have been in use for the production of glucoamylase (and acid amylase) by Wallerstein Laboratories since the sixties. Wallerstein was acquired by Baxter-Travenol and later on divested to Gist-brocades, now part of DSM.

Strain DS03043 was used as a suitable host strain to over-express enzymes of interest for DSM. For over-expression of phytase, the phytase gene was cloned from the donor strain *A. niger* NRRL3135 (also known as *Aspergillus ficuum*), put in an expression cassette under

the control of the host-own *gla*A promoter. Multiple copies of the phytase *phy*A gene were randomly incorporated into the genome of the host. The research yielded two phytase production strains, designated *A. niger* DS25956 and DS27301. These strains are being used in large-scale production of phytase, and marketed under the brandname Natuphos since 1991.

Using the same procedures the gene encoding endo-1,4-β-xylanase gene from a xylanase overproducing strain of *A. niger* (strain DS16813) was over-expressed in DS03043 by random integration of multiple copies of the xylanase expression cassette. The resulting strain *A. niger* DS26538 is used since 1995 for larger scale production of xylanase, marketed under the brandname Natugrain Wheat.

In recent years the strain DS03043 was genetically modified to serve as a suitable host for targeted integration of genes of interest to DSM and over-expression of the genes. Genetic analysis of the classical improved GAM lineage of strains showed that the increase in glucoamylase production capacity is, at least partly, due to an increase in the number of gene copies in the strain. Whereas NRRL3122 only contained one gene copy of the glucoamylase *gla*A gene, the DS03043 strain contains seven *gla*A gene copies. Many of the production strains that have undergone strain improvement by classical mutation and selection techniques have appeared to contain multiple copies of the gene of interest.

A recombinant strain was derived from DS03043, in which the seven glaA loci (i.e., the promoter and coding sequences) were deleted, creating so-called Δ*gla*A loci. Each of these loci was designed in such a way that it can be individually detected on a gel electrophoresis system (Fig. 1). The strain, in which all seven loci had been deleted, was used as the primary DSM host strain for over-expression of other enzymes. From this strain other host strains were derived with additional modifications such as inactivation of the major protease *pep*A and with improved enzyme production capability. As specific demands require new host strains will be derived from this *A. niger* strain lineage. The present set of *A. niger* host strains have been approved as GMO self-clones[1] by the Dutch competent authorities.

In order to obtain accurate integration and expression of any desired gene in one of the above-mentioned host strains, standard expression units are used, where the gene of interest (call it gene X) is inserted between the host-own glucoamylase promoter and glucoamylase terminator elements in a proprietary *Escherichia coli* vector. Also the *amd*S gene, to be used as a dominant selection marker, was put in such a standard unit. For details, see Selten et al. (1995, 1998).

[1] Self-cloning as defined in the European Directive on the Contained Use of Genetically Modified Micro-organisms 98/81/EC (revision of 90/219/EC).

Prior to the transformation the two vectors (the amdS vector and the vector containing gene X) are linearized using the appropriate restriction enzymes and all sequences derived from *E. coli* are removed by purification on agarose gels. The linearized expression cassettes for gene X and the *amd*S marker are then introduced into the host strain. The gene X and *amd*S expression units—both completely free of any *E. coli* DNA—are integrated into the genome of the host by co-transformation. Due to the homology in the 3′- and 3″-*gla*A parts of the two expression units, these expression units are targeted to one of the seven Δ*gla*A loci. Transformants are selected on agar plates containing acetamide as the sole carbon source. Only those clones are able to grow that have acquired at least one copy of the *amd*S expression unit.

Transformants are selected that contain an *amd*S expression unit with several in tandem integrated gene X expression units in one of the seven Δ*gla*A loci. The selection of these transformants is done by PCR analyses, applying gene X and *gla*A specific primers. Subsequently, by counter-selection on fluoro-acetate containing plates, a natural variant is selected in which the *amd*S selection marker is deleted as a result of a natural internal recombination event. This is schematically depicted in Fig. 2. In the recombination event the strain also may lose a number of gene X expression units. The absence of the *amd*S marker is confirmed by Southern analysis. The resulting organism is thus not only totally free of *E. coli* DNA, but also of the *amd*S selection marker.

For further multiplication of the remaining gene X expression units in this marker-free organism, the naturally occurring gene conversion is used to fill up other Δ*gla*A loci. Gene conversion is a natural spontaneous process, not involving any mutagenic treatment of the strain (Selten et al., 1998).

Strains can be selected in which up to all seven Δ*gla*A loci are occupied with multiple copies of the expression unit in question, arranged in tandem (see Fig. 3).

Since the integration of new gene copies into the genome is targeted there is no disturbance of other parts in the genome, so there is no possibility of e.g. activation of mycotoxin gene(s).

Due to the fact that the newly introduced gene copies are under the genetic regulation of the glucoamylase promoter the production processes for new enzyme products can be kept the same with respect to the raw materials used. This eliminates the chances of new safety hazards for the enzyme product brought in to the system by the raw materials.

This innovative technology has been used by DSM to design and build a lineage of novel marker free production strain lineages, including the following:

- The ABF lineage, e.g., strain DS34552, over-expressing the *abf*A arabinofuranosidase gene from the DSM classical arabinofuranosidase production strain *A. niger* DS06846.

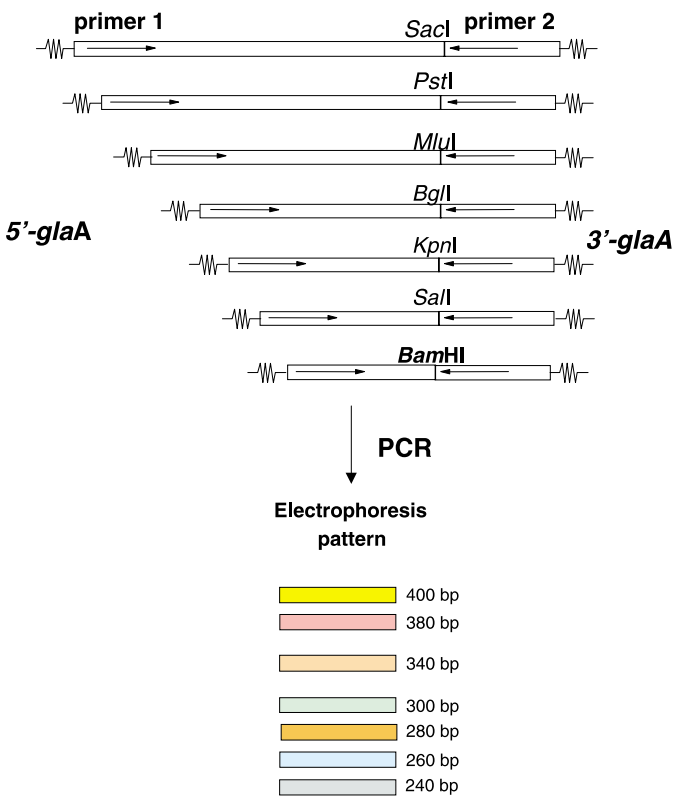

Fig. 1. Visualization of all seven marked *gla*A loci of *A. niger* strains, in which the *gla*A genes were removed. All gene sequences of the seven glucoamylase loci were removed by using proprietary technology. Subsequently they were partly filled using the same methodology with sequences from the 3′ region of the original locus. Due to the fact that these sequences varied in length and were decorated with a specific restriction site the seven marked loci can detected individually by PCR using a set of specific primers and separation on a gel electrophoretic system.

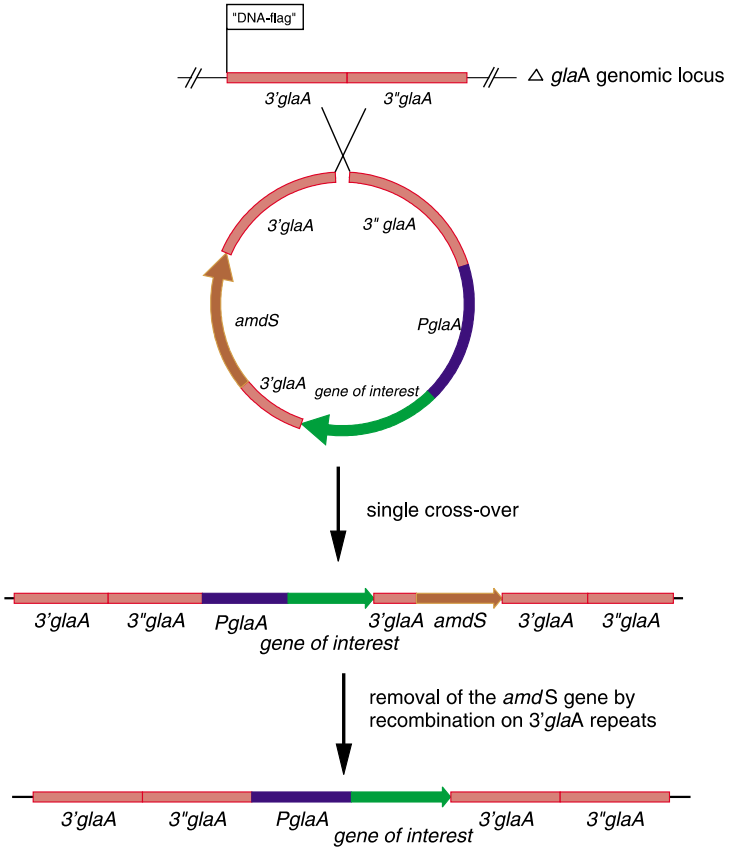

Fig. 2. Example of the marker-gene free insertion of an expression unit. The expression unit, a linear piece of DNA, is integrated into one of the seven marked loci by homology of the 3′ regions of the loci. By varying the conditions of transformation of the expression units multiple copies of the gene of interest arranged in tandem can be integrated in a marked locus. Current practise is that both the gene of interest and the *amd*S selection marker are on different expression units. So the transformation is carried out with a mixture of expression units carrying the gene of interest and expression units carrying the *amd*S marker. By selection on agarplates containing acetamide as sole carbon source the transformants are selected. By counter-selection on agarplates containing fluoro-acetate variants can be selected from these transformants which have lost the *amd*S marker but which have retained (multiple copies of) the gene of interest.

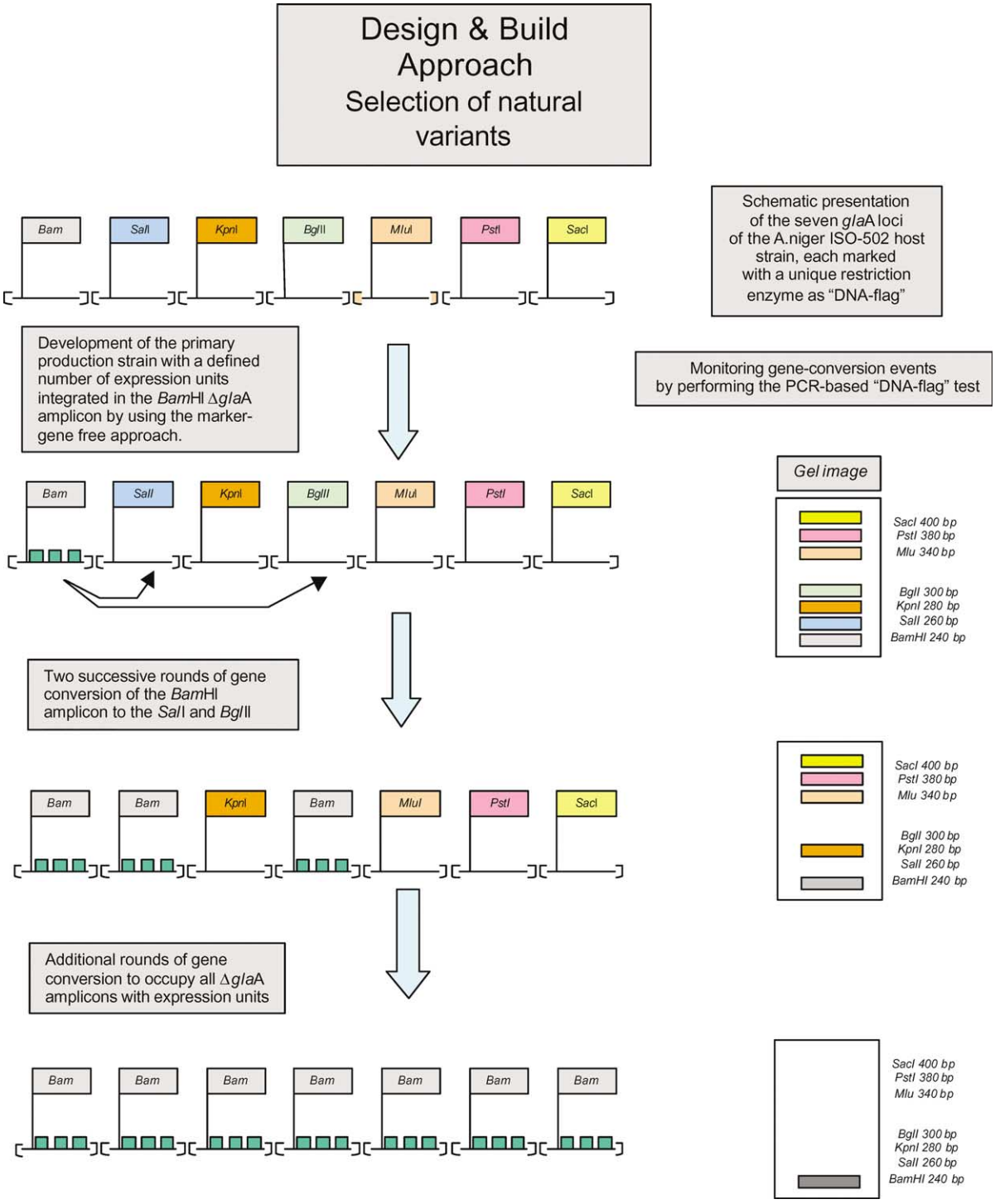

Fig. 3. Selection of natural variants. The natural process of gene conversion is used to select for variants that have acquired a set of multiple gene copies of the gene of interest in any the remaining empty marked loci. The filling-up process in the several selection rounds can be followed by analysis in a gel electrophoretic system.

- The NPH lineage, e.g., strain DS35387, over-expressing the *phy*A phytase gene from *A. niger* NRRL3135 (also known as *Aspergillus ficuum*).
- The PME lineage, e.g., strain DS34553, over-expressing the *pme*A pectin methyl esterase gene from the DSM classical *A. niger* pectinase production strain.
- The PLA lineage, e.g., strain DS35496, over-expressing the phospholipase A_2 gene obtained as cDNA from porcine pancreas.
- The GLA and AMY lineages, e.g., strains DS36728 and DS37099, respectively, over-expressing the *gla*A glucoamylase gene and the *amy*A acid amylase gene

from *A. niger* DS03043, the DSM glucoamylase production strain and parental strain of the above described novel host strains.
- The XEA lineage, e.g., strain DS38163, over-expressing a heterologous xylanase gene.

Several new production strain lineages are under development.

The GMO strains from the PME, ABF, NPH, GLA, and AMY lineages have been approved by the Dutch competent authorities as GMO self-cloned strains.

Based on the genetic modifications performed, there are no reasons to assume that the new recombinant production strains should be less safe than the original *A. niger* DS03043 strain.

4. Secondary metabolites produced by strains from the GAM lineage

It has been shown (Van Dijck et al., 2002) that the *A. niger* ancestor strain NRRL3122 has the potency to produce nigragillin and naphtho-γ-pyrones as secondary metabolites. This potency remains more or less the same with the classically improved strains from the GAM lineage, although the number and the amount of secondary metabolites decrease. Introduction of genes by random integration of phytase genes (DS25956 and DS27301) or xylanase genes (DS26538) into the genome of DS03043 does not change this pattern.

We expect that targeted introduction of genes into the genome of the novel *A. niger* host strains, by design should not have any influence on the pattern of secondary metabolites. That this indeed is the case was demonstrated for all production strains that were developed so far. All these GMO strains, if tested under conditions optimal for mycotoxin production, showed a pattern of secondary metabolites similar to that of the original host strain DS03043. In none of the strains could aflatoxin, trichothecenes, or ochratoxin A be detected. (Van Dijck et al., 2002).

The strains were also tested under conditions representative for large-scale production conditions. All secondary metabolites, normally found in the strains under stress conditions, such as nigragillin and naphtho-γ-pyrones, were not detectable either in broth samples, taken at the end of the fermentation, or in the final (unformulated) enzyme products (Van Dijck et al., 2002).

5. Safety studies on the classical strain

The toxicological studies performed on various enzyme preparations from *A. niger* provided the basis for a safety evaluation by the Joint Expert Committee on Food Additives (JECFA) of the FAO/WHO. Since the enzyme preparations tested were of different activities and forms, and of most of the organic materials in the preparations are not the enzyme per se JECFA estimated a numerical Acceptable Daily Intake (ADI) expressed in terms of total organic solids (TOS). Although all toxicological studies for enzyme preparations of *A. niger* showed no-observed-effect levels greater than 100 mg TOS/kg bw/day in 90-day studies in rats, JECFA nevertheless allocated an ADI of 0–1 mg TOS/kg bw for each enzyme preparation. This was merely based on the fear that some strains may produce unknown toxins (JECFA, 1988). However, in view of the long history of use as an enzyme source, the numerous toxicological studies and two expert reports that showed that toxin production is very unlikely (Bennett, 1988; Moss, 1988), JECFA reconsidered its decision and changed the ADI for enzyme preparations derived from *A. niger* into "not specified" (JECFA, 1991).

Among the safety studies evaluated by JECFA (1975) were Baxter-Travenol's unpublished safety studies carried out on a carbohydrase product (Garvin and Merubia, 1959; unpublished report submitted to WHO by Baxter Laboratories; Garvin et al., 1972; unpublished report from Travenol Laboratories submitted to WHO by Gist-brocades). This study was a 90-day oral toxicity study with rats and showed a NO(A)EL (No Observed Adverse Effect Level) of 10% in the diet. In the early seventies strain numbers were not mentioned in the reports of the safety studies. We can only assume but not prove that the safety studies were done on a carbohydrase product obtained from a fermentation with *A. niger* NRRL3122. Besides amyloglucosidase the product also exhibited α-amylase activity. Since the DSM GAM-lineage of strains originates from the Wallerstein (= Baxter-Travenol) amyloglucosidase production strain, the result of the study bears relevance for the DSM GAM lineage of strains. Table 1 gives an overview of all the safety studies performed on the DSM GAM lineage of strains.

6. Safety studies on strains obtained by random integration of genes

Representative batches of phytase produced either with strain DS25956 or strain DS27301 or xylanase produced with strain DS26538 have been subjected to safety studies as required by the guidelines of the EU Scientific Committee for Food (1992). Two mutagenicity tests (Ames test and an in vitro chromosome aberration study) and a 90-day subchronic oral toxicity test in rats have been done. In all three cases the NOAEL was the highest enzyme dose tested and was above the 100 mg TOS/kg bw/day, which was the threshold for safe enzyme products according to JECFA. For an overview of the safety data, see Table 1. From these data we conclude that strains derived from strains from the GAM

Table 1
Overview of oral toxicity studies on enzyme preparations produced with strains from the *A. niger* GAM strain lineage

Enzyme	Production organism	Type of integration	Oral toxicity study		
			Year of study	Duration	NO(A)EL[a] (mg TOS/kg bw/day)
General (JECFA)	*A. niger*		—	90	>100
Glucoamylase	*A. niger* NRRL-3122	—	1972	90	
Phytase	*A. niger* DS25956	Random	1991	90	>1260
Phytase	*A. niger* DS27301	Random	2000	90	>1206
Xylanase	*A. niger* DS26538	Random	1995	90	>4095
Phytase	*A. niger* DS35387	Targeted	2000	90	>833
Phosholipase A2	*A. niger* DS35496	Targeted	2000	90	>1350
Xylanase	*A. niger* DS38163	Targeted	2001	90	>1850
Pectin methylesterase	*A. niger* DS34553	Targeted	2000	14	>133
Arabinofuranosidase	*A. niger* DS34552	Targeted	2000	14	>103
Amyloglucosidase	*A. niger* DS36728	Targeted	2001	14	>1640

[a] In all cases the NOAEL (No-observed-adverse-effect level) was the highest dose level tested.

lineage do not produce any harmful compounds that may end up in the enzyme product.

7. Safety studies on the novel DSM *A. niger* strains

With enzyme products produced with the novel DSM *A. niger* strains in three cases, besides the two mutagenicity studies, a 90-day subchronic oral toxicity study in rats was carried out. This was done for phytase, produced with *A. niger* DS35387, phospholipase A2 produced with a strain from the *A. niger* PLA lineage and xylanase, produced with *A. niger* DS38163. In all three cases the NOAEL was the highest enzyme dose tested and was above the 100 mg TOS/kg bw/day.

For other enzyme products made with these novel strains only a 14-day oral toxicity study was carried out. This holds for arabinofuranosidase, produced with DS34552, pectin-methyl esterase, produced with DS35387, and gluco-amylase, produced with a strain from the GLA lineage. Also with these three products the results showed that the NOAEL was the highest enzyme dose tested (i.e., >100 mg TOS/kg/day). In all cases the mutagenicity studies were negative.

8. Discussion

In this paper we have shown that the *A. niger* GAM lineage and the derived GMO strains can be considered as a safe strain lineage. There are safety data available from the classical ancestor strain, from the DS03043 derived GMO strains, where the expression cassettes were randomly integrated into the genome of DS03043 and from the novel GMO strains, where the expression cassettes were integrated into the genome of the host at a targeted site.

The finding that in the strains obtained by random integration of expression cassettes no change in the pattern of secondary metabolites is found makes it highly unlikely that random integration of genetic material in the genome, due to pleiotropic effects, induces the formation of novel toxic metabolites. This often is raised as a hypothetical possibility by regulatory authorities. In strains obtained by targeted integration of expression cassettes, both *cis* and *trans* activations of toxin producing genes are hard to imagine and can be ruled out for all practical purposes.

The fact that the pattern of secondary metabolites does not change can also be taken as an indication that the over-expression of enzymes does not lead to induction of metabolic stress to such an extent that secondary metabolites are formed. Metabolic stress due to over-expression of proteins has been found in bacterial and yeast systems (Gill et al., 2000; Patil and Walter, 2001). With the DSM *A. niger* GMO strains the over-expression occurs in a strain that is derived from an industrial strain, which has already the capacity to over-produce and secrete copious amounts of enzyme protein. Any stress that is inflicted upon these GMO strains apparently does not lead to any detectable toxin production. This can be concluded from the analyses of the secondary metabolites under actual large-scale fermentation conditions.

The fact that the genes of interest are over-expressed under the same genetic regulatory system enables the fermentation process of all these products to be constant with respect to the raw materials composition of the fermentation medium. Consequently no new safety hazards are introduced into the product background. This is supported by the safety studies performed on these novel enzymes. In all cases the highest dose level tested was the NOAEL. Using standard methods for the calculation of the expected human consumption (Douglas et al., 1997;

Löwik et al., 1998) the Margin of Safeties were 250 up to 400,000 in case of a 90-day toxicity study and 500 up to 2750 for the 14-day toxicity studies.

We conclude that with all safety data available we cover the safety of new enzymes to be produced using a GMO strain derived from the novel DSM *A. niger* strain lineage. Provided of course that with the gene of interest we do not introduce additional non-characterized gene sequences into the strain. PCR techniques allow us to restrict the DNA fragment to be transformed only to the coding sequence of the gene of interest. When we apply the case of an enzyme product belonging to a class of enzymes which have a history of safe use in food processing (see Table 1 in the Pariza and Johnson paper) made according to the principles described in the previous pages to the Decision Tree put forward by Pariza and Johnson (2001) it is clear that the production strain is genetically modified and that recombinant techniques have been used in the introduction (Q1 and 2). The question, whether the expressed enzyme product, which is encoded for by the introduced DNA, has a history of safe use (Q3a), can be answered with yes. Since the novel DSM strains do not contain any heterologous markers such as antibiotic resistance markers (Q3c) and all introduced DNA is well characterized and free of attributes that would render it unsafe of constructing micro-organisms to be used in food-grade products (Q3e) the Decision Tree takes us to Q4. The introduced DNA is integrated in the genome of the host in a targeted manner (in one or more of the seven glucoamylase loci), which brings us to question 6: is the production strain derived from a safe strain lineage? For the GAM lineage of strains we have shown in the preceding paragraphs that sufficient data are available from safety studies on enzyme products made with strains from this lineage that the *A. niger* GAM lineage of strains truly can be called a safe strain lineage.

This affirmative answer to Q6 concludes the passage through the Decision Tree with the statement: the test article is ACCEPTED.

Thus, when assessing the potential safety hazards of a new enzyme product it would make no difference as to from what background the enzyme encoding gene of interest is isolated with PCR, provided that the enzyme itself has a history of safe use in food. The donor organism can be a safe micro-organism but it would not matter if the donor strain is pathogenic, toxicogenic or when the background of the gene is not known. So even in case of direct isolation of DNA by PCR techniques from a biological source, without the intermediary step of isolating and cultivating the donor micro-organism, the existing safety studies would cover the derived enzyme product and no new safety studies would be required. Consequently unnecessary use of testing animals would be prevented.

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