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Original Submission

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January 9, 2003

Linda S. Kahl, Ph.D.
Regulatory Policy Branch, HFS-206
Center for Food Safety and Applied Nutrition
Food and Drug Administration
200 C Street, S.W.
Washington, D.C. 20204

Dear Dr. Kahl,

We are hereby submitting, in triplicate, a generally recognized as safe (GRAS) notification, in accordance with proposed 21 C.F.R. § 170.36, for Novozymes' laccase enzyme preparation produced by laccase preparations produced by submerged fermentation of *Aspergillus oryzae* expressing the gene encoding a laccase from *Myceliophthora thermophila*. The laccase enzyme preparation is intended for use in the food industry as an ingredient in breath-freshening products such as breath mints, chewing gum and other food products.

Please contact me by direct telephone at 919 494-3152 or direct fax at 919 494-3420 if you have any questions or require additional information.

Sincerely,

Denise Bernstein
Senior Regulatory Specialist

Enclosures (3 binders)

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Novozymes North America, Inc.
77 Perry Chapel Church Road
P.O. Box 576
Franklinton, North Carolina 27525

Tel: 919-494-3000 *Fax: 919-494-3450* *Internet: www.novozymes.com*

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January 9, 2003

RE: GRAS Notification - Exemption Claim

Dear Sir or Madam:

Pursuant to the proposed 21C.F.R.§ 170.36 (c)(1) Novozymes North America Inc. hereby claims that laccase preparations produced by submerged fermentation of *Aspergillus oryzae* expressing the gene encoding a laccase from *Myceliophthora thermophila* are Generally Recognized as Safe; therefore, they are exempt from statutory premarket approval requirements.

The following information is provided in accordance with the proposed regulation:

Proposed § 170.36 (c)(1)(i) *The name and address of the notifier.*

Novozymes North America Inc.
77 Perry Chapel Church Rd., Box 576
Franklinton, NC 27525

Proposed § 170.36 (c)(1)(ii) *The common or usual name of notified substance.*

Laccase enzyme preparation produced by *Aspergillus oryzae* expressing the gene encoding a laccase from *Myceliophthora thermophila.*

Proposed § 170.36 (c)(1)(iii) *Applicable conditions of use.*

The laccase is intended for use in the in the food industry as an ingredient in breath-freshening products such as breath mints, chewing gum and other food products. The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements for normal production following Good Manufacturing Practices.

Proposed §170.36 (c)(1)(iv) *Basis for GRAS determination.*

This GRAS determination is based on scientific procedures.

Proposed § 170.36 (c)(1)(v) *Availability of information.*

A notification package providing a summary of the information which supports this GRAS determination is enclosed with this letter. The package includes a safety evaluation of the production strain, the enzyme, and the manufacturing process, as well as an evaluation of dietary exposure. A published article on the safety of laccase from *Myceliophthora thermophila* expressed in *A. oryzae* is also included in the notification package. Complete data and information that are the basis for this GRAS determination are available to the Food and Drug Administration for review and copying upon request.

10 January, 2003
Date

John Carroll
Director, Regulatory Affairs

Novozymes North America, Inc.
77 Perry Chapel Church Road
P.O. Box 576
Franklinton, North Carolina 27525

Tel: 919-494-3000 *Fax: 919-494-3450* *Internet: www.novozymes.com*

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LUNA No. 2003-01638-01

GRAS NOTIFICATION

LACCASE

A laccase produced by *Aspergillus oryzae* expressing the gene encoding a laccase from *Myceliophthora thermophila*

Date: January 2003

Denise Bernstein, Regulatory Affairs, Novozymes North America, USA
Rie Tsuchiya, Safety and Regulatory Affairs, Novozymes A/S, Denmark

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1. INTRODUCTION

The Laccase enzyme is produced by submerged fermentation of *Aspergillus oryzae* carrying a gene encoding a laccase from *Myceliophthora thermophila.* The enzyme preparation is to be used in the food industry as an ingredient in breath-freshening products such as breath mints, chewing gum and other food products.

Laccases are widely distributed among fungi and plants. Laccases (E.C. 1.10.3.2) are oxidases that catalyse the oxidation of a wide range of phenols and other substrates with the concomitant reduction of oxygen (O_2) to water. Reactions between laccases and phenols are commonly found in nature. One prominent example is the oxidation of phenols in fruits and vegetables by laccases to form brown polymers, which is known as the "bruising or enzymatic browning" of fruits and vegetables. Since laccase has the capability of catalyzing the oxidation and the polymerisation of phenols and other substances, laccase is receiving increasing interest in various applications such as in wine clarification and to improve the storage of beer (i.e., reduce or eliminate the development of off-flavours during storage).

The applications of the present submission are directed to breath-freshening products such as breath mints, chewing gum, toothpaste, and mouthwash that contain laccase enzyme that facilitate a reaction of naturally-occurring polyphenolic compounds existing in food and food extracts to form reactive compounds that interact with odor-causing compounds located in the oral cavity. Laccase oxidizes a phenolic compound to form a quinone that reacts with volatile sulphur compounds that can cause bad breath. In essence, the laccase facilitates the formation of compounds that bind to compounds responsible for the bad breath, which creates a deodorizing effect.

The safety of the production organism must be a prime consideration in assessing the probable degree of safety of an enzyme preparation intended for use in food (Ref. 2, 3). The production organism for this laccase, *Aspergillus oryzae*, is discussed in Sections 2 and 7. Sections 4, 5, and 6 describe the production process, the product specification and application of the laccase. Section 7 outlines the safety evaluation of the product including the toxicology program, which has been carried out confirming the safety of the product for its intended use.

It should be noted that in some reports, the laccase is described as batch "PPX 5720," "Novozym 809," "SP 966," or Novozymes 18043. SP 966 and Novozym 809 are internal names used before marketing. PPX 5720 is the internal code of the test batch used for safety testing. Novozymes 18043 is a trade name.

2. PRODUCTION MICROORGANISM

2.1 Production Strain

The Aspergillus oryzae strain, designated Mt-3, was constructed by transformation of the recipient strain, designated HowB711/RaMB17.WT, (see Section 2.2), with a purified DNA fragment. This genetically modified production organism complies with the OECD (Organization for Economic Cooperation and Development) criteria for GILSP (Good Industrial Large Scale Practice) microorganisms (Ref. 1). It also meets the criteria for a safe production microorganism as described by Pariza and Foster, Pariza and Johnson, and several expert groups (Ref. 2-9).

The DNA fragment used in the strain construction contains strictly defined fungal chromosomal DNA fragments and synthetic DNA linker sequences. The specific DNA sequences include a laccase enzyme gene from *Myceliopthora thermophila* strain E421 (CBS 117.65); an Aspergillus nidulans selectable marker gene, amdS (acetamidase) (Ref. 10); well-characterized non-coding regulatory sequences including the *Aspergillus niger strain* BO-1 terminator (Ref. 11), the TAKA-amylase promoter from *Aspergillus oryzae* strain HW325 (Ref. 12), and a fragment from a well known sequence from *Escherichia coli* plasmids pUC19 and pUC18 (Ref. 13).

2.2 Recipient Organism

The recipient microorganism, designated How B711/RaMB17.WT, used in the construction of the laccase production strain is a genetically modified derivative of A. oryzae A1560 by means of site-directed gene disruption to cause interruption of the three resident TAKA amylase genes. The obtained strain A. oryzae How B711 is a derivative of the fully characterized, well-known industrial production strain of A. oryzae (Ahlburg) Cohn. The strain was obtained from the Institute for Fermentation, Osaka, Japan (IFO) and is designated strain IFO 4177 (synonym A1560).

The taxonomic characteristics of the host strain of *A. oryzae* A1560 are the following:

Name:	*Aspergillus oryzae*
Class:	Blastodeuteromycetes (Hyphomycetes)
Order:	Phialidales (Moniliales)
Genus:	*Aspergillus*
Species:	*Oryzae*

This classification has been confirmed by the Centraalbureau voor Schimmelculutres, Baarn, Holland (Ref. 16).

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2.3 Laccase Gene Isolated from *Myceliopthora thermophila*

2.3.1 Taxonomy

Myceliophthora thermophila (Apinis) van Oorschot belongs to the hyphomycetes fungi. It is a true thermophilic fungus and a common component of decaying manure, silage, wood chips and pulp etc. in Europe and North America (Ref. 79).

Classification:
Basionym: *Sporotrichum thermophilum* Apinis. Teleomorph: *Corynascus heterothallicus* (van Klopotek) von Arx.
Ascomycota, Pyrenomycetes, Sordiales (Sphaeriales *(Ainsworth)*), Corynascus (Thielavia)

It was originally described as *Sporotrichum thermophilum* (Ref. 80). *Sporotrichum* is a hyphomycete genus with basidiomycetous teleomorphs. Therefore, when von Klopotek described the ascomycetous teleomorph *Thielavia heterothallica* (Ref. 81), *S.thermophilum* was transferred to the genus *Chrysosporium*. The fungus was examined by van Oorshot (Ref. 82), who reintroduced the genus *Myceliophthora* as a segregate of *Chrysosporium,* based on different conidial structures. Finally, the teleomorph of *Myceliophthora thermophila* was re-examined by von Arx (Ref. 48), and transferred to the genus *Corynascus* as *C. heterothallicus* (van Klopotek) von Arx.

2.3.2 Cloning of the Enzyme Gene

An *M. thermophila* genomic DNA library was constructed in the bacteriophage cloning vector lambda-EMBL4. The library was screened by hybridization with a radiolabel *Neurospora crassa* laccase DNA fragment using non-stringent conditions.

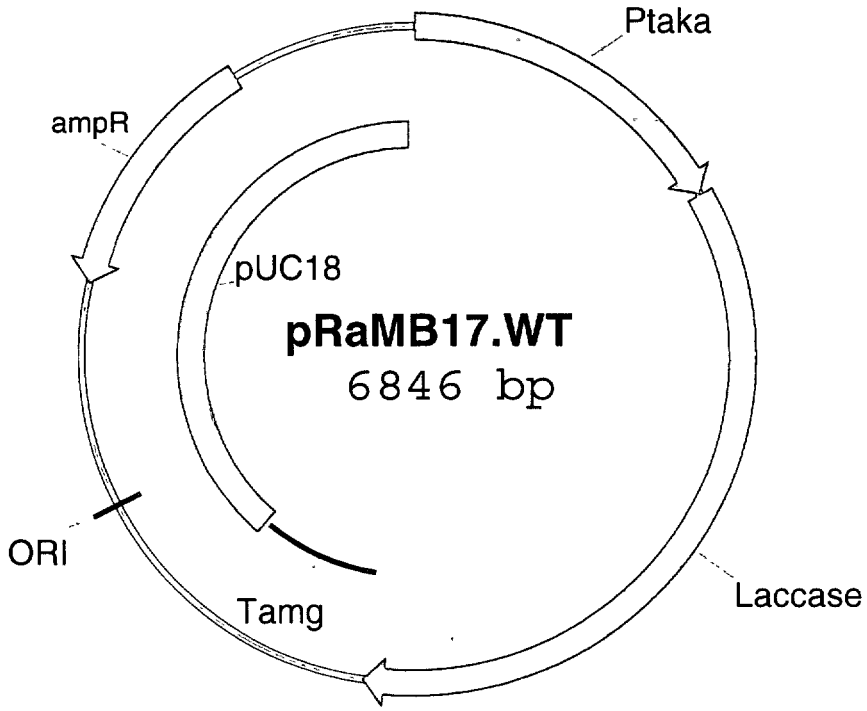
Eight plaques were identified that hybridized strongly to the *N. crassa* laccase gene probe. A 3.2 kb *Nhe*I–*Bgl*II segment harboring the entire laccase gene was sub-cloned into pUC119.

2.4 Construction of the Expression Vector and the Selection Plasmid

The laccase expression plasmid pRaMB17.WT was constructed through the following steps: 1) Isolation of 1.5 kb *M. thermophila* laccase gene resulting in the plasmid pInt2.22; 2) Isolation of *A. oryzae* TAKA-amylase promoter resulting in the plasmid pInt1; 3) Isolation of *A. niger* glucoamylase terminator resulting in the plasmid pUC::AMGTerm; and 4) The expression plasmid pRaMB17.WT was constructed with the 1.5 kb fragment containing the laccase gene which is under transcriptional control of the TAKA-promoter by a three part ligation.

Structure of the expression vector pRaMB17.WT.

pRaMB17.WT consists of the following elements:



Position (bp)	Size (bp)	Element	Origin
1-1143	1143	Ptaka	A. oryzae hW325
1144-1146	3	Linker	Synthetic
1147-3584	2438	Laccase	M. thermophila
3585-3591	7	Linker	Synthetic
3592-4176	585	Tamg	A. niger BO1
4177-4198	22	Linker	Synthetic
4199-6833	2635	PUC18	E. coli
6834-6846	13	Linker	Synthetic

Ptaka is the TAKA amylase promoter of *A. oryzae*.
Laccase is the genomic laccase gene of *M. thermophila*.
Tamg is the amyloglycosidase terminator of *Aspergillus niger*.
pUC18 is a general *E. coli* vector (Ref. 18) that features an origin of replication (ORI, position 4615) and the ampicillin resistance gene (ampR, position 6234 - 5377, size 858 bp).

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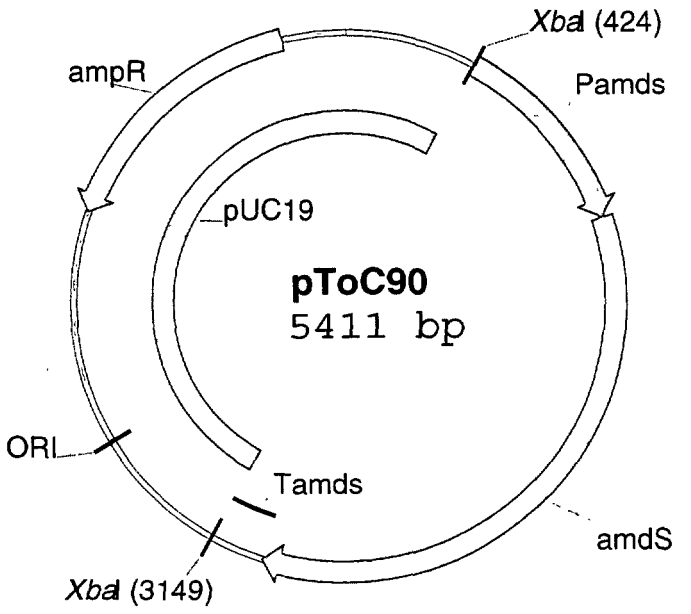
Structure of the selection plasmid pToC90

pToC90 consist of the following elements:



Position (bp)	Size (bp)	Element	Origin
424-1072	649	Pamds	*A. nidulans.*
1073-2972	1900	amdS	*A. nidulans.*
2973-3148	176	TamdS	*A. nidulans.*
3149-423	2686	pUC19	*E. coli.*

Pamds, amdS and **Tamds** are the promoter, coding sequence and terminator of the Aspergillus nidulans amdS gene.
pUC 19 is a general *E. coli* vector (Ref. 18) that features an origin of replication (ORI, position 3592) and the ampicillin resistance gene (ampR, position 5211-4354, size 858 bp).

2.5 Construction of the Recombinant Production Organism

The recombinant DNA molecule was introduced into the recipient *A. oryzae* How B711 by incubating protoplasts with pRaMB17.WT (laccase expression vector) and pToC90 (selectable marker). The selection of transformants was achieved by growing on a medium with acetamide as the sole nitrogen source and screening for co-expression of the *M. thermophila* laccase. A single colony of transformant HowB711/RaMB17.WT was selected.

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The production strain is obtained from transformant HowB711/-RaMB17.WT following mutation with nitrosoguanidine (NTG) and screening for high yield of the *Myceliophthora thermophila* laccase.

2.6 Stability of the Introduced Genetic Sequences

The presence and configuration of the introduced DNA sequences were determined by Southern hybridization to assess the stability and potential for transfer of genetic material as a component of the safety evaluation of the production microorganism (Ref. 4-9). The transforming DNA is stably integrated into the A. oryzae chromosome and, as such, is poorly mobilizable for genetic transfer to other organisms and is mitotically stable (Ref. 1).

2.7 Antibiotic Resistance Gene

A gene coding for resistance to ampicillin has been introduced into the production organism as a result of the genetic modification. However, since the expression signals for the resistance gene are not present in the construction, the gene is not expressed in the production organism.

2.8 Absence of Production Organism in Product

The absence of the production organism is an established specification for the commercial product as specified in Section 5.2. The production organism does not end up in food and therefore the first step in the safety assessment as described by IFBC (Ref. 3) is satisfactorily addressed.

3. ENZYME IDENTITY

3.1 Enzyme Identity

Key enzyme and protein chemical characteristics of the laccase are given below:

Classification:	Laccase (generic name)
IUB nomenclature:	Urishiol oxidase; urushiol oxidase; *p*-diphenol oxidase
Systematic name:	Benzenediol: oxygen oxidoreductase
IUB No.:	1.10.3.2
CAS No.:	80498-15-3
Molecular weight:	85 kDa
Amino acid sequence:	The total nucleotide and amino acid sequences have been determined; the enzyme has 3 internal disulfide bonds and 4 copper atoms

Laccase is a polyphenol oxidase, which belongs to the family of blue multi-copper oxidases (Ref. 74). The substrate oxidized by laccase is regarded as an electron donor. "Oxidase" is used only where O_2 is an acceptor. These enzymes catalyse the oxidation of a wide range of diphenols and other substrates with the concomitant reduction of dioxygen (O_2) to water. In general, there are two types of phenol oxidases found in plants and fungi. The first type is a laccase (EC 1.10.3.2), which oxidizes o-, p-diphenol to their corresponding quinones in the presence of molecular oxygen (Ref. 45, 47). Laccases as phenol oxidases are widely distributed in fruits and fungi. Since laccase has the capability of catalyzing the oxidation and the polymerisation of phenolic substances, the laccase is receiving increasing interest in various applications (see Section 6). The second type comprises catechol oxidase and monophenol monooxygenase (EC 1.10.3.1 and EC 1.14.18.1) that oxidizes o-diphenols to their corresponding quinones in the presence of molecular oxygen.

The enzyme protein is well characterized (Ref. 18, 30). The first report of the cloning and heterologous expression of a thermostable laccase was published in 1997. This article characterizes a gene encoding an extracellular laccase of *Myceliopthora thermophila* and the analysis of a recombinant laccase as expressed in A. oryzae (Ref. 30).

3.2 Enzymatic Activity

A typical laccase product has an estimated activity of 250 LAMU/g. LAMU is an acronym for LAccase *Myceliophthora* Unit.

Definition of laccase activity unit: 1 LAMU is the amount of enzyme which under the given analytical conditions catalyses the conversion of 1 micromole syringaldazine per minute.

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Appendix A shows Novozymes' method to determine the enzyme activity (LAMU).

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4. MANUFACTURING PROCESS

This section describes the manufacturing process for the laccase, which follows standard industry practices (Ref. 21-23). The manufacturing process is composed of a fermentation process, a purification process, a formulation process, and finally a quality control of the finished product (Ref. 23). This section describes the processes used in the manufacturing of the Novozymes 18043 laccase.

The laccase is manufactured in accordance with current Good Manufacturing Practices, Food. The quality management system used in the manufacturing process complies with ISO 9001. The raw materials are Food Grade Quality and have been subjected to appropriate analysis to ensure their conformity with the specifications.

4.1 Raw Materials

All raw materials used in the fermentation and recovery process for the laccase enzyme concentrate are standard ingredients used in the enzyme industry (Ref. 21-23). The raw materials conform to Food Chemicals Codex specifications except those raw materials which do not appear in the FCC. For those not appearing in the FCC, internal specifications have been made in line with FCC requirements. On arrival at Novozymes A/S, the raw materials are sampled by the Quality Control Department and subjected to the appropriate analyses to ensure their conformance to specifications.

The antifoam used in fermentation and recovery are used in accordance with the Enzyme Technical Association submission to FDA on antifoams and flocculants dated April 10, 1998. The maximum use level of these antifoams in the Laccase product is less than 1%.

4.2 Fermentation Process

Novozymes 18043 is produced by submerged fed-batch pure culture fermentation of the genetically modified strain of *Aspergillus oryzae*, described in Section 2. All equipment is carefully designed, constructed, operated, cleaned and maintained so as to prevent contamination by foreign microorganisms. During all the steps of fermentation, physical and chemical control measures are taken and microbiological analyses are done to ensure absence of foreign microorganisms and confirm strain identity.

4.2.1 Production Organism

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Each batch of the fermentation process is initiated with either a lyophilized stock culture or in glycerol at –80°C stock culture of the production organism, A. orzyae, described in Section 2. Each new batch of the stock culture is thoroughly controlled

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for identity, absence of foreign microorganisms, and enzyme-generating ability before use.

4.2.2 Criteria for the Rejection of Fermentation Batches

Growth characteristics during fermentation are observed both macroscopically and microscopically. Samples are taken from both the seed fermentor and the main fermentor before inoculation, at regular intervals during cultivation, and before transfer/harvest. These samples are tested for microbiological contamination by microscopy and by plating on a nutrient agar followed by a 24-48 hour incubation period.

The fermentation is declared "contaminated" if one of the following conditions is fulfilled:

1. Infection is observed in 2 or more samples by microscopy; or
2. Infection is observed in two successive agar plates at a minimum interval of 6 hours.

Any contaminated fermentation is rejected.

4.3 Recovery Process

The recovery process is a multi-step operation that starts immediately after the fermentation process. The process is designed to separate the desired enzyme from the microbial biomass and partially purify, concentrate, and stabilize the enzyme.

4.3.1 Purification

The enzyme is recovered from the culture broth by the following series of operations:

The steps of this process involve a series of unit operations:

1. **Pretreatment -** pH Adjustment: Inorganic acids and bases are used for pH adjustments.

2. **Primary Separation** – Vacuum Drum Filtration: The cell mass and other solids are separated from the broth by well-established techniques such as pre-coat vacuum drum filtration or centrifugation. The pre-coat used in the filter and the filter aid used in the process is diatomaceous earth.

3. **Concentration** – Ultrafiltration and Evaporation: A combination of ultrafiltration and evaporation is applied for concentration and further purification. The ultrafiltration is applied to fractionate high molecular weight components from low molecular weight impurities and is used to increase the activity/dry matter

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ratio. Evaporation is used to increase the activity. The pH and temperature is controlled during the concentration step, which is performed until the desired activity and activity/dry matter ratio has been obtained.

4. **Germ Filtration**: Germ filtration is performed for removal of any residual production strain organisms and as a general precaution against microbial degradation. The filtrations are performed at well-defined pH and temperature intervals, and results in an enzyme concentrate solution free of the production strain and insoluble substrate components from the fermentation.

5. **Stabilization**: The enzyme concentrate is blended with water, sodium chloride, and sucrose and, if necessary, adjusted to the desired pH.

6. **Final Concentration**: The product is standardized according to the product Specification (see Section 5). Evaporation is conducted if enzyme concentration is too low to reach target yield.

4.4 Micro Granulation Process

A salt such as NaCl is sprayed with the liquid concentrate in a fluid bed process. A binder such as maltodextrin is added during the process when necessary to reach a specified activity. The product is discharged continuously after sieving.

4.5 Quality Control of Finished Product

The final products are analyzed according to the specifications given in Section 5.2.

5 COMPOSITIONS AND SPECIFICATIONS

5.1 TOS and Composition

The laccase will be available as a Food Grade Micro Granulate formulation with a typical activity of 250 LAMU/g.

The laccase has the typical composition:

Enzyme solids (TOS)	approx.	1-5%
Dextrin or maltodextrin	approx.	0-10%
Sodium chloride	approx.	85-99%

TOS = Total Organic Solids, defined as: 100% - water - ash – diluents

5.2 Specifications

The laccase conforms to the general and additional requirements for enzyme preparations as described in Food Chemicals Codex, 4th edition, 1996 (Ref. 24). In addition, the laccase also conforms to the General Specifications for Enzyme Preparations Used in Food Processing as proposed by the Joint FAO/WHO Expert Committee on Food Additives in Compendium of Food Additive Specifications (Ref. 25).

The established Novozymes' specifications are set forth in the table below. All final laccase preparations must conform to the following complete specification:

Composition Analyses of Final Product	Contents
Enzyme activity	According to declaration
Heavy Metals (excluding Cu)	Not more than 30 ppm
Lead	Not more than 5 ppm
Arsenic	Not more than 3 ppm
Total viable count/g	Not more than 1 x 10^4
Total coliforms/g	Not more than 30
Enteropathogenic E. coli/25 g	Negative by test
Salmonella/25 g	Negative by test
Antibiotic activity	Negative by test
Production organism	Negative by test
Mycotoxins	Negative by test

Heavy metals = sum of Ag, As, Bi, Cd, Hg, Ni, Pb, Sb, Sn

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The heavy metals and lead specifications meet FCC and exceed JECFA requirements. The arsenic, total viable count and *E. coli* specifications meet JECFA requirements and are not included in FCC. The total coliforms and *Salmonella* specifications meet both FCC and JECFA. The antibiotic activity and mycotoxins specifications meet JECFA and are not included in FCC (although FCC mentions

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mycotoxins but has not established tolerances). The production microorganism specification is a Novozymes' specification and is not mentioned in FCC or JECFA.

The activity of this enzyme is measured in Laccase Myceliopthora Units (LAMU/g) using Novozymes Laccase Activity Determination Manual Method (SOP No. EB-SM-0506.02/01). One Laccase Myceliopthora Unit (LAMU) is defined as the amount of enzyme which degrades 1 µmol syringaldazine per minute forming tetrametoxy-azp bis-methylene quinine (see Section 3.2 and Appendix A).

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6 APPLICATION

6.1 Technological Function & Uses

6.1.1 Summary

The applications of the present submission are directed to breath-freshening products such as breath mints, chewing gum, toothpaste, and mouthwash that contain laccase enzyme that facilitate a reaction of naturally-occurring polyphenolic compounds existing in food and food extracts to form reactive compounds that interact with odor-causing compounds located in the oral cavity.

The basic concept involves the laccase directly catalyzing a reaction of diphenols to create highly reactive quinones, which is followed by a non-enzymatic second reaction where quinones interact with odor-causing compounds. More specifically, in the first reaction the laccase catalyzes the oxygen-dependent oxidation of phenolic substrates such as o-diphenols and p-diphenols to form their corresponding quinones; and then in the second reaction these quinones quickly react with odor causing compounds such as sulfides, thiols, and amines to eliminate odors in the oral cavity (Ref. 14, 67).

6.1.2 Naturally Occurring Compounds

Laccase enzymes and phenolic compounds are naturally occurring components common in the human diet as they are found in abundant sources of foods such as fruits, vegetables, fungi, herbs, spices, teas, and coffees (Ref. 17, 52, 55, 56, 64, 75, and 79). Bananas exhibit high levels of laccase activity throughout growth and ripening processes (Ref. 59). Reports show that the laccase in bananas, tea leaves, tobacco leaves, and peaches have exclusive activity on o-diphenols and no ability to hydroxylate mono-phenols. Foods such as potatoes and apples, for example, have both types of activity. Nuts, tea and coffee beans are some examples of rich sources of polyphenols. Teas vary in amounts of polyphenols from about 33,000 to 270,000 ppm and nuts have an estimate of 140,000 ppm.

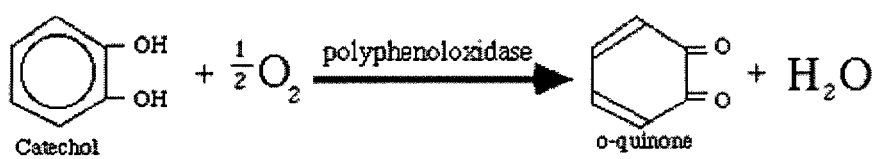
The fermentation of many foods or beverages such as coffee or tea by their endogenous enzymes, polyphenoloxidases, allows for the monomers, catechins, to form the polymeric materials, which is described more fully in Section 6.1.3. It is these materials that give rise to the astringent properties of tea and the brown or copper colors for coffee and black tea. Fresh tea leaf is especially rich in catechins, which is a flavanol group of polyphenols. Catechins may constitute up to 30% of the dry leaf weight. Another polyphenol, which is unique to tea, is theogallin (3-galloyquinic acid). Other flavanols and their glycosides and depsides are also common in other foods (Ref. 60). Such tea catechins and other polyphenolic compounds or o-quinones are well known for their deodorizing activity against methyl mercaptan (CH_3SH) (Ref. 14, 67).

Interactions involving laccase and phenolic substrates are responsible for the well known browning of freshly peeled or bruised fruit and vegetables (Ref. 65, 69). The reaction is initiated when endogenous laccase is released from bruised tissue. Laccase oxidizes phenols in the presence of oxygen to produce dopachromes which are polymerized to melanins as a final product (Ref. 58). In some cases, the attributes of browning are highly desirable. Browning is important for flavor and color development, for example. Browning produces a higher quality palm dates, prunes, raisins, cocoa, and tea. Browning also has a beneficial role in the curing of tea, coffee, and tobacco (Ref. 57).

6.1.3 Laccase

Laccases are single chain "blue" proteins that contain three or four copper ions and thus are often called multi-copper enzymes or (blue) copper oxidases. Laccases catalyze the oxidation of a variety of phenolic substrates with the concomitant reduction of O_2 to H_2O as shown in the laccase enzyme reaction depicted in Figure 1 (Ref. 30, 61).

Figure 1: The Laccase Enzyme Reaction



The reaction converts catechol (a polyphenol) to an oxidized form with the aid of a polyphenoloxidase (e.g., laccase). In this depiction, atmospheric oxygen is reduced to oxygen in 1 molecule of water, liberating 4 electrons, without hydrogen peroxide produced as an intermediary step. Laccase catalyses the oxidation of a range of reducing substances (especially phenols and aniline derivatives) associated with a concomitant reduction of oxygen. The reaction involves the removal of hydrogens from the *o*-diphenol to give an *o*-quinone. The reaction may then further proceed leading to a polymerisation of the reaction products as seen with the soluble polyphenols naturally occurring in extracts of plants and fruits, eventually leading to a precipitation as depicted in Figure 3 below.

Although the specificity for the electron donor is low, the specificity for the acceptor is essential. The acceptor has to be oxygen. Peroxides are not produced in the reaction. The reactions catalyzed by laccases proceed by bimolecular phenoxy radical coupling – see Figure 2 below:

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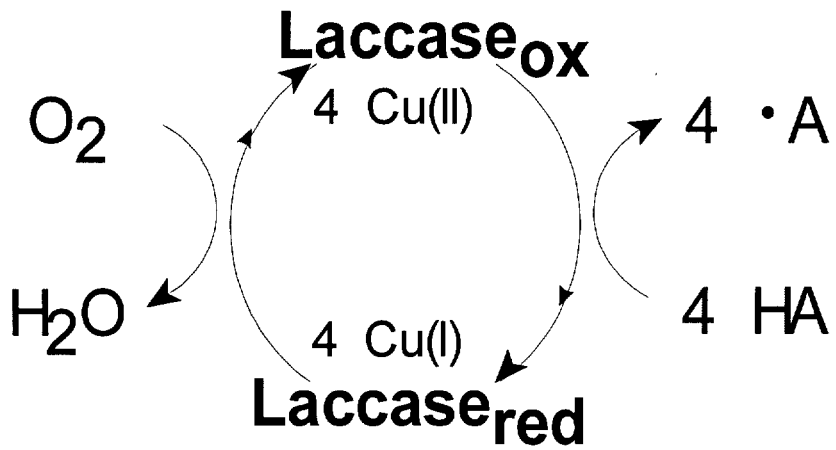
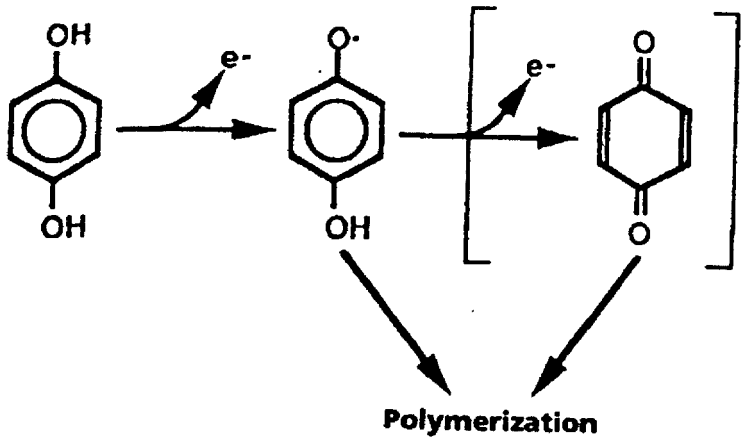
Figure 2: Mechanism for oxidation catalysed by laccase.



Substrate oxidation by laccase is a one-electron reaction generating a free radical. The initial product is typically unstable and may undergo 1) a second enzyme-catalysed oxidation converting phenol to quinone 2) non-enzymatic reactions such as hydration or disproportionation 3) and/or may partake in a polymerisation reaction giving an amorphous insoluble melanin-like product as shown below. As one-electron substrate oxidation is coupled to four-electron reduction of oxygen the reaction mechanism must be rather complicated enabling the laccase to store electrons from the intermediate reactions.

The typical laccase reaction is summarized in Figure 3 below. A diphenol such as hydroxyquinone undergoes a one-electron oxidation to form an oxygen-centered free radical. This species can be converted to the quinone in a second enzyme-catalysed step or by spontaneous disproportionation. Quinone and free radical products undergo polymerisation. The semiquinone product may react further either enzymatically or non-enzymatically.

Figure 3. Typical Laccase Reaction.



The action of phenol oxidases upon polyphenolic substrates resulting in the formation of highly reactive o-quinone species then reacts with nucleophilic malodorous materials (sulfides, thiols, amines) to produce covalent complexes of extremely low volatility. This process effectively removes the volatile, malodorous sulfides, thiols, and amines from the headspace thereby effecting deodorization (Ref. 41). The proposed mechanism of action of a laccase enzyme and a phenolic compound is illustrated in the scheme in Figure 4 below.

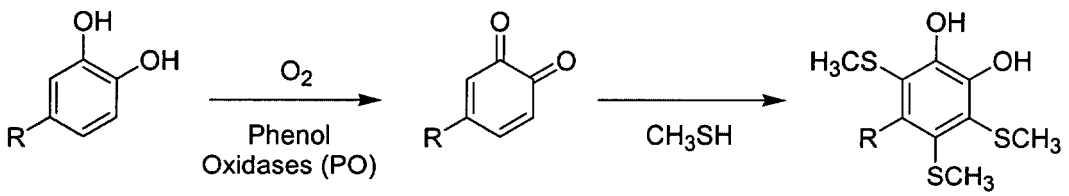


Figure 4.

Action of the appropriate enzymes such as laccase upon these polyphenols in food residue in the oral cavity accelerates the formation of the requisite *o*-quinones, and since this first step is the rate limiting step in the entire deodorization process, this results in much more rapid and efficient capture of volatile malodorous materials.

The processes discussed above represent a very rapid and efficient method for the sequestering of sulphur and nitrogen containing molecules to form covalent complexes of low volatility and, hence, low odor. The end result is the effective removal of those chemical species responsible for the vast majority of malodors.

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6.1.4 Other Industrial Uses

The ability of laccase to oxidize polyphenols makes them particularly valuable as potential industrial enzymes (Ref. 18, 43). These industrial uses illustrate the extremely versatile nature of laccases, although these specified uses are not a subject of this Notification. Laccases are useful in the stabilization of fruit juices (Ref. 54), dough improvement for the production of bread (Ref. 80), ethanol production (Ref. 63), drug analysis to distinguish morphine from codeine (Ref. 53), wine, beer, and juice clarification (Ref. 73), and bioremediation such as the degradation of environmental pollutants (Ref. 66, 70, 81). Fungal laccases are used to speed up the polymerization of polyphenols in the production of beverages such as beer, wine, and apple juice. In wine clarification, laccase is used to remove phenols from white grape must (Ref. 62, 73). An example of an oxygen scavenging application is the prevention of the formation of off-flavor precursors in beer (Ref. 83, 84). Thus, a potential application of laccases can be oxygen scavenging during processing of foodstuffs. Low levels of oxygen, especially when processing foods at elevated temperatures, prevents oxidation of sensitive substances while preserving aromatic compounds or preventing the formation of off-flavours.

6.2 Use Levels

The enzyme preparation is used at minimum levels necessary to achieve the desired effect. The recommended use level of the laccase would be:

a. up to 40 LAMU per stick of chewing gum.
b. up to 100 LAMU per g of mouthwash.
c. up to 20 LAMU per breath mint tablet.
d. up to 20 LAMU per application of toothpaste.

6.3 Enzyme in Final Food

The enzyme is added to the chewing gum, mouthwash, breath mint tablets, and toothpaste. Estimates of the enzyme exposure are set forth in Section 7.6.

7 SAFETY EVALUATION

7.1 Safety of the Production Strain

The issues relevant to a safety evaluation of an enzyme preparation are the safety of the enzyme source, the enzyme component, the manufacturing process, and a consideration of dietary exposure (Ref. 2, 3, 19, 20). The safety of the production organism must be the prime consideration in assessing the probable degree of safety of an enzyme preparation intended for use in food (Ref. 2, 3). If the organism is non-toxigenic and non-pathogenic, then it is assumed that food or food ingredients produced from the organism, using the current Good Manufacturing Practice (cGMP) Regulations, is safe to consume (Ref. 4). Pariza and Foster (Ref. 2) define a non-toxigenic organism as "one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure" and a non-pathogenic organism as "one that is very unlikely to produce disease under ordinary circumstances." A. oryzae meets these criteria for non-toxigenicity and non-pathogenicity. In addition, A. oryzae is not considered pathogenic by JEFCA (Ref. 26).

Barbesgaard et al. reviewed the safety of *A. oryzae* and describe it as having a very long history of safe industrial use, being widely distributed in nature, and being commonly used for production of food grade enzymes (Ref. 27). *A. oryzae* is accepted as a constituent of foods (Ref. 26). *A. oryzae* has been used to produce soy sauce in the United States since before 1958 (Ref. 4, 27). Therefore, *A. oryzae* meets the criterion of "common use in foods in the US before 1958" and derived products can be considered "generally recognized as safe", GRAS (Ref. 4, 36).

A GRAS petition, 3G0016, proposing affirmation that enzyme preparations from *A. oryzae* are GRAS for use in food was submitted to FDA in 1973 (Ref. 28), and subsequently converted-in-part to a GRAS notice (GRN 000090) (Ref. 46) in accordance with the agency's proposed regulation, proposed 21 CFR 170.36 (Ref. 36). GRAS Notice No. GRN 000090 includes carbohydrase and protease from *A. oryzae*, and carbohydrase from *Rhizopus oryzae*. Each of the enzyme preparations described in GRN 000090 meets the general and additional requirements as set forth by the Food and Chemicals Codex (Ref. 24). Further, enzyme preparations from *A. oryzae* have been marketed in the U.S. as GRAS by Novozymes and other companies since that time. Therefore, enzyme preparations from *A. oryzae* are also considered GRAS (Ref. 2, 3, 26).

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An evaluation of the genetically modified production microorganism for the laccase, embodying the concepts initially outlined by Pariza and Foster in 1983 (Ref. 2), and further developed by IFBC in 1990, the EU SCF in 1991, the OECD in 1992, ILSI Europe Novel Food Task Force in 1996, FAO/WHO in 1996, JECFA in 1998, and Pariza and Johnson in 2001 (Ref. 3) demonstrates the safety of this genetically modified production microorganism strain. The components of this evaluation: the identity of the host strain, a description of the plasmids used, the sources and

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functions of the introduced genetic material, an outline of the genetic construction of the production strain, and some characteristics of the production strain and the enzyme derived from it are given in Section 2. Since the genetic modifications are well characterized and specific, and the incorporated DNA does not encode and express any known harmful or toxic substances, the laccase enzyme preparation derived from the genetically modified *A. oryzae* is considered safe (Ref. 2-4, 29). To confirm the safety of the enzyme, safety studies were performed on the enzyme preparation and are described in Section 7.6.

7.2 Safety of the Laccase Enzyme

Enzyme proteins themselves do not generally raise safety concerns (Ref. 2, 31-32). As indicated in Section 3, the laccase is an Urishiol oxidase (benzenediol: oxygen oxidoreductase), IUB EC 1.10.3.2, which is a group of multi-copper proteins of low specificity acting on both o- and p-quinols, and often acting on aminophenols and phenylenediamine. The semiquinone may react further either enzymatically or non-enzymatically.

A typical reaction is: 4 Benzenediol + O_2 = 4 Benzosemiquinone + 2 H_2O.

The laccase enzyme is approved for brewing in Denmark (Ref. 85). In the brewing industry the storage stability of the beer is a growing concern as the major producers want to sell world wide. By applying laccase during the mashing process precursors for trans-2-nonenal will not be produced, and the resulting beer will not develop off flavour when stored.

7.3 Safety of the Donor

The donor for the laccase gene is *Myceliophthora thermophila* strain E421. Opportunistic infections in humans caused by *Myceliophthora thermophila* have been reported. In recent years, three different cases of human infection caused by this species have been reported (Ref. 49-51). However, this species is not regarded as a true pathogen and it is not related to any known pathogenic fungi. *Myceliophthora thermophila* does not present any risk to healthy persons. An infection only proceeds in severely debilitated patients subsequent to a traumatic inoculation. The literature does not contain any information about toxic metabolites produced by *Myceliophthora*.

7.4 Laccases

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Microbial enzymes have been used in food production since 1952 (Ref. 33, 34). Laccases are naturally occurring in a wide variety of fruits, vegetables, fungi, coffee, and tea as discussed in Section 6. As discussed above in Section, 7.2, the laccase is approved for the brewing industry in Denmark (Ref. 85). Laccase has also been used for wine clarification. Laccase immobilized on a copper-chelate carrier was used successfully to remove phenols from white grape must (Ref. 47).

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7.5 Safety of the Manufacturing Process

The laccase meets the general and additional requirements for enzyme preparations as outlined in the monograph on Enzyme Preparations in the Food Chemicals Codex. As described in Section 4, the laccase preparation is produced in accordance with current Good Manufacturing Practices using ingredients that are acceptable for general use in foods, and other conditions that ensure a controlled fermentation. These methods are based on generally available and accepted methods used for production of microbial enzymes (Ref. 21-23).

7.6 Safety Studies

This section describes the studies and analyses performed to evaluate the safety of the use of Novozymes 18043.

7.6.1 Description of the Test Material

The safety studies described below were conducted on the batch of laccase, PPX 5720, comprising a mixture of three test batches that are produced in the same manner as in the production scale (see description given in Section 4), omitting stabilization and standardization. Key characteristics of the test batches, including the composition of batch PPX 5720, are described in a published safety paper entitled "Toxicological studies on laccase from *Myceliophthora thermophila* expressed in *Aspergillus oryzae*" (Regulatory Toxicology and Pharmacology, June 2002; Appendix B) and in the Summary of Toxicity Data (Appendix C) (see Section 7.5.2).

7.6.2 Laccase Safety Studies

The following studies were performed:

1. Acute oral toxicity in rats.
2. Acute inhalation toxicity in rats.
3. Acute dermal toxicity in rats
4. Acute toxicity, skin irritation in rabbits.
5. Acute toxicity, eye irritation in rabbits.
6. Skin sensitization: delayed contact hypersensitivity in guinea pigs.
7. Human skin sensitization.
8. Subacute/subchronic toxicity: two week oral dose range finder in rats.
9. Subacute/subchronic toxicity: 13 week oral toxicity in rats.
10. Gene mutation (Ames test with *S. typhimurium/E.coli*).
11. Chromosome aberrations (*in vitro* cytogenetics with human lymphocytes).
12. Acute toxicity for *Daphnia*.

The safety paper summarizes the safety studies and evaluates the safety in use of the laccase enzyme preparation produced by submerged fermentation of *Aspergillus oryzae* that contains a laccase gene originating from *Myceliophthora thermophila* (Appendix B). This paper concludes that there are no safety concerns when using this laccase enzyme preparation from the selected strain of A. oryzae expressing the gene encoding the M. thermophila in different oral care products.

The main conclusions of the safety studies can be summarized as follows:

- Oral administration to rats of up to 10.0 mL/kg body weight/day, which is equivalent to a total organic solids dosage of 1.72 g/kg body weight/day, for 13 weeks did not cause any adverse effects related to treatment, and this dosage represents the No-Observed-Adverse-Effect-Level (NOAEL) in this study.
- Laccase (PPX 5720) was not found to be mutagenic in the Salmonella typhimurium reverse mutation assay and did not cause chromosomal aberrations in cultured human lymphocytes. Thus, it was concluded that Laccase batch PPX 5720 is not genotoxic as no mutagenic activity was found in either Ames test or the human lymphocyte test.
- There was no evidence of inhalation toxicity or skin and eye irritation.
- There was no evidence of possible skin sensitization in a human skin sensitization test when laccase was tested at 10% (w/v). Thus, Laccase would appear to have a low skin sensitization potential.
- Laccase was tested in a Human Repeat Insult Patch Test (HRIPT) and it was concluded that Laccase has a low sensitization potential.

7.7 Estimates of Human Consumption and Safety Margin

The laccase enzyme product has an activity of 250 LAMU/g and an approximate content of 1.33% TOS (Total Organic Substances from the fermentation, mainly protein and carbohydrate components).

The laccase enzyme is an ingredient of chewing gum, mouthwash, breath mints, and toothpaste. The examples provided below serve to illustrate estimates of human consumption and safety margin for each application.

7.7.1 Estimated Human Consumption

7.7.1.1 Chewing Gum

The average human intake of chewing gum is estimated from the following references:

Market Research Corporation of America
Market Research Corporation of America, 14-Day Food Diary Data, 1991-1992.

USDA
USDA, Household Food Consumption Survey, *Food Intake and Nutritive Value of the Diets of Men, Women and Children in the United States*, 1969.
Based upon the Market Research Corporation of American survey in conjunction with the USDA survey, the mean intake of chewing gum per person per day is estimated to be 0.2 g.

21 CFR §101.12
According to 21 CFR §101.12, *Reference amounts customarily consumed per eating occasion (Table 2)*, consumption of chewing gum per person per eating occasion is 3 g.

Forbes, November 8, 1993 (p. 208)
According to Forbes, U.S. per capita consumption of chewing gum was 215 sticks in 1978, 168 sticks in 1986, and 183 sticks in 1992.

Average Chewing Gum Consumption:
Assumptions used to determine average chewing gum consumption in grams per person per day and its equivalence of chewing gum sticks per day:

i) It is assumed that all chewing gum products are produced using laccase, which contains the highest recommended dosage of Laccase.
ii) The calculation is made assuming that all TOS is released from the chewing gum product. That is, all the material in the gum is consumed.
iii) Chewing gum serving size varies from about 2 g to about 3 g. For calculation purposes, 1 stick of chewing gum weighs 3 g.
iv) Average consumption of chewing gum in g per person per year assuming 3 g per stick of chewing gum * 215 sticks per year (Forbes, 1993) is 645 g.
v) Thus, as a result of the assumptions given above, then an average consumption of chewing gum in g per person per day (645 g chewing gum per person per year / 365 days per year) is 1.77 g or 1.8 g chewing gum per person per day, which is equivalent to 0.6 sticks of chewing gum per day for the average consumer.

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95th Percentile Chewing Gum Consumption:
In order to demonstrate a worst case calculation, the 95th percentile is determined based on the average consumption determined above and these following assumptions:

It is assumed that the heavy users of chewing gum are at approximately the 95th percentile. For instance, some smokers may be considered to be heavy consumers of chewing gum and would fall into this category. Examination of food frequency and other types of food consumption surveys conducted in the U.S. show that the 95th percentile is roughly four times the mean intake (www.cfsan.fda.gov/~dms/opa-cg8.html). Therefore, the 95th percentile corresponds roughly to 7.2 g chewing gum per person per day (1.8 g chewing gum per person per day * 4 = 7.2 g), which is equivalent to 2.4 sticks of chewing gum per day.

These are exaggerations since:

- A significant amount of the TOS may remain in the gum matrix. The worse case scenario used assumes ingestion of the gum product and/or 100% release of the TOS.
- Not everyone chews gum and those who do use chewing gum will not always choose to use breath freshening chewing gum products. For example, according to analyzed consumption habits in Georgia, approximately 42% of respondents consumed chewing gum (Institute for Polling and Marketing, IPM Group Newsletter, May-June 2000).

The maximum dosage of laccase per stick of chewing gum is 40 LAMU, which corresponds to 0.16 g of laccase. Laccase contains 1.33% TOS. This gives a theoretical TOS exposure of content of 2.1 mg TOS per stick of chewing gum.

Based on the highest daily intake of 2.4 sticks of chewing gum per person per day, the daily intake per person of laccase corresponds to 2.1 mg * 2.4 = 5.0 mg TOS per day.

For an average person weighing 60 kg this corresponds to 8.3×10^{-5} g TOS/ kg body weight per day.

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7.7.1.2 Mouthwash

The average human intake of mouthwash is estimated from the following reference:

ECETOC
ECETOC, Centre for Ecotoxicology, Monograph No. 20, Percutaneous Absorption, Appendix B, August 1993, (http://www.ecetoc.org) .

In accordance with the ECETOC, the expected frequency of mouthwash use is 1-3 times per day and the average quantity per application is 12 g.

In order to demonstrate a worst case calculation, an exaggerated human intake is estimated using the following assumptions.

i) It is assumed that all mouthwash products contain laccase at the highest recommended dosage.
ii) The calculation assumes that all the mouthwash is swallowed and that all the TOS is ingested.
iii) It is assumed that the maximum frequency of use will be 3 times per day, which corresponds to 36 g mouthwash per person per day.

These are exaggerations since:

- According to the manufacturer's label, users of mouthwash are advised not to swallow the mouthwash.

The maximum dosage of laccase per mouthwash application is 100 LAMU, which corresponds to 0.4 g of laccase. Laccase contains 1.33% TOS. This gives a theoretical TOS exposure of content of 5.3 mg TOS per mouthwash application.

Based on the highest frequency of 3 daily uses, the daily intake per person of laccase corresponds to 5.3 * 3 = 15.9 mg TOS per day.

For an average person weighing 60 kg this corresponds to 2.7×10^{-4} g TOS per kg body weight per day.

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7.7.1.3 Breath Mints

The average human intake of breath mints is estimated from the following references:

Market Research Corporation of America
Market Research Corporation of America, 14-Day Food Diary Data, 1991-1992.

USDA
USDA, Household Food Consumption Survey, *Food Intake and Nutritive Value of the Diets of Men, Women and Children in the United States*, 1969.
Based upon the Market Research Corporation of American survey in conjunction with the USDA survey, the mean intake of breath mint per person per day is estimated to be 0.6 g per day.

21 CFR §101.12
According to 21 CFR §101.12, *Reference amounts customarily consumed per eating occasion* (Table 2), consumption of breath mint tablets per person per eating occasion is 2 g.

Average Breath Mint Consumption
Assumptions used to determine average breath mint consumption in grams per person per day and its equivalence of breath mint tablets per day:

i) It is assumed that all breath mint tablets are produced using laccase, which is used at the highest recommended dosage.
ii) The calculation is made assuming that all TOS is released from the breath mint tablet. That is, all the material in the breath mint is consumed.
iii) Breath mint tablet serving size varies from about 0.4 g to about 2 g. For calculation purposes, 1 breath mint weighs 2 g.
iv) Half of breath mint consumers eat between two and five rolls of breath mints per month. A roll of mints contains 12 mint tablets. Thus, half of all breath mint consumers eat between 24 and 60 mints per month or between 0.8 to 2 mint tablets per day.
v) Thus, for calculation purposes, it is assumed that the average consumption is 2 breath mint tablets per day for the average consumer.

95th Percentile Breath Mint Consumption
In order to demonstrate a worst case calculation, the 95th percentile is determined based on the average consumption determined above and these following assumptions:

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It is assumed that the heavy users of breath mint tablets are at approximately the 95th percentile. Examination of food frequency and other types of food consumption surveys conducted in the U.S. show that the 95th percentile is roughly four times the mean intake (www.cfsan.fda.gov/~dms/opa-cg8.html). Accordingly,

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the 95th percentile corresponds roughly to 2 breath mints per person per day * 4, which is 8 breath mints per day.

These are exaggerations since:

- Not everyone consumes breath mints. An estimated 63% of Americans consume breath mints on a monthly basis.

The maximum dosage of laccase per breath mint tablet is 20 LAMU, which corresponds to 0.08 g of laccase. Laccase contains 1.33% TOS. This gives a theoretical TOS exposure of content of 1.1 mg TOS per breath mint.

Based on the highest daily intake of 8 breath mints per person per day, the daily intake per person of laccase corresponds to 1.1 mg * 8 g = 8.8 mg TOS per day.

For an average person weighing 60 kg this corresponds to 1.5×10^{-4} g TOS/ kg body weight per day.

7.7.1.4 Toothpaste

The average human intake of toothpaste is estimated from the following reference:

ECETOC, Centre for Ecotoxicology, Monograph No. 20, Percutaneous Absorption, Appendix B, August 1993, (http://www.ecetoc.org).
In accordance with the ECETOC, the expected frequency of toothpaste use is 1-2 times per day and the average quantity per toothpaste application is 1.5 g.

In order to demonstrate a worst case calculation, an exaggerated human intake is estimated using the following assumptions.

i) It is assumed that all toothpaste products contain laccase at the highest recommended dosage.
ii) The calculation is made assuming that the toothpaste is swallowed and all the TOS is ingested.
iii) It is assumed that the frequency of use will be 2 times per day, which corresponds to 3 g toothpaste per person per day.

These are exaggerations since:

- According to the manufacturer's label, users of toothpaste are advised not to swallow the toothpaste after brushing.

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The maximum dosage of laccase per toothpaste application is 20 LAMU, which corresponds to 0.08 g of laccase. Laccase contains 1.33% TOS. This gives a theoretical TOS exposure of content of 1.1 mg TOS per toothpaste application.

Based on the highest frequency of 2 daily uses, the daily intake per person of laccase corresponds to 1.1 * 2 = 2.2 mg TOS per day.

For an average person weighing 60 kg this corresponds to 3.7×10^{-5} g TOS/ kg body weight per day.

7.7.2 Safety Margin

The safety margin is calculated as dose level with no adverse effect (NOAEL) divided by the estimated human consumption.

The NOAEL dose level in the 13 weeks oral toxicity study in rats was 10 ml/kg/day corresponding to 1.72 g TOS/kg/day.

The estimated human consumption is:

Chewing Gum:	8.3×10^{-5} g TOS/kg/day
Mouthwash:	2.7×10^{-4} g TOS/kg/day
Breath Mints:	1.5×10^{-4} g TOS/kg/day
Toothpaste:	3.7×10^{-5} g TOS/kg/day

The safety margin can thus be calculated to be:

Chewing Gum:	$1.72/8.3 \times 10^{-5} = 2.1 \times 10^{4}$
Mouthwash:	$1.72/2.7 \times 10^{-4} = 6.4 \times 10^{3}$
Breath Mints:	$1.72/1.5 \times 10^{-4} = 1.1 \times 10^{4}$
Toothpaste:	$1.72/3.7 \times 10^{-5} = 4.6 \times 10^{4}$

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7.8 Copper

7.8.1 Laccase and Copper

Laccase contains 4 Cu atoms per molecule. Copper sulphate is added to the fermentation broth to provide sufficient amounts of this essential co-factor (see Section 4). The safety studies described in Section 7 were all performed on the enzyme batch PPX 5720. This batch was obtained by mixing of 3 sub batches, each produced according to the description given in section 3, omitting stabilization and standardization.

The following chemical analyses have been established for the Laccase batch PPX 5720:

Composition analyses of PPX 5720	Unit	Contents
MtL Laccase Activity	LAMU/g	3130
Heavy Metals	ppm	233
Pb	ppm	<1
As	ppm	<0.3
Cd	ppm	<0.05
Hg	ppm	<0.05
Cu	ppm	229

Heavy Metals = Sum of Ag, As, Bi, Cd, Cu, Hg, Ni, Pb, Sb, Sn.

The concentration of Heavy Metals as defined above is an unusually high value for heavy metals in the batch concentrate analysis. This is due to the elevated copper content.

The laccase batch PPX 5720 has a copper content of 229 ppm for a laccase activity of 3130 LAMU/g. When the concentrates are formulated and standardised the copper content is 18.3 ppm for a 250 LAMU/g product.

7.8.2 Copper – An Essential Nutrient

Copper is a metal that has a role in the activation of molecular oxygen. Copper is an active participant in oxidoreductase enzymes such as laccase. Copper is an essential nutrient required for a number of metalloenzymes involved in hemoglobin function, carbohydrate metabolism, catecholamine biosynthesis, and cross-linking of collagen, elastin, and hair keratin. As a result of copper's involvement in these biochemical processes, adequate copper is an essential requirement of a diet for good health. Many foods naturally contain copper; foods rich in copper include liver, potatoes, seafood, grains and cereal products. The copper concentration of various foods ranges from 20 to 400 ppm (Ref. 68).

According to International Programme on Chemical Safety, copper is an essential element for good health (Ref. 31). Adverse health effects are related to deficiency as well as excess. The main source of copper for humans is their diet. However, particularly in Europe and the Americas, there is greater risk of health effects from deficiency of copper intake than from excess copper intake. Most dietary surveys show that humans consume about a milligram of copper everyday through food and drink. In the U.S., the median intake of copper from food is 0.93-1.3 mg/day for adults (0.016-0.021 mg Cu/kg/day using a 60-kg reference body weight). Although copper balance studies conducted on human volunteers suggest a minimum requirement of 1.0-2.6 mg/day, many people run a greater risk from a deficiency of copper intake than its excess. The Recommended Daily Allowance (RDA) has been set at 1.5-3 mg/day. The lower limit of the acceptable range of oral intake (AROI) is 20 µg Cu/kg body weight per day.

The greatest potential source of copper exposure is though drinking water such as water consumed from copper piping that sits overnight. EPA has established that drinking water should not contain more than 1.3 mg copper per liter of water. Copper concentrations in drinking water vary from a few ppb to 10 ppm. Biochemical mechanisms exist for controlling uptake and excretion of copper.

7.8.3 Current Regulations

- The standard specification for heavy metals (cited above as 233 ppm) is for safeguarding against the toxic metals Hg, Cd, Pb and As.

- The international body, JECFA, Fifty-third meeting, Rome, 1-10 June 1999, Summary and Conclusions, Annex 1 p. 18 (http://www.fao.org/ es/esn/ jecfa/ report53.pdf) has decided to replace the heavy metal limit test with specific limits for individual metals of concern, that is lead, cadmium, mercury and – when needed – arsenic. Limits for copper have not been indicated by JECFA, probably because there is no need for it.

- The United States of America's Code of Federal Regulations, Title 21 – Food and Drugs, Chapter I -- Food and Drug Administration, Department of Health and Human Services, Part 101-- Food Labelling, Sec. 101.9 Nutrition labelling of food, states a recommended daily intake (RDI) of 2 mg Cu to safeguard against copper deficiency.

- The European Council Directive 98/83/EC, 3 November 1998, states that the quality of water intended for human consumption stipulates a upper limit of 2 mg per litre drinking water to protect human health against toxic effects.

- The United States of America's Code of Federal Regulations, Title 21 – Part 184, Sec. 1261 - Direct food substance affirmed as generally recognized as safe when used as a nutrient supplement or as a processing aid.

7.8.4 Other Commercial Products Containing Copper

Two examples of commercial products containing copper gluconate are Certs® Cool Mint Drops® and Listerine's ListerinePocketPaks® – Oral Care Strips. Certs containing Retsyn® (a combination of partially hydrogenated cottonseed oil, copper gluconate, and flavouring) was introduced in 1960.

7.8.5 Estimated Human Consumption of Copper from Laccase Containing Breath Freshening Products

In order to illustrate "worst case" situations of copper exposure from laccase containing breath freshening products for chewing gum, mouthwash, breath mints, and toothpaste, the following calculations and conditions are set forth.

Assuming a use level of laccase corresponding to 100 LAMU per mouthwash application, 40 LAMU per stick of chewing gum, 20 LAMU per breath mint, and 20 LAMU per toothpaste application, the copper content is as follows:

Conditions:

Laccase Activity:	1 g of laccase = 250 LAMU
Cu Content/250 LAMU:	0.0183 mg Copper (Cu)
Assumption:	100% release of the copper.

Application	LAMU Dosage	Weight of Laccase (g)	Cu Exposure/ Application	Number of Applications/ Day (95th Percentile)	Maximum Cu Exposure/ Day	Maximum Cu Exposure (mg Cu/ kg/day)*
Chewing Gum	40/stick	0.16	0.0029 mg Cu	2.4 sticks	0.007 mg Cu	0.0001
Mouthwash	100/appl.	0.40	0.0073 mg Cu	3 appl.	0.022 mg Cu	0.0004
Mint tablets	20/mint tablet	0.08	0.0015 mg Cu	8 mint tablets	0.012 mg Cu	0.0002
Toothpaste	20/appl.	0.08	0.0015 mg Cu	2 appl.	0.003 mg Cu	0.00005

***Based on a 60-kg reference body weight**

One gram of a representative enzyme product contains 0.0183 mg Cu and 250 LAMU. A breath mint tablet is expected to contain up to 20 LAMU and 0.0015 mg Cu per tablet, therefore it would take 1,333 breath mint tablets/day to achieve FDA's recommended daily intake (RDI) of 2 mg Cu.

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Based on the results shown in the table above, the maximum copper exposure (mg Cu/ kg/day) for the 95th percentile user is 0.0001 mg Cu/kg/day for chewing gum, 0.0004 mg Cu/kg/day for mouthwash, 0.0002 mg Cu/kg/day for breath mints, and 0.00005 mg Cu/kg/day for toothpaste.

Based upon the information presented above, we conclude that the copper content in laccase-containing breath-freshening products is insignificant.

7.9 Results and Conclusion

The basic conclusions and guidelines for determining the safety of enzymes used in foods as stated by Pariza and Foster in 1983 (Ref. 2) and reiterated by FDA (Ref. 36) can be applied to laccase. In particular, enzyme preparations derived, using cGMPs, from a non-pathogenic and non-toxigenic microorganism, which does not produce antibiotics, would not ordinarily present a basis for a safety concern and will be safe to consume at the low levels encountered in processed foods.

The information presented in the previous sections and the safety evaluation discussed in this section demonstrate that laccase, a laccase enzyme preparation from a selected non-toxigenic strain of *Aspergillus oryzae* expressing the gene encoding a laccase from *Myceliophthora thermophila*, meets these criteria and is safe for use in the breath-freshening products.

8 LIST OF APPENDICES

A. Standard Method: LAMU – Laccase activity determination manual method. SOP No. EB-SM-0506.02/01.

B. Safety Paper: Brinch, D.S., and Pedersen, P.B. Toxicological Studies on Laccase from *Myceliopthora thermophila* Expressed in *Aspergillus oryzae*, Regulatory Toxicology and Pharmacology, 35:296-307, 2002 (June).

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Appendix A

Novo Nordisk

STANDARD METHOD

SOP No.: EB-SM-0506.02/01	COPY No.:
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APPROVING OFFICIALS	DATE
Prepared by: (GI) _____ Method Responsible	_____
Approved by: (SCIn) _____ Manager	_____
Approved by: (FIF) _____ Director of EBQM	_____

LAMU

Laccase activity determination
Manual method

SCOPE

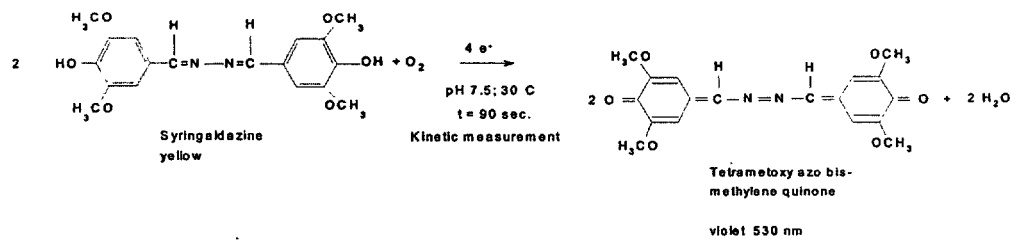
External services.

1. APPLICATION FIELD

Finished goods samples.

2. PRINCIPLE

Laccase (E.C. 1.10.3.2), p-diphenol: O_2 oxidoreductase, containing copper in the prosthetic group, uses molecular oxygen as a final electron acceptor. Atmospheric oxygen is directly reduced to 2 H_2O, during liberation of 4 electrons, without hydrogenperoxide being an intermediate step.
Laccase will under aerobic conditions catalyse the oxidation of syringaldazine forming tetrametoxy-azo bis methylene quinone. See reaction principle below.



2.1 REACTION MIXTURE CONDITIONS

Substrate	:	Syringaldazine, 6 mg/L
Buffer	:	Tris, 23 mM
pH	:	7.50
Temperature	:	30°C
Time of reaction	:	120 sec.
Enzyme working area	:	0.0011 - 0.0068 LAMU/mL
Wavelength	:	530 nm

3. DEFINITION OF UNITS

Laccase Myceliopthora Unit, LAMU is defined relatively to an enzyme standard under the conditions described in this method.
One LAMU was originally defined as the amount of enzyme which degrades 1 µmol syringaldazine per minute.

4. SPECIFICITY AND SENSITIVITY

Limit of detection LOD:	0.007 LAMU/mL
Limit of quantification LOQ:	0.05 LAMU/mL
Range:	0.05 - 0.30 LAMU/mL

5. APPARATUS

Spectrophotometer, cuvette thermostable	e.g.: Shimadzu UV-1601 PC
Thermo stable water bath	e.g.: Heto
Automate diluter	e.g.: Hamilton
Analytical balance	e.g.: Mettler, AT200
pH meter	e.g.: Radiometer, PHM 82

6. REAGENTS/SUBSTRATES

6.1 Maleic acid, 1.0 M

Maleic acid 37% paM *) 800380 ..... 23.2 g
Milli-Q water or similar quality ..... up to 200 mL

Maleic Acid is weighed out and added 150 mL water in a 200 mL volumetric flask. Stir until dissolved. Add up to the mark with water.

The reagent should be contained in a dark bottle properly labelled: reagent name, producers initials, production date, expiry date and safety label: **none**.

Storage stability at room temperature: 1 month.

*) pro analysi Merck

6.2 Tris, 1.0 M

Tris[hydroxymethyl]aminomethane Sigma T-1378 ..... 121.1 g
Milli-Q water or similar quality ..... up to 1000 mL

Tris is weighed out and added 800 mL water in a 1000 mL volumetric flask. Stir until dissolved. Add up to the mark with water.

The reagent should be contained in a bottle properly labelled: reagent name, producers initials, production date, expire date and safety label: **none**.

Storage stability at room temperature: 1 month

6.7 Triton X-100, 10%

Triton X-100 Sigma T-9284 ..... 25.0 g
Milli-Q water or similar quality ..... up to 250 mL

Weigh out 25.0 g Triton X-100 and add 150 mL water in a 250 mL volumetric flask. Stir until dissolved under weak heating. Add up to the mark with water.

Storage stability at 1-10°C: 1 month

6.4 Buffer, pH 7.50

Tris, 1.0 M (6.2) ..... 25.0 mL
Maleic acid, 1.0 M (6.1) ..... 10.0 mL
Triton X-100, 10% (6.3) ..... 5.0 mL
Milli-Q water or similar quality ..... up to 1000 mL

Pour 25 mL Tris 1.0 M into a 1000 mL volumetric flask and add about 700 mL water. Now add 10.0 mL Maleic acid, 1 M and 5.0 mL Triton X-100, 10%. Adjust pH to 7.50 ± 0.05 and add up to the mark with water. (**pH may not be adjusted with HCl**, because of Cl^- inhibiting effect on the laccase enzyme.)

The reagent should be labelled: reagent name, pH, producers initials, production date and safety label: **none**.

Prepare fresh daily.

6.5 Diluent (PEG 6000 solution 50g/L)

PEG 6000 paM 807491 ..... 250.0 g
Milli-Q water or similar quality ..... up to 5000 mL

PEG is weighed out and added 4500 mL water into a 5000 mL volumetric flask. Stir until dissolved. Add up to the mark with water.

The reagent should be labelled: reagent name, producers initials, production date and safety label: **none**.

Prepare fresh daily.

6.6 Syringaldazine, 0.56 mM

Syringaldazine anh Sigma S-7896 ..... 10.0 mg
Ethanol 96% ..... up to 50 mL

Rinse a 50 mL volumetric flask by Milli-Q water and ethanol to avoid traces of soap. Weight out 10.0 mg syringaldazine and add ethanol up to the mark. Stir until dissolved (app. 3 hours).

The reagent should be kept in a dark bottle properly labelled: reagent name, producers initials, production date, expire date and safety label: **very flammable**.

Storage stability at 1-10°C: 5 days.

6.7 Syringaldazine, 0.25 mM

Syringaldazine, 0.56 mM (6.6) ..... 4.40 mL
Milli-Q water or similar quality ..... up to 10 mL

Rinse a 10 mL volumetric flask by Milli-Q water and ethanol. Transfer 4.40 mL syringaldazine, 0.56 mM and add up to the mark with Milli Q-water.

The reagent should be kept in a dark bottle properly labelled: reagent name, producers initials, production date, expire date and safety label: **very flammable**.

Storage stability: up to 2 hours at room temperature.

7. SAMPLES AND STANDARDS

7.1 Standard curve

An enzyme standard dilution series in diluent (6.5) is used for calibration of each series of the assay. Six dilutions of 0.05 – 0.10 – 0.15 – 0.20 – 0.25 – 0.30 LAMU/mL were applied.

Example: Preparation of LAMU standard curve

Batch no.:	61-1100 (= 00HMJ0004)
Laccase activity:	276 LAMU/g
Weighing:	0.9056 g ± 0.0009 g
Flask:	50 mL
Solvent:	Diluent (reagent 6.5)

Dilution no.	Enzyme solution [µL]	Solvent (reagent 6.5) [µL]	Dilution factor	Concentration of Laccase [LAMU/mL]
1	10	990	100	0.05
2	20	980	50	0.10
3	30	970	33.33	0.15
4	40	960	25	0.20
5	50	950	20	0.25
6	60	940	16.67	0.30

7.2 Level Control

A stable, homogeneous sample that is representative for the product is assayed as level control.

7.3 Unknown samples

The Laccase samples are diluted to an expected activity of 0.25 LAMU/mL with the diluent (6.5).
Working range: 0.05 - 0.30 LAMU/mL.
For best precision limit the range to 0.2-0.3 LAMU/mL.

8. PROCEDURE

Reagents and the samples: standards, level control and unknown samples are prepared. Each weigh out is analyzed in duplicate.

Syringaldazine, 0.25 mM (reagent 6.7)	240 µL
30 °C	
Sample (7.1, 7.2 and 7.3)	80 µL
Mixed by dilutor pipetting with	
Buffer, pH 7.5 (reagent 6.4)	3.20 mL
Reading 60-120 sec at 530 nm	

Table.1 LAMU procedure

Place buffer (6.4) in thermo state bath for preheating to 30 ± 1 °C. Transfer 240µL syringaldazine, 0.25 mM (6.7) to a cuvette. Place the cuvette in a 30 ± 1 °C thermo stated cell. Add by dilutor direct into the cuvette 80µL sample simultaneous mixed with 3.20 mL buffer, pH 7.5 (6.4), preheated to 30 ± 1 °C. This mixes the entire reaction mixture. Avoid air in the cuvette. The absorption is followed at 530 nm, and the linear absorption increase per minute from 60-120 sec is calculated.

Alternatively the reaction mixture is mixed in the same proportions outside the cuvette and aspirated into a 30 ± 1 °C thermo stated photometer cell.
The detection might be simplified to reading off the absorbances at 60 and 120 sec only, followed by subtraction of the 60' from the 120'.

If 530 nm absorbance is >1.0 at 60 sec {against a blank where sample is replaced by diluent (6.5)} the sample should be diluted further and re-analyzed.

9. CALCULATION

The, almost linear, standard curve polynomial 2.order fit is calculated. The absorbance increase per minute of the sample reaction is entered and the LAMU/mL is read off from the standard curve. This is entered into further calculations as shown below:

$$LAMU/g = \frac{A \cdot V \cdot D}{W}$$

Example:

$$= \frac{0.2489 \, x \, 100 \, x \, 10}{0.8673} = 287 \text{ LAMU/g}$$

Where:

A:	result from standard curve [LAMU/mL]	0.2489
V:	dissolving volume [mL]	100

D:	further dilution of dissolved sample [mL/mL]	10
W:	weighing out [g]	0.8673

10. ACCURACY AND PRECISION

The accuracy is 99.7% and reproducibility is 5%.

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8. Validation of LAMU assay: F9600415, internal report

Appendix B

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Appendix C

Toxicology

Date : 19th November 2002
Ref.: NiB/DSc
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SUMMARY OF TOXICITY DATA (Edition 3)

Laccase from *Myceliophthora thermophila* expressed in *Aspergillus oryzae*

Author :
Ninna Berg

Issued by :
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Novo Allé
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Denmark

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1. ABSTRACT

The enzyme, laccase (IUB No. 1.10.3.2) is an oxido-reductase catalysing the oxidation of phenolic substrates by oxygen to quinones. In this summary "Laccase" means the enzyme produced by submerged fermentation of *Aspergillus oryzae* containing a gene coding for laccase from *Myceliophthora thermophila*. In the following, toxicity studies carried out with Laccase are summarised.

All animal studies were carried out in accordance with current EU and OECD guidelines and in compliance with the OECD principles of Good Laboratory Practice (GLP). The studies were carried out at Huntingdon Life Sciences, UK, in-house at Novo Nordisk or at TNO Nutrition and Food Research Institute, Delft, The Netherlands during the period September 1996 to August 1998. The Human Repeat Insult Patch Test was performed in compliance with the OECD principles of Good Clinical Practice (GCP) at IS Consultancy Ltd., Scotland, in the period from August 1999 to February 2000.

The main conclusions of the safety studies can be summarised as follows :

Laccase is non-toxic, given as a single orally administered dose of up to 12 ml/kg body weight (~ 39,025 LAMU/kg or 2.07 g TOS/kg) to rats.

The LC_{50} in rats after 4 hours inhalation of an aerosol of Laccase is in excess of 5.16 mg/l (~ 16.12 LAMU/l or 0.85 mg TOS/l).

The acute percutaneous median lethal dose (LD_{50}) of Laccase to rats was greater than 12 ml/kg body weight (~ 39,025 LAMU/kg or 2.07 g TOS/kg).

Laccase can be classified as non irritant to both rabbit skin and to the rabbit eye.

Oral administration to rats of up to 10 ml/kg body weight/day (~ 32520 LAMU/kg/day or 1.72 g TOS/kg/day) for 13 weeks has not revealed any significant toxic effects attributable to the test substance Laccase.

Laccase has shown no mutagenic activity in either Ames' test or the human lymphocyte test.

Laccase is non-toxic to Daphnia magna.

Laccase was not tested in a skin sensitisation test in guinea pigs, please read section about skin sensitisation for explanation.

Laccase was tested in a Human Repeat Insult Patch Test (HRIPT) and it was concluded that Laccase has a low sensitisation potential.

2. TEST SUBSTANCE

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2.1 Production of test substance

The batch of Laccase, PPX 5720, used for the present toxicological programme was a mixture of 3 sub-batches and was produced by submerged fermentation of *Aspergillus oryzae* containing a gene coding for laccase from *Myceliophthora thermophila*.

2.2 **Characterisation** (cf. table 1)
One batch was used in the toxicological studies and the characterisation is presented in table 1.

Batch PPX 5720 was a dark brown liquid.

The various dose levels are mostly given in Total Organic Substance (TOS = 100% ÷ water % ÷ ash % ÷ diluent %) per kg body weight (bw).

Table 1. Characterisation of the Laccase batch used in the toxicological studies.

	Batch No. PPX 5720
Activity, LAMU/g[1]	3130
Ash (600°C)	0.3%
Water (Karl-Fisher)	83.1%
Diluents	None
TOS	16.6%
Specific gravity (g/ml)	1.039

[1] Laccase units/g (LAMU/g). LAMU is the amount of enzyme which under the given analytical conditions catalyses the conversion of 1 μmol syringaldazine per minute. The laccase specific activity is 80 LAMU/mg pure active enzyme protein.

3. TOXICITY DATA

3.1 General toxicity

3.1.1 Acute toxicity

3.1.1.1 Acute Oral Toxicity (Limit Test) in Rats

In accordance with OECD guidelines No. 401, a limit test was performed with one group of rats consisting of 5 females and 5 males.

The dose was administered by gavage in a total volume of 12 ml undiluted Laccase, batch PPX 5720/kg bw (equivalent to 39,025 LAMU/kg or 2.07 g TOS/kg).

The animals were observed 1½ and 4 hours after administration and subsequently every day during the entire 14-day observation period. After the observation period, the animals were sacrificed and examined pathologically.

Neither body weight, clinical signs nor gross pathological examination revealed any treatment related effects.

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In conclusion Laccase causes no toxic effects, given as a single orally administered dose of up to 12 ml/kg bw (equivalent to 39,025 LAMU/kg or 2.07 g TOS/kg) to rats.

3.1.1.2 Acute Inhalation Toxicity (Limit Test) in Rats

In accordance with OECD guideline No. 403 and EEC B2, a Limit Test was performed with one group of rats consisting of 5 females and 5 males.

The animals were exposed by snout only exposure for 4 hours to air containing aerosolised Laccase, batch PPX 5720, at a concentration of 5.16 mg/l (equivalent to 16.12 LAMU/l or 0.85 mg TOS/l).

Particle size measurements revealed that the respirable fraction (% of aerosol mass < 7 μm) was 90%. The mass median aerodynamic diameter was 2.7 μm, in total a very high respirable fraction. The animals were observed during exposure, immediately after exposure and also with half hourly intervals during the first 2 hours after the exposure and then every day in the subsequent 14-day observation period. After the observation period, the animals were sacrificed and examined pathologically.

Immediately following exposure, the rats exhibited a wet and unkempt appearance. All rats showed recovery within 2 h of exposure. Reduced food consumption and body weight gain were recorded for 1 day following exposure. No animals died during the observation period, and the pathological examination revealed no abnormalities related to the exposure.

In conclusion, Laccase causes only minimal evidence of toxicity in rats after 4 hours of inhalation of a concentration of 5.16 mg/l (~ 16.12 LAMU/l or 0.85 mg TOS/l). The LC_{50} for Laccase is in excess of 5.16 mg/l of air.

3.1.1.3 Acute Dermal Toxicity (Limit Test) in Rats

In accordance with current EU guidelines, a limit test was performed with one group of rats consisting of 5 females and 5 males.

The dose was applied to the closely clipped dorsum of each animal in a total volume of 12 ml undiluted Laccase, batch PPX 5720/kg bw (equivalent to 39,025 LAMU/kg or 2.07 g TOS/kg), and was covered by an occlusive dressing for 24 hours.

The animals were observed after dosing and subsequently every day during the entire 14-day observation period. After the observation period, the animals were sacrificed and examined pathologically.

No deaths occurred and no clinical effects were seen relating to the test substance, however, weight loss was observed in one female and a very low weight gain was observed in another female, for which there was no explanation. There was no abnormal findings at necropsy.

Under the conditions of this study, the acute percutaneous median lethal dosage (LD_{50}) of Laccase, batch No. PPX 5720, was greater than 2070 mg TOS/kg.

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3.1.1.4 Acute Toxicity, Skin Irritation

In accordance with the current OECD and EU guideline, a primary skin irritation test in rabbits was performed with Laccase, batch PPX 5720.

On the intact skin of the clipped back of each the 3 rabbits, 0.5 ml of the test substance was applied under a patch of gauze (2.5 x 2.5 cm). A semiocclusive bandage was utilised and the adjacent areas of untreated skin served as control.

After 4 hours exposure the patches and the remaining test substance were removed by gentle washing, and the skin reaction, erythema-, eschar- and edema formation was evaluated after ½-1, 24, 48 and 72 hours after application. None of the rabbits exhibited any dermal reactions at the test side.

In conclusion, Laccase can be classified as non-irritant to the skin according to the criteria of EU.

3.1.1.5 Acute Toxicity, Eye Irritation

In accordance with current OECD and EU guidelines, a test for eye irritation in rabbits was performed with Laccase, batch PPX 5720.

The eyes of each animal were checked for defects or irritation before instillation and only animals without defects were used. 0.1 ml of batch PPX 5720 was placed in the conjunctival sac of the left eye by pulling the lower eyelid away from the eyeball. The lids were gently held together for about one second after instillation. The untreated eye served as control.

The ocular reactions were recorded at ½-1, 24, 48 and 72 hours after instillation according to the specified guidelines. Additionally, one rabbit was read 96 hours after instillation. Only slight reactions on conjunctiva were observed after instillation of the test substance with no reactions on cornea or iris. The conjunctival reactions were fully reversible within 96 hours after instillation.

In conclusion Laccase can be classified as a "non irritant" to the rabbit eye according to the criteria of EU.

3.1.1.6 Skin Sensitisation, comment to guinea pig models

Enzymes are recognized to be potential respiratory allergens and should be classified as R42 "may cause sensitisation by inhalation" in the context of the Dangerous Substances Directive (92/32/EEC). However, enzymes are not regarded as skin sensitisers. A review by the Association Internationale de la Savonerie et de la Détergence/ Association of Manufacturers of Fermentation Enzyme Products (AIS/AMFEP) AD-hoc group in 1995 concluded that enzymes should not be classified as skin sensitisers in the context of the Dangerous Substances directive.

There is no animal model that has been developed or validated for assessing proteins as skin sensitisers. It is clear that any foreign protein has the ability to cause an immune response, largely by the formation of a specific antibody. Thus to assess the potential to cause a T-cell mediated response, it is vital to ensure that the model is not biased towards B-cell responses. It has been the experience of the AIS/AMFEP that all foreign proteins, e.g. enzyme preparations, can be made to generate skin reactions in suitably treated animals, including the OECD/EU recognised guinea pig maximization test and the Buehler test. The predominance of the antibody response in the guinea pig makes it impossible to discern to what extent, if any, a T-cell mediated type IV hypersensitivity reaction takes place. Therefore it is necessary to rely heavily on risk assessment and human experience.

Therefore, since there is no animal model developed or validated for assessing proteins as contact skin sensitisers and enzymes are well documented to not be skin sensitisers, we consider testing enzymes in guinea pig sensitisation models as unnecessary and a guinea pig sensitisation study has therefore not been performed.

According to the AIS/AMFEP document, there has not been a case of allergic contact dermatitis in the enzyme production industries and in a period of 25 years, billions of consumers have been exposed to enzymes through detergent and other cleaning products without any evidence of skin sensitisation.

3.1.1.7 Human Skin Sensitisation

A total of one hundred and four volunteers took part in the human repeat insult patch test (HRIPT), of whom one hundred fully completed the study.

Laccase, batch PPX 5720, was applied at a concentration of 10.0% w/v in distilled water in aliquots of 0.50 ml to each occlusive Webril® / Blenderm® patch (2 cm x 2 cm). In the induction phase, all test subjects had 9 induction patches (occlusive) applied, each for 24 hours, on a Monday-Wednesday-Friday schedule lasting for 3 weeks. As induction progressed a gradual build-up of mild erythema was seen. Two weeks after application of the final induction patch, each subject had one patch soaked with the present test material at a concentration of 10% w/v in distilled water applied to each arm, removed after 24 hrs and graded after 48 and 96 hrs.

There was evidence of possible skin sensitisation observed on three of the one hundred subjects who completed the study (D440, D1005, D1032). In all three subjects there were similar responses to one or both of the other two enzymes tested at the same time in this study. A consultant dermatologist was present to confirm the reactions observed at the 96 h challenge stage.

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Three subjects were re-challenged at two concentrations (10% w/v and 1% w/v) after approx. one month to conclude on reactions at the first challenge.

The re-challenge patch test confirmed that none of these subjects (D440, D1005, D1032) gave any evidence of skin sensitisation to the Laccase test material, when tested alone at both 10.0% w/v and 1.0% w/v.

It can be concluded that on the evidence of the present study the enzyme Laccase would appear to have a low sensitisation potential.

3.1.2 Subacute / Subchronic Toxicity

3.1.2.1 Two Week Oral Dose Range Finder in Rats

A subacute toxicity study in rats with a duration of two weeks served as a dose range finding study to the 13-week study. The study was performed according to the current guideline in EU.

Groups of 5 male and 5 female CD rats were dosed for 2 weeks with Laccase, batch PPX 5720, orally *via* gavage at dose levels of 0, 0.17, 0.57 and 1.72 g TOS/kg body weight/day, at a constant dose volume of 10 ml per kg body weight.

At the end of 2 weeks treatment, all animals were killed and subjected to necropsy.

There were no adverse effects on mortality, clinical signs, body weights, food and water consumption, necropsy findings or organ weights.

In conclusion there were no obvious effects of treatment with Laccase at dose levels up to 1.72 g TOS/kg bw/day.

3.1.2.2 13 Week Oral Toxicity in Rats

A subchronic toxicity study in rats with a duration of 13 weeks was performed at dose levels chosen from the results in the 2 week dose range finder. The study was conducted in accordance with the current guideline in OECD.

Groups of 10 male and 10 female CD rats were dosed for 13 weeks with Laccase, batch PPX 5720, orally *via* gavage at dose levels of 0, 17, 172 and 1720 mg TOS/kg body weight/day, at a constant dose volume of 10 ml per kg bodyweight.

During week 13 of the study blood samples were taken from all animals for laboratory investigations.

At the end of 13 weeks treatment, all surviving animals were killed and subjected to necropsy. All animals were given a detailed post mortem examination with major organs being weighed and placed in fixative.

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There were no effects of treatment on clinical signs, body weight, food and water consumption, food conversion efficiency, ophthalmic findings, haematology and clinical chemistry profiles, or on organ weights and macroscopic or microscopic findings.

In conclusion administration of Laccase at dosages of up to 1720 mg TOS/kg body weight/day to CD rats orally via gavage for 13 weeks was well tolerated and without toxic effects. The no-observed-adverse-effect level (NOEAL) was 10 mL or 1720 mg TOS per kg body weight per day.

3.1.3 Mutagenic Potential

3.1.3.1 Testing for Mutagenic Activity (Ames Test)

Laccase, batch PPX 5720, was examined for mutagenic activity in four histidine-dependent strains of *Salmonella typhimurium*, strains TA98, TA100, TA1535 and TA1537 using the "treat and plate" procedure, and the tryptophan-dependent strain *Escherichia coli* WP2uvrA using the direct plate incorporation method.

The study was conducted in accordance with OECD Guideline for testing of chemicals, No. 471 : "Bacterial Reverse Mutation Assay" (July 1997), European commission Annex V. Test Method B14, and the Japanese guideline : "Concrete Operation Procedure of Mutagenicity Study Using Bacteria. Ministry of Labour, 1988", concerning the general specifications of the test. But the exposure of test bacteria in liquid culture ("treat and plate"), as it is applied in this study with strains of *Salmonella*, is not specifically described in any guidelines.
The study was conducted in the presence and absence of an activating system derived from rat liver (S-9 mix). All tests included solvent (purified water) and positive controls with and without S-9 mix.

Each test with each strain, was conducted on two separate occasions. In both these experiments bacteria were exposed to 6 doses separated with bi-sections. The highest dose level applied was 5 mg per ml with *Salmonella* strains and 5 mg per plate with the *E. coli* strain (5 mg test substance is equivalent to 0.83 mg TOS).

Marked increases in the number of revertant colonies were induced by the standard mutagens when examined under similar conditions as the test substance, confirming the sensitivity of the test.
Treatment of bacteria with Laccase, batch PPX 5720 in the absence and the presence of S9 did not result in significant and dose related increases in mutation levels compared to those seen in concurrent solvent controls.

It was concluded that Laccase did not induce gene mutations in bacteria in either the absence or presence of S9, when tested under the conditions employed in this study.

3.1.3.2 Human Lymphocyte Cytogenetic Assay

The study was designed to follow the current guidelines of OECD and EU.

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Chromosomal aberration assays were performed with duplicate, human peripheral blood incorporating a preliminary and 2 main tests. The tests were conducted with and without S-9 mix.

Cultures, established approx. 48 h before testing, were treated for 3 h in the presence or continuously for 19 or 43 hrs in the absence of S-9 mix. Cultures were harvested after 19 and 43 hours.

The tests were performed using a range of concentrations of Laccase, PPX 5720, to cover the appropriate range of toxicity :

- 19 hour sampling time, without S-9 mix : 25, 50 and 100 µg TOS/ml
  with S-9 mix : 1000, 3000 and 5000 µg TOS/ml
- 43 hour sampling time, without S-9 mix : 100 µg TOS/ml
  with S-9 mix : 5000 µg TOS/ml

Small, but statistically significant increases in the frequency of metaphases with chromosomal aberrations were observed at several test points, compared to the solvent control values. These increases were not considered to be biologically significant for the following reasons :

- When gap-type aberrations were included in the analysis, four cultures had frequencies of aberrant metaphases which narrowly exceeded the historical control range at this laboratory (0-9%). However, gaps were of questionable significance.
- When gaps were excluded, only one culture had a frequency value (5%) which exceeded the historical control range (0-4%).
- The increases, excluding gaps, were poorly reproducible between the two main tests.

The results fit the stated criteria for a negative response.

It was concluded that Laccase under the conditions of test, did not show any evidence of clastogenic activity.

3.1.4 Ecotoxicology

3.1.4.1 Acute Toxicity for Daphnia (Limit Test)

The acute toxicity of Laccase, batch PPX 5720, to the fresh-water crustacean *Daphnia magna* was determined according to the current OECD and EU Guideline.

A limit test at a concentration of 100 mg TOS/l was carried out with 20 Daphnia divided in 4 batches with the same number of Daphnia in the controls. The test duration was 48 hours.

The test solution was clear throughout the test. The results of the test were as follows :

	Nominal concentration mg TOS/l
48 h EC50 (mobility)	> 100
48 h EC100 (mobility)	≥ 100
48 h NOEC (condition)	≥ 100

In conclusion, Laccase is not toxic to *Daphnia magna*.

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4. REFERENCES

4.1 Study Reports

Laccase, SP 809, batch No. PPX 5720. Acute Oral Toxicity (Limit Test) in Rats. Novo Nordisk Study No. 968232, 1996. File No. QR-9616937.

Laccase. Acute Inhalation Study in Rats (4-hour snout only exposure). HLS Study No. NLE/187. Novo Nordisk Study No. 966028, 1997.File No. ED-9713101.

Laccase, batch PPX 5720. Acute Dermal Toxicity (Limit test) in Rats. Novo Nordisk Study No. 968233, 1997. File No. QR-9616938.

Laccase, batch No. PPX 5720. Acute Dermal Irritation/Corrosion in Rabbits. Novo Nordisk Study No. 968234, 1996. File No. QR-9616846.

Laccase, batch No. PPX 5720. Eye Irritation/Corrosion in Rabbits. Novo Nordisk Study No. 968235, 1997. File No. QR-9616861.

Laccase, PPX 5720. Preliminary Toxicity Study by Oral Gavage Administration to CD rats for 2 weeks. HLS Study No. NLE/185. Novo Nordisk Study No. 966029, 1997. File No. 1998-00550-01.

Laccase, PPX 5720. Toxicity Study by Oral (Gavage) Administration to CD rats for 13 Weeks. HLS Study No. NLE/186. Novo Nordisk Study No. 966030, 1997. File No. 1998-00563-01.

Laccase (Batch Number : PPX 5720) : Test for Mutagenic Activity with strains of *Salmonella typhimurium* and *Escherichia coli*. Novo Nordisk Study No. 988041, 1998. File No. 1998-07387-01.

Laccase, batch PPX 5720. Lymphocyte Cytogenetic Study. HLS Study No. NLE/188. Novo Nordisk Study No. 966031, 1997. File No. 1998-00571-01.

Report : Static acute toxicity test with Laccase, batch PPX 5720, and *Daphnia magna*. TNO Report No. V96.744. Novo Nordisk Study No. 966032, 1996. File No. ED-9710141.

H.R.I.P.T. on Enzyme Laccase (batch PPX 5720). ISC Project No. 0539. Novo Nordisk Study No. 996043, 2000. File No. 2000-01433-01.

4.2 Guidelines

EEC, 1992. Annex to Commission Directive 92/69/EEC. *The Journal of the European Communities* **L383A**, 29 December.

OECD, Guidelines for testing of Chemicals. Section 3 and 4 : Health effects. Organisation for Economic Co-operation and Development, Paris.

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End Submission

M

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Fax

To: Susan J. Carlson, Ph.D. **From:** Denise Bernstein

Co.: FDA, Division of Biotechnology and

GRAS Notice Review **Dept:** Regulatory Affairs

Fax: 202-418-3179 **Pages:** (25) including cover sheet

cc: **Date:** May 16, 2003

☐ Urgent ☒ For Review ☐ Please Reply/Comment ☐ For Your Information

Subject: GRAS Notice No. GRN 000122

Message: A draft response to your emails dated April 16 & 17, 2003 is being forwarded to you by facsimile for your review. Please note that Appendices 1, 2, 5 & 7 are enclosed and only the top page of the remaining Appendices is included in this package. Once we've discussed the draft, a complete package will be forwarded by overnight mail.

I look forward to speaking with you early next week.

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Confidential information may be contained in this fax and is intended only for the use of the addressee. If you are not the addressee, please do not copy or deliver this to anyone else. If you receive this telefax by mistake, please telephone the sender. Thank you.

Novozymes North America, Inc.
Regulatory Affairs
77 Perry Chapel Church Road
P.O. Box 576
Franklinton, North Carolina 27525

Tel:919-494-3152 *Fax: 919-494-3420* *Internet: www.novozymes.com*

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DRAFT

May 16, 2003

Susan J. Carlson, Ph.D.
Division of Biotechnology and
GRAS Notice Review, HFS-255
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

Re: GRAS Notice No. GRN 000122

Dear Susan J. Carlson, Ph.D.:

In response to your recent emails of April 16 and 17th, thank you for the opportunity to clarify the lineage of the production strain Mt-3; and to clarify and provide additional information on the nontoxigenicity and nonpathogenicitiy of *Aspergillus oryzae*, particularly *Aspergillus oryzae* A1560.

Lineage of the production strain Mt-3
The production strain is designated Mt-3. A flowchart of the lineage of the production strain is attached as suggested in your email (**Appendix 1**). For your convenience, an updated version of Sections 2.1, 2.2, and 2.5 is enclosed (**Appendix 2**).

Non-toxigenicity & non-pathogenicity of Aspergillus oryzae, including strain A1560
The information presented below addresses the nontoxigenicity and nonpathogenicity of *A. oryzae*, including *A. oryzae* A1560.

Section 7.1 of the original submission sets forth the definitions of nontoxigenicity and nonpathogenicity - a nontoxigenic organism was defined by Pariza and Foster (1983) (**Appendix 3**) as "one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure" and a nonpathogenic organism as "one that is very unlikely to produce disease under ordinary circumstances." According to the definitions set forth by Pariza and Foster, *A. oryzae*, including *A. oryzae* A1560, meets these criteria for nontoxigenicity and nonpathogenicity.

Novozymes North America, Inc.
77 Perry Chapel Church Road
P.O. Box 576
Franklinton, North Carolina 27525

Tel: 919-494-3000 *Fax: 919-494-3450* *Internet: www.novozymes.com*

According to a second review article (Pariza and Johnson, 2001) **(Appendix 4)**, "the safety of the production strain should be the primary consideration in evaluating enzyme safety" and is the basis for establishing the "Safe Strain Lineage" concept - "[t]horoughly characterized nonpathogenic, nontoxigenic microbial strains, particularly those with a history of safe use in food enzyme manufacture, are logical candidates for generating a *safe strain lineage...*" *A. oryzae* has a long history of safe use and is considered a safe strain as set forth by the original submission and by further information provided in this response.

- The taxonomic characteristics of the host strain *A. oryzae* A1560 as set forth in Section 2.2 of the original submission was confirmed by the Centraalbureau voor Schimmelcultures. Reference 16 of the original submission, "Identification Report on Strain A1560 Centraalbureau voor Schimmelcultures, Baarn, Holland, 1998," is enclosed to support that this is indeed *A. oryzae* **(Appendix 5)**.

- The laccase is manufactured in accordance with current Good Manufacturing Practices for food **(Section 4 of the original submission)**. Further, the quality management system used in the manufacturing process complies with ISO 9001. ISO is an acronym for International Organization for Standardization (www.iso.ch/), which is a network of national standards institutes from 145 countries working in partnership with international organizations, governments, industry, business and consumer representatives. ISO serves as a bridge between public and private sectors.

- Section 7.1, page 21, of the original submission discusses the review article of Barbesgaard et al. regarding the safety of *A. oryzae* (Barbesgaard, P., et al., Mini-review. On the safety of *Aspergillus oryzae*: a review, *Appl. Microbiol. Biotechnol.*, 36:569-572, 1992 **(Appendix 6)**. The authors conclude that "*A. oryzae* does not produce aflatoxins or any other cancerogenic metabolites. The absence of significant levels of mycotoxins in industrial products is regularly checked." This reference strongly supports the safety of *A. oryzae*, namely its nonpathogenic and nontoxigenic nature.

- The pathogenic potential of *A. oryzae* strain A1560 was evaluated. One pathogenicity study entitled "Report - Pathogenicity of *Aspergillus oryzae* to rats after oral administration, Novo Nordisk Study No. 1288" concluded that "*A. oryzae* A1560 Is non pathogenic to rats after oral administration" **(Appendix 7)**.

- Four batches of laccase have been analyzed for toxins according to the former JECFA specifications to document the absence of these toxins. The results are shown below in **Table 1**.

Table 1: Mycotoxin analyses of four laccase batches produced with *A. oryzae* Mt-3

	OMF 0005	OMF 0006	OMF 0008	OMF 0009
Aflatoxin B_1	ND	ND	ND	ND
Ochratoxin A	ND	ND	ND	ND
Sterigmatocystein	ND	ND	ND	ND
T2-toxin	ND	ND	ND	ND
Zearalenon	ND	ND	ND	ND

ND = Not Detected

The production strain *A. oryzae* Mt-3 does not produce aflatoxin. *A. oryzae* has been used in the food industry for centuries and has not been shown to produce aflatoxins (*Aspergillus oryzae*, TSCA Section 5(h)(4) Exemption: Final Decision Document, U.S. Environmental Protection Agency, Biotechnology Program Under Toxic Substances Control Act (TSCA), Section IIIC, http://www.epa.gov/opptintr/ biotech/fra/ fd007.htm) (**Appendix 8**).

- *A. oryzae* has a history of safe use in the food industry. *A. oryzae* has been used for hundreds of years in the production of food products such as soy sauce, miso, and sake "without recorded incidents" (*Aspergillus oryzae*, TSCA Section 5(h)(4) Exemption: Final Decision Document, U.S. Environmental Protection Agency, Biotechnology Program Under Toxic Substances Control Act (TSCA), Section I, (**Appendix 8**). More specifically, *A. oryzae* has been used to produce soy sauce in the United States since before 1958 and meets the criterion of "common use in foods in the US before 1958" and, as a result, [products derived from *A. oryzae*] can be considered "generally recognized as safe" – GRAS (**Appendices 6 & 9** - IFBC Ref- Ch4, 1990). Further, according to a 1987 report by JECFA (Joint FAO/WHO Expert Committee on Food Additives), "different varieties of *A. oryzae* are used in certain parts of the world in the preparation of foods" and enzymes derived from *A. oryzae* "should be considered as normal constituents of food" (**Appendix 10**, pages 16 &17).

- The Summary of Toxicity Data (**Appendix C of the original submission**) and the published safety paper (**Appendix B of the original submission**) document the safety of laccase by conducting a series of toxicological tests including that it was not found to be mutagenic in the *Salmonella typhimurium* reverse mutation assay, nor did it cause chromosomal aberrations in cultured human lymphocytes.

- Section 2.8 of the original submission states that "the production organism does not end up in food", which is "an established specification for the commercial product as specified in Section 5.2."

- Novozymes has received Agency Response Letters for enzymes derived from the well-known industrial production strain *A. oryzae* A1560 (e.g., GRAS Notice Nos. GRN 000008, 000034, 000043, 000075, 000103 and 000106).

Conclusion

Based upon full consideration of the laccase GRN Notification and the information presented above, we conclude that *Aspergillus oryzae* A1560 is nonpathogenic and nontoxigenic.

We would like to take this opportunity to thank you for reviewing our submission. We hope this package addresses your outstanding concerns. Should you have additional questions or comments, please contact me directly at ((b) (6)

Sincerely yours,

Denise Bernstein
Sr. Regulatory Specialist

Enclosures: List of Appendices
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GRAS Notice No. GRN 000122

May 16, 2003

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List of Appendices

Appendix 1	Flow Chart - Lineage of A. oryzae production strain Mt-3.
Appendix 2	Laccase GRAS Notification - Updated Version of Sections 2.1, 2.2 & 2.5.
Appendix 3	Pariza, M.W. and Foster, E.M. Determining the Safety of Enzymes Used in Food Processing, *Journal of Food Protection*, 46:5:453-468, 1983.
Appendix 4	Pariza, M.W. and Johnson, E.A. Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing: Update for a New Century, *Regulatory Toxicology and Pharmacology*, 33:173-186, 2001.
Appendix 5	Report on Strain A1560, Centraalbureau voor Schimmelcultures, Baarn, The Netherlands, 1988.
Appendix 6	Barbesgaard, Peder, Heldt-Hansen, Hans Peter and Diderichsen, Børge. On the Safety of *Aspergillus oryzae:* a Review, *Appl. Microbiol. Biotechnol.*, 36:569-572, 1992.
Appendix 7	Report - Pathogenicity of *Aspergillus oryzae* to rats after oral administration. Novo Nordisk Study No. 1288, 1997.
Appendix 8	*Aspergillus oryzae*, TSCA Section 5(h)(4) Exemption: Final Decision Document, U.S. Environmental Protection Agency, Biotechnology Program Under Toxic Substances Control Act (TSCA), Section IIIC, http://www.epa.gov/ opptintr/biotech/fra/ fd007.htm).
Appendix 9	IFBC (International Food Biotechnology Council). Chapter 4: Safety Evaluation of Foods and Food Ingredients Derived from Microorganisms in Biotechnologies and Food: Assuring the Safety of Foods Produced by Genetic Modification. Regulatory Toxicology and Pharmacology 12:S1-S196, 1990.
Appendix 10	JEFCA, Evaluation of certain food additives and contaminants, Thirty-first report of the Joint FAO/WHO Expert Committee on Food Additives. Technical Report Series 759. World Health Organization, Geneva, 1987.

GRAS Notice No. GRN 000122

May 16, 2003

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Appendix 1

Lineage of A. oryzae production strain Mt-3

A 1560 (IFO 4177)	Host
↓	Delete *pyrG* gene.
HowB 101 (pyrG mutant)	
↓	Disrupt TAKA amylase gene; decreased amylase activity. Southern blot analysis demonstrates that two of the three resident TAKA amylase genes have been disrupted.
#18 (reduced TAKA amylase activity)	
↓	Selection of spontaneous pyrG mutant by plating of spores on agar plates containing 5-fluoro-orotic acid (FOA) (cells having an intact pyrG gene copy are killed by FOA).
#18P1 (reduced TAKA activity, pyrG mutant)	
↓	Disrupt the final resident TAKA amylase gene.
HowB711 (TAKA amylase negative)	
↓	The plasmid pRaMB17.WT (laccase expression vector) is transformed into the host strain HowB711 using selection on acetamide.
HowB711/RaMB17.WT	
↓	Grow transformants on medium with acetamide as the sole nitrogen source and screening for co-expression of the laccase. A single colony is selected.
Mt-3	The resultant production strain from HowB711.RaMB17.WT following mutation with nitrosoguanidine and screening for high yield of laccase to produce an amylase negative, *M. thermophila* laccase expressing strain.

Appendix 2

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Laccase GRAS Notification - Updated Version of Sections 2.1, 2.2 & 2.5

2. PRODUCTION MICROORGANISM

2.1 Production Strain

The *Aspergillus oryzae* strain, designated Mt-3, was constructed by transformation of the recipient strain, designated HowB711, by incubating protoplasts with pRaMB17.WT (laccase expression vector) and pToC90 (selectable marker). This genetically modified production organism complies with the OECD (Organization for Economic Cooperation and Development) criteria for GILSP (Good Industrial Large Scale Practice) microorganisms (Ref. 1). It also meets the criteria for a safe production microorganism as described by Pariza and Foster, Pariza and Johnson, and several expert groups (Ref. 2-9).

The DNA fragment used in the strain construction contains strictly defined fungal chromosomal DNA fragments and synthetic DNA linker sequences. The specific DNA sequences include a laccase enzyme gene from *Myceliopthora thermophila* strain E421 (CBS 117.65); an *Aspergillus nidulans* selectable marker gene, *amdS* (acetamidase) (Ref. 10); well-characterized non-coding regulatory sequences including the *Aspergillus niger strain* BO-1 terminator (Ref. 11), the TAKA-amylase promoter from *Aspergillus oryzae* strain HW325 (Ref. 12), and a fragment from a well known sequence from *Escherichia coli* plasmids pUC19 and pUC18 (Ref. 13).

2.2 Recipient Organism

The recipient microorganism, designated How B711, used in the construction of the laccase production strain is a genetically modified derivative of *A. oryzae* A1560 by means of site-directed gene disruption to cause interruption of the three resident TAKA amylase genes. The obtained strain *A. oryzae* How B711 is a derivative of the fully characterized, well-known industrial production strain of *A. oryzae* (Ahlburg) Cohn. The strain was obtained from the Institute for Fermentation, Osaka, Japan (IFO) and is designated strain IFO 4177 (synonym A1560).

The taxonomic characteristics of the host strain of *A. oryzae* A1560 are the following:

Name:	*Aspergillus oryzae*
Class:	Blastodeuteromycetes (Hyphomycetes)
Order:	Phialidales (Moniliales)
Genus:	*Aspergillus*
Species:	*Oryzae*

This classification has been confirmed by the Centraalbureau voor Schimmelculutres, Baarn, Holland (Ref. 16).

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2.5 Construction of the Recombinant Production Organism

The recombinant DNA molecule was introduced into the recipient *A. oryzae* How B711 by incubating protoplasts with pRaMB17.WT (laccase expression vector) and pToC90 (selectable marker). The selection of transformants was achieved by growing on a medium with acetamide as the sole nitrogen source and screening for co-expression of the *M. thermophila* laccase. A single colony of transformant HowB711/RaMB17.WT was selected.

The production strain is obtained from transformant HowB711/RaMB17.WT following mutation with nitrosoguanidine (NTG) and screening for high yield of the *Myceliophthora thermophila* laccase.

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Pages 000094 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

CENTRAALBUREAU VOOR SCHIMMELCULTURES

Postal address:
P.O. Box 273,
3740 AG BAARN
The Netherlands

Visiting address:
Oosterstraat 1,
3742 SK BAARN

Telephone:
02154-11841

Appendix 5

BEST ORIGINAL COPY

Uw/Your Ref:
Onze/Our Ref: RAS/as

F-9501135

Dr. R.I. Nielsen
Novo BioKontrol

NOVO Industri A/S
Novo Alle
DK-2880 BAGSVAERD

BAARN, 07.01.1988

Dear Dr. Nielsen,

Enclosed you find our identification report of the Aspergillus strain (A 1560).
We did only observe sparse sclerotium development on our Czapek Yeast medium.
Enclosed you will find the invoice for this service.
I am happy to use this opportunity to show the great importance of taxonomic investigations and its implication for the applied mycology like your own experiments in NOVO. However particularly the genera Penicillium ans Aspergillus are still problematic and a general consensus is strongly needed. When I organized the First International Penicillium and Aspergillus Workshop in May 1985 a first step was made to start the international dialogue and some progress could be made (see also R.A. Samson & J.I Pitt, (1985). Advances in Penicillium and Aspergillus Systematics Plenum Publishers, New York). At present I am planning to organize a second workshop in May 1989 and I hope the same successful output could be reached. Since the results of our taxonomic consensus have a direct implication to your research at NOVO, I wonder if your company would be interested to support the second workshop with (your) sponsorship.
I would very much appreciate to hear your response. If necessary I can supply more detailed data about the meeting, the participants and the support needed.

My personal wishes for the New Year.

Yours sincerely,

Dr. R.A. Samson

CENTRAALBUREAU VOOR SCHIMMELCULTURES - P.O. BOX 273 - BAARN

Identification report

Strain A1560 sent by NOVO Industri A/S
Novo Alle, DK-2880 Bagsvaerd, Denmark

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Aspergillus oryzae (Ahlburg) Cohn

Colonies on Czapek agar attaining a diameter of 4.0-4.5 cm within 7 days at 24°C, greenish yellow near Olive Yellow (Ridgway, Pl. XXX), Dark Olive Buff (Pl. XL) or Yellowish Citrine (Pl. XVI). Somewhat floccose near centre often with long conidiophores intermixed with some aerial mycelium. Reverse not coloured. Conidial heads radiate, 100-300 µm diam. Conidiophores arising from the substrate, mostly 3-4 mm long and 8-20 µm in diam., finely roughened. Vesicles mostly subglobose to slightly clavate, variable in size but mostly 50-70 µm in diam. Phialides flask shaped, directly borne on vesicle or sometimes on metulae. Abortive or atypical phialidic development often present. Conidia globose to subglobose, often ellipsoidal to pyriform when young, variable in size, but mostly 3-6 µm in diam., when ellipsoidal up to 7-8 µm in long axis, at first smooth-walled, becoming slightly to finely roughened with age.
Colonies on 2% Maltextractagar growing faster (6-7 cm in diam) but thinner, yellow-green near Vetiver-green (Pl. XLVII). Morphology similar as on Czapek. Sclerotia were not observed in 14 day-old cultures (only sparsely sclerotium development observed on Czapek Yeast Agar).

Note: The taxonomy used is in accordance to Raper & Fennell (1965) - The genus Aspergillus, Williams & Wilkens Co, Baltimore. The species concept in the Aspergillus flavus group is still problematic, because of the great variability. The strain is classified in Aspergillus oryzae based on colony colour, floccose growth, uni- and biseriate conidial heads, size and ornamentation of the conidia. Identification was only based on morphological criteria and not on biochemical properties (e.g. mycotoxin-production.

Legends to figures.

Fig 1-5 Conidial heads 1-2 Young heads showing uniseriate and biseriate pattern 3-4 Atypical phialidic development, some phialides not producing conidia but with elongated growth. 5. Abortive conidial head with sparse conidiation. 6. Conidia Micrographics taken at 7 days, all 1000x.

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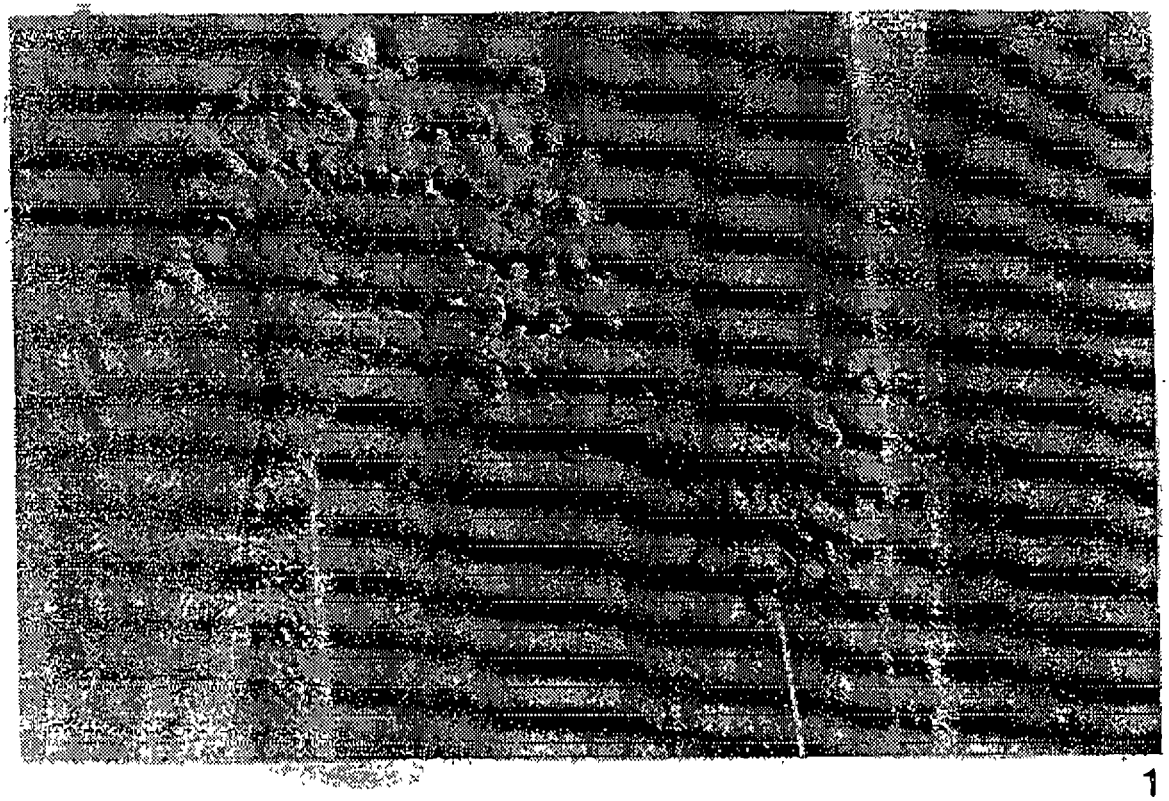

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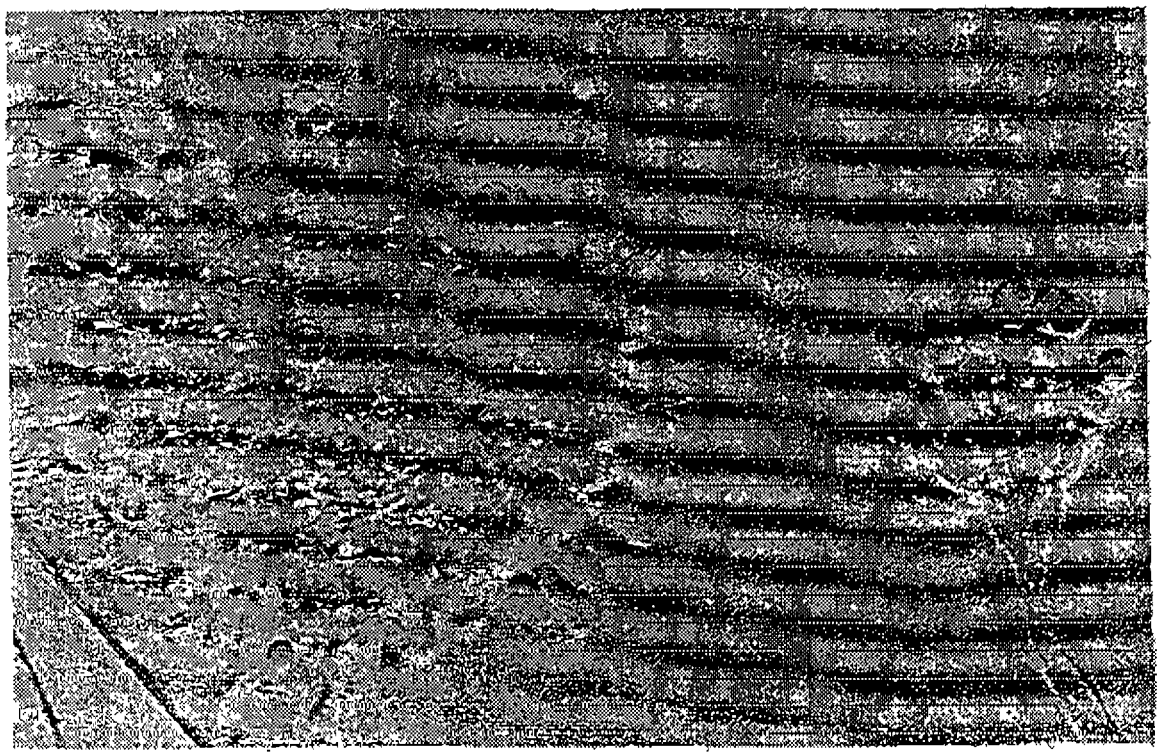

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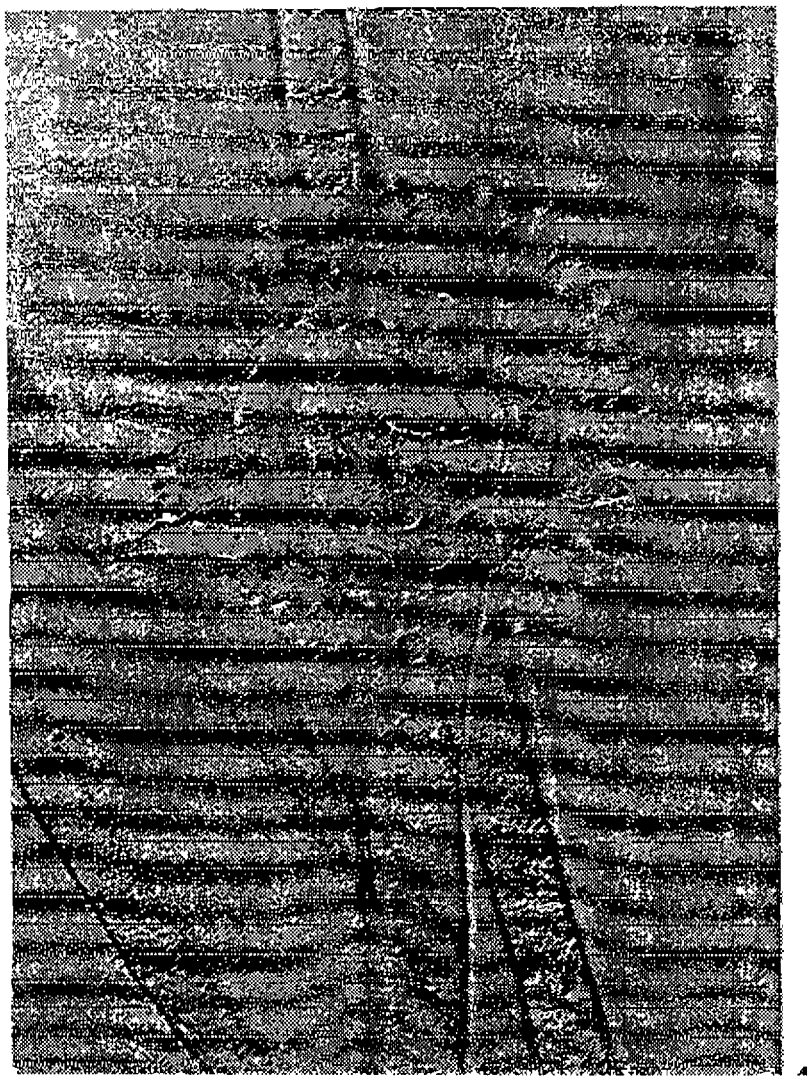

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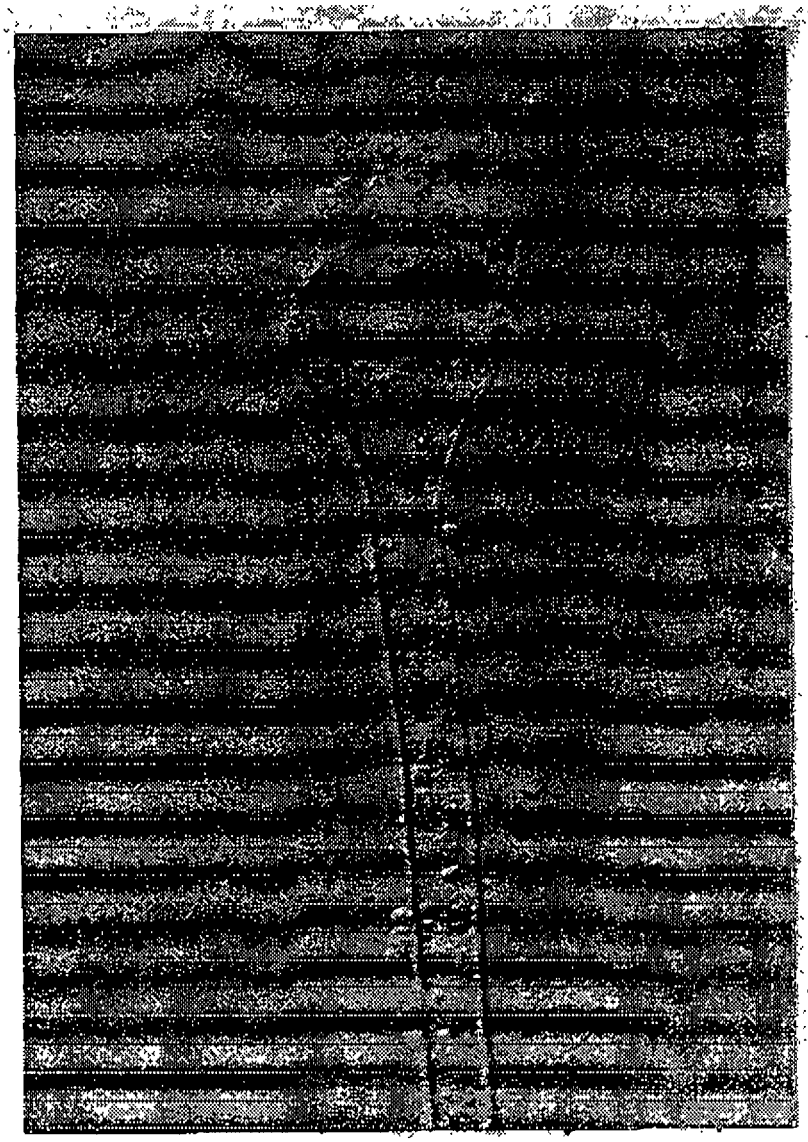

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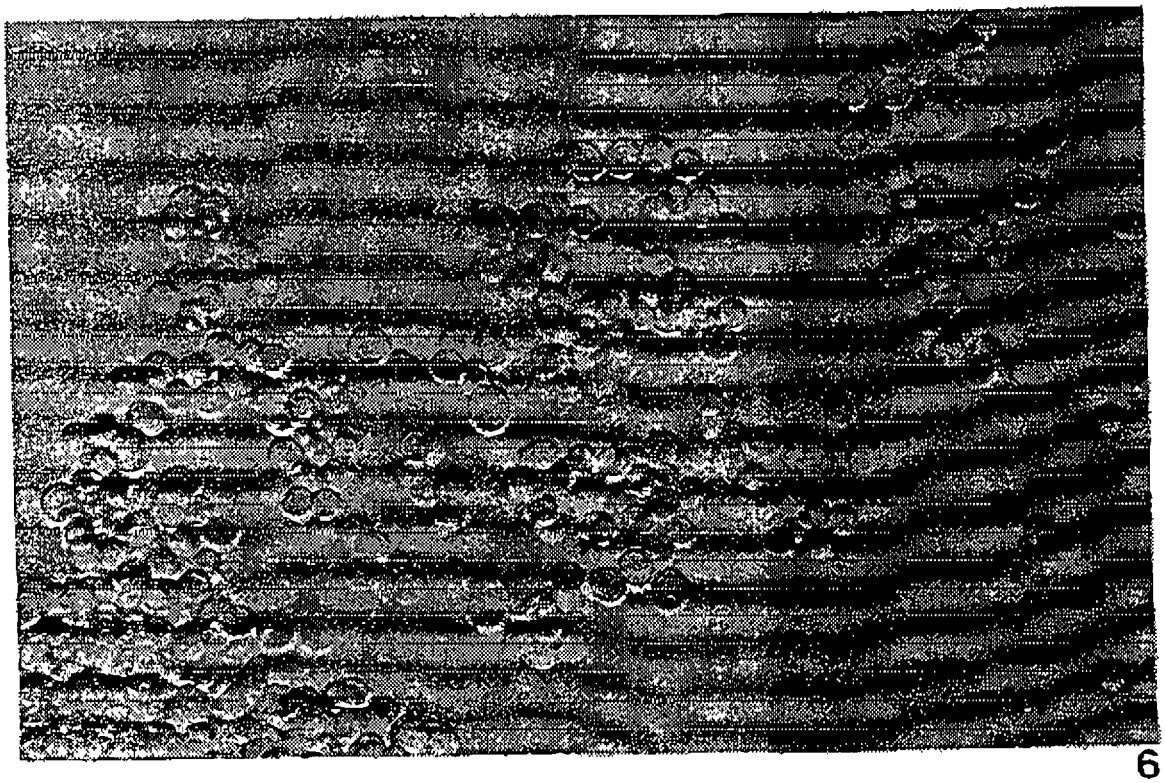

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Appendix 7

Novo Nordisk

Enzyme Business Toxicology

Date : 1997-09-08
File: ED-9713942
Ref.: ASB/DSc

REPORT

Pathogenicity of *Aspergillus oryzae* to rats after oral administration

Novo Nordisk Study No: 1288

This report is a translation of the original report on the study 1288.
Tables and appendices are not included.

Author:
Anne Sietske de Boer

Issued by:
Novo Nordisk A/S
Novo Allé
2880 Bagsvaerd
Denmark

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000102

1. GENERAL INFORMATION

TEST FACILITY	Novo Nordisk A/S Novo Allé DK - 2880 Bagsvaerd
DEPARTMENTS INVOLVED	EB Toxicology Animal Unit, Bagsvaerd
ARCHIVE	Enzyme Business Information & Documentation (EB INFODOC) Novo Nordisk A/S Novo Allé DK - 2880 Bagsvaerd

PERSONNEL INVOLVED IN THE STUDY

STUDY DIRECTOR	Anne Sietske de Boer, D.V.M. Hans Peter Heldt-Hansen
LABORATORY ASSISTANT	Jens Bak

DATE OF FINAL REPORT

Date : 1997-09-09

Anne Sietske de Boer
D.V.M.
Study Director

000103

2. SUMMARY

Study design:
The aim of the study was to evaluate the pathogenicity of the production strain *Aspergillus oryzae* A1560 and to investigate the possibility of this organism to colonise in the gastrointestinal tract of rats.

The test strain was grown in a nutrient broth. The nutrient broth and the fungi was homogenised prior to dosing.

The fungi was dosed orally to a group of five male and five female Wistar rats. The animals were observed for any clinical reactions to treatment for 10 days. All rats were subjected to macroscopic pathological investigation after the terminal kill at day 10. During the ten day observation period faeces from each animal was collected daily and examined for the presence of the dosed micro-organism.

Result:
The oral administration of *Aspergillus oryzae* to rats did not cause any clinical reaction to the test animals during the ten day observation period. The micro-organism was recovered the day after dosing in only one animal.

Conclusion:
It is concluded that *Aspergillus oryzae* A1560 is non pathogenic to rats after oral application and that the micro-organism is unable to colonise in the gastrao-intestinal tract of the rat.

3. AIM OF THE STUDY

The aim of this study was to investigate the pathogenic potential of *Aspergillus oryzae* to rats when dosed orally and to investigate the capability of the micro-organism to colonise in the gastro-intestinal tract.

4. MATERIAL

4.1 Test material

Aspergillus oryzae was cultured in nutrient broth for 3 day at 30° C. At the termination of the growth period the broth was blended to achieve a homogenous suspension of the test material.

The viable count of the broth before administration of the test substance to the rats was 3×10^6 colony forming units/ml.

4.2 Animals

Male and female Wistar rats were received from Møllegaard Breeding Centre. Lille Skensved, Denmark.

The animals were acclimatised for four days in cages holding five animals of same sex. Thereafter the rats were caged singly for three days in cages having grid floors. The rats were fed standard rat maintenance diet (Brood Stock Feed for Rats and Mice - R3 - Ewos).

During the acclimatisation period and the test period the animals were weighed at the arrival and at termination of the acclimatisation and there after once each fifth day.

4.3 Dosing

The rats were dosed orally a single dose of the micro-organism after depriving of food for 20 hours. All animals were dosed either concentrated test material or the control substance (0.9% NaCl) according to the dosing scheme:

Test substance	Dosage viable cells/kg bw	Animal number Males	Females
Sodium chloride	-	1 - 5	6 - 10
A. oryzae A 1560	3×10^7	21 - 25	26 - 30

4.4 Observation
The rats were observed daily during the acclimatisation period and the test period. The animals observed 30 minutes and 2 hours after dosing and at least once daily the following 10 days.

4.5 Termination of the study
All animals were killed by CO_2+O_2 anaesthesia and bleeding and autopsy.

5. RESULTS FROM THE ANIMAL EXPERIMENTS

5.1 Mortality and clinical symptoms
No animals died or showed clinical symptoms during the study.

5.2 Body weight
The body weight development was not affected by treatment.

5.3 Gross pathology
No macroscopic alterations were observed at the autopsy.

6. MICROBILOGICAL RECOVERY. METHOD AND RESULTS

6.1 Method
The faeces of the rats was collected daily and examined immediately after.

The faecal sample was diluted 5 times by sterile water and mixed until a complete suspension was present.

From each suspension 1 ml was transferred to each of 4 different Petri dishes (ø=13 cm) and was incubated for 30°C for 4-6 days.

At the evaluation the number of colonies of filamentous fungi and the number of these which have morphological similarities with the test organism. The final identification is performed by RIE (rocket immunoelectroforesis).

6.2 Results
The micro-organism was recovered for faeces from one animal one day after dosing, only.

7. DISCUSSION AND CONCLUSION

Based upon the experimental procedures it can be concluded that *A. oryzae* A1560 is non pathogenic to rats after oral administration. Furthermore it is concluded that the micro-organism is unable to colonise in the gastro-intestinal tract of the rat.

Appendix 8

U.S. Environmental Protection Agency

Biotechnology Program Under Toxic Substances Control Act (TSCA)

Aspergillus oryzae TSCA Section 5(h)(4) Exemption: Final Decision Document

Fax-On-Demand:

Telephone: (202) 401-0527

Item No.: 3172

FINAL DECISION DOCUMENT:

TSCA SECTION 5(H)(4) EXEMPTION FOR

ASPERGILLUS ORYZAE

I. BACKGROUND

In the September 1, 1994, Federal Register (59 FR 45526), the Environmental Protection Agency (EPA) proposed at 40 CFR Part 700 under section 5(h)(4) of the Toxic Substances Control Act (TSCA), Tier I and Tier II exemptions. These exemptions, which would be found at § 725.400, are exemptions from EPA review and expedited EPA review, respectively, for certain microorganisms under certain use conditions. EPA proposed to include Aspergillus oryzae at § 725.420 as a candidate recipient microorganism for the tiered exemptions. Aspergillus oryzae is an asexual, ascomycetous fungus used for hundreds of years in the production of soy sauce, miso and sake without recorded incidents. It has also been used in the fermentation industry for production of enzymes and other organic compounds.

This final decision document describes the basis for EPA's decision to include Aspergillus oryzae as a recipient microorganism at § 725.420.

II. CONDITIONS OF EXEMPTION

EPA recognizes that some microorganisms present a low risk when used under specific conditions at general commercial use. Therefore, EPA proposed to institute expedited regulatory processes for certain microorganisms under these specific conditions at the general commercial use stage. Microorganism uses that are exempt would meet criteria addressing: (1) performance based standards for minimizing the numbers of microorganisms emitted from the manufacturing facility; (2) the introduced genetic material; and (3) the recipient microorganism. Microorganisms that qualify for these exemptions, termed Tier I and Tier II, must meet a standard of no unreasonable risk in the exempted use.

To evaluate the potential for unreasonable risk to human health or the environment in developing these exemptions, EPA focuses primarily on the characteristics of the recipient microorganisms. If the recipient is shown to have little or no potential for adverse effects, introduced genetic material meeting the specified criteria would not likely significantly increase potential for adverse effects. As further assurance that risks would be low, EPA is also specifying procedures forminimizing numbers of organisms emitted from the facility. When balanced against resource savings for society and expected product benefits, these exemptions will not present unreasonable risks.

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AM

Fax

To: Susan J. Carlson, Ph.D. **From:** Denise Bernstein

Co.: FDA, Division of Biotechnology and GRAS Notice Review **Dept:** Regulatory Affairs

Fax: 202-418-3179 **Pages:** (2 x 45) including cover sheet

cc: **Date:** May 19, 2003

☐ **Urgent** ☒ **For Review** ☐ **Please Reply/Comment** ☐ **For Your Information**

Subject: GRAS Notice No. GRN 000122

Message: A draft response to your emails dated April 16 & 17, 2003 is being forwarded to you by facsimile for your review. Once we've discussed the draft, a complete package will be forwarded by overnight mail.

I look forward to speaking with you early this week.

Confidential information may be contained in this fax and is intended only for the use of the addressee. If you are not the addressee, please do not copy or deliver this to anyone else. If you receive this telefax by mistake, please telephone the sender. **Thank you.**

Novozymes North America, Inc.
Regulatory Affairs
77 Perry Chapel Church Road
P.O. Box 576
Franklinton, North Carolina 27525

Tel:919-494-3152 *Fax: 919-494-3420* *Internet: www.novozymes.com*

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DRAFT

May 16, 2003

Susan J. Carlson, Ph.D.
Division of Biotechnology and
GRAS Notice Review, HFS-255
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

Re: GRAS Notice No. GRN 000122

Dear Susan J. Carlson, Ph.D.:

In response to your recent emails of April 16 and 17th, thank you for the opportunity to clarify the lineage of the production strain Mt-3; and to clarify and provide additional information on the nontoxigenicity and nonpathogenicitiy of *Aspergillus oryzae*, particularly *Aspergillus oryzae* A1560.

Lineage of the production strain Mt-3
The production strain is designated Mt-3. A flowchart of the lineage of the production strain is attached as suggested in your email (**Appendix 1**). For your convenience, an updated version of Sections 2.1, 2.2, and 2.5 is enclosed (**Appendix 2**).

Non-toxigenicity & non-pathogenicity of Aspergillus oryzae, including strain A1560
The information presented below addresses the nontoxigenicity and nonpathogenicity of *A. oryzae*, including *A. oryzae* A1560.

Section 7.1 of the original submission sets forth the definitions of nontoxigenicity and nonpathogenicity - a nontoxigenic organism was defined by Pariza and Foster (1983) (**Appendix 3**) as "one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure" and a nonpathogenic organism as "one that is very unlikely to produce disease under ordinary circumstances." According to the definitions set forth by Pariza and Foster, *A. oryzae*, including *A. oryzae* A1560, meets these criteria for nontoxigenicity and nonpathogenicity.

Novozymes North America, Inc.
77 Perry Chapel Church Road
P.O. Box 576
Franklinton, North Carolina 27525

Tel: 919-494-3000 *Fax: 919-494-3450* *Internet: www.novozymes.com*

According to a second review article (Pariza and Johnson, 2001) (**Appendix 4**), "the safety of the production strain should be the primary consideration in evaluating enzyme safety" and is the basis for establishing the "Safe Strain Lineage" concept - "[t]horoughly characterized nonpathogenic, nontoxigenic microbial strains, particularly those with a history of safe use in food enzyme manufacture, are logical candidates for generating a *safe strain lineage...*" *A. oryzae* has a long history of safe use and is considered a safe strain as set forth by the original submission and by further information provided in this response.

- The taxonomic characteristics of the host strain *A. oryzae* A1560 as set forth in Section 2.2 of the original submission was confirmed by the Centraalbureau voor Schimmelcultures. Reference 16 of the original submission, "Identification Report on Strain A1560 Centraalbureau voor Schimmelcultures, Baarn, Holland, 1998," is enclosed to support that this is indeed *A. oryzae* (**Appendix 5**).

- The laccase is manufactured in accordance with current Good Manufacturing Practices for food (**Section 4 of the original submission**). Further, the quality management system used in the manufacturing process complies with ISO 9001. ISO is an acronym for International Organization for Standardization (www.iso.ch/), which is a network of national standards institutes from 145 countries working in partnership with international organizations, governments, industry, business and consumer representatives. ISO serves as a bridge between public and private sectors.

- Section 7.1, page 21, of the original submission discusses the review article of Barbesgaard et al. regarding the safety of *A. oryzae* (Barbesgaard, P., et al., Mini-review. On the safety of *Aspergillus oryzae*: a review, *Appl. Microbiol. Biotechnol.*, 36:569-572, 1992 (**Appendix 6**). The authors conclude that "*A. oryzae* does not produce aflatoxins or any other cancerogenic metabolites. The absence of significant levels of mycotoxins in industrial products is regularly checked." This reference strongly supports the safety of *A. oryzae*, namely its nonpathogenic and nontoxigenic nature.

- The pathogenic potential of *A. oryzae* strain A1560 was evaluated. One pathogenicity study entitled "Report - Pathogenicity of *Aspergillus oryzae* to rats after oral administration, Novo Nordisk Study No. 1288" concluded that "*A. oryzae* A1560 is non pathogenic to rats after oral administration" (**Appendix 7**).

- Four batches of laccase have been analyzed for toxins according to the former JECFA specifications to document the absence of these toxins. The results are shown below in **Table 1**.

Table 1: Mycotoxin analyses of four laccase batches produced with *A. oryzae* Mt-3

	OMF 0005	OMF 0006	OMF 0008	OMF 0009
Aflatoxin B_1	ND	ND	ND	ND
Ochratoxin A	ND	ND	ND	ND
Sterigmatocystein	ND	ND	ND	ND
T2-toxin	ND	ND	ND	ND
Zearalenon	ND	ND	ND	ND

ND = Not Detected

The production strain *A. oryzae* Mt-3 does not produce aflatoxin. *A. oryzae* has been used in the food industry for centuries and has not been shown to produce aflatoxins (*Aspergillus oryzae*, TSCA Section 5(h)(4) Exemption: Final Decision Document, U.S. Environmental Protection Agency, Biotechnology Program Under Toxic Substances Control Act (TSCA), Section IIIC, http://www.epa.gov/opptintr/ biotech/fra/ fd007.htm) (**Appendix 8**).

- *A. oryzae* has a history of safe use in the food industry. *A. oryzae* has been used for hundreds of years in the production of food products such as soy sauce, miso, and sake "without recorded incidents" (*Aspergillus oryzae*, TSCA Section 5(h)(4) Exemption: Final Decision Document, U.S. Environmental Protection Agency, Biotechnology Program Under Toxic Substances Control Act (TSCA), Section I, (**Appendix 8**). More specifically, *A. oryzae* has been used to produce soy sauce in the United States since before 1958 and meets the criterion of "common use in foods in the US before 1958" and, as a result, [products derived from *A. oryzae*] can be considered "generally recognized as safe" – GRAS (**Appendices 6 & 9** - IFBC Ref- Ch4, 1990). Further, according to a 1987 report by JECFA (Joint FAO/WHO Expert Committee on Food Additives), "different varieties of *A. oryzae* are used in certain parts of the world in the preparation of foods" and enzymes derived from *A. oryzae* "should be considered as normal constituents of food" (**Appendix 10, pages 16 &17**).

- The Summary of Toxicity Data (**Appendix C of the original submission**) and the published safety paper (**Appendix B of the original submission**) document the safety of laccase by conducting a series of toxicological tests including that it was not found to be mutagenic in the *Salmonella typhimurium* reverse mutation assay, nor did it cause chromosomal aberrations in cultured human lymphocytes.

- Section 2.8 of the original submission states that "the production organism does not end up in food", which is "an established specification for the commercial product as specified in Section 5.2."

- Novozymes has received Agency Response Letters for enzymes derived from the well-known industrial production strain *A. oryzae* A1560 (e.g., GRAS Notice Nos. GRN 000008, 000034, 000043, 000075, 000103 and 000106).

Conclusion

Based upon full consideration of the laccase GRN Notification and the information presented above, we conclude that *Aspergillus oryzae* A1560 is nonpathogenic and nontoxigenic.

We would like to take this opportunity to thank you for reviewing our submission. We hope this package addresses your outstanding concerns. Should you have additional questions or comments, please contact me directly at (919) 494-3152.

Sincerely yours,

Denise Bernstein
Sr. Regulatory Specialist

Enclosures: List of Appendices
10 Appendices

GRAS Notice No. GRN 000122

May 16, 2003

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List of Appendices

Appendix 1	Flow Chart - Lineage of A. oryzae production strain Mt-3.
Appendix 2	Laccase GRAS Notification - Updated Version of Sections 2.1, 2.2 & 2.5.
Appendix 3	Pariza, M.W. and Foster, E.M. Determining the Safety of Enzymes Used in Food Processing, *Journal of Food Protection*, 46:5:453-468, 1983.
Appendix 4	Pariza, M.W. and Johnson, E.A. Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing: Update for a New Century, *Regulatory Toxicology and Pharmacology*, 33:173-186, 2001.
Appendix 5	Report on Strain A1560, Centraalbureau voor Schimmelcultures, Baarn, The Netherlands, 1988.
Appendix 6	Barbesgaard, Peder, Heldt-Hansen, Hans Peter and Diderichsen, Børge. On the Safety of *Aspergillus oryzae:* a Review, *Appl. Microbiol. Biotechnol.*, 36:569-572, 1992.
Appendix 7	Report - Pathogenicity of *Aspergillus oryzae* to rats after oral administration. Novo Nordisk Study No. 1288, 1997.
Appendix 8	*Aspergillus oryzae*, TSCA Section 5(h)(4) Exemption: Final Decision Document, U.S. Environmental Protection Agency, Biotechnology Program Under Toxic Substances Control Act (TSCA), Section IIIC, http://www.epa.gov/ opptintr/biotech/fra/ fd007.htm).
Appendix 9	IFBC (International Food Biotechnology Council). Chapter 4: Safety Evaluation of Foods and Food Ingredients Derived from Microorganisms in Biotechnologies and Food: Assuring the Safety of Foods Produced by Genetic Modification. Regulatory Toxicology and Pharmacology 12:S1-S196, 1990.
Appendix 10	JEFCA, Evaluation of certain food additives and contaminants, Thirty-first report of the Joint FAO/WHO Expert Committee on Food Additives. Technical Report Series 759. World Health Organization, Geneva, 1987.

GRAS Notice No. GRN 000122

May 16, 2003

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Appendix 1

Lineage of A. oryzae production strain Mt-3

A 1560 (IFO 4177)	Host
↓	Delete *pyrG* gene.
HowB 101 (pyrG mutant)	
↓	Disrupt TAKA amylase gene; decreased amylase activity. Southern blot analysis demonstrates that two of the three resident TAKA amylase genes have been disrupted.
#18 (reduced TAKA amylase activity)	
↓	Selection of spontaneous pyrG mutant by plating of spores on agar plates containing 5-fluoro-orotic acid (FOA) (cells having an intact pyrG gene copy are killed by FOA).
#18P1 (reduced TAKA activity, pyrG mutant)	
↓	Disrupt the final resident TAKA amylase gene.
HowB711 (TAKA amylase negative)	
↓	The plasmid pRaMB17.WT (laccase expression vector) is transformed into the host strain HowB711 using selection on acetamide.
HowB711/RaMB17.WT	
↓	Grow transformants on medium with acetamide as the sole nitrogen source and screening for co-expression of the laccase. A single colony is selected.
Mt-3	The resultant production strain from HowB711.RaMB17.WT following mutation with nitrosoguanidine and screening for high yield of laccase to produce an amylase negative, *M. thermophila* laccase expressing strain.

GRAS Notice No. GRN 000122

May 16, 2003

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Appendix 2

Laccase GRAS Notification - Updated Version of Sections 2.1, 2.2 & 2.5

2. PRODUCTION MICROORGANISM

2.1 Production Strain

The *Aspergillus oryzae* strain, designated Mt-3, was constructed by transformation of the recipient strain, designated HowB711, by incubating protoplasts with pRaMB17.WT (laccase expression vector) and pToC90 (selectable marker). This genetically modified production organism complies with the OECD (Organization for Economic Cooperation and Development) criteria for GILSP (Good Industrial Large Scale Practice) microorganisms (Ref. 1). It also meets the criteria for a safe production microorganism as described by Pariza and Foster, Pariza and Johnson, and several expert groups (Ref. 2-9).

The DNA fragment used in the strain construction contains strictly defined fungal chromosomal DNA fragments and synthetic DNA linker sequences. The specific DNA sequences include a laccase enzyme gene from *Myceliopthora thermophila* strain E421 (CBS 117.65); an *Aspergillus nidulans* selectable marker gene, *amd*S (acetamidase) (Ref. 10); well-characterized non-coding regulatory sequences including the *Aspergillus niger strain* BO-1 terminator (Ref. 11), the TAKA-amylase promoter from *Aspergillus oryzae* strain HW325 (Ref. 12), and a fragment from a well known sequence from *Escherichia coli* plasmids pUC19 and pUC18 (Ref. 13).

2.2 Recipient Organism

The recipient microorganism, designated How B711, used in the construction of the laccase production strain is a genetically modified derivative of *A. oryzae* A1560 by means of site-directed gene disruption to cause interruption of the three resident TAKA amylase genes. The obtained strain *A. oryzae* How B711 is a derivative of the fully characterized, well-known industrial production strain of *A. oryzae* (Ahlburg) Cohn. The strain was obtained from the Institute for Fermentation, Osaka, Japan (IFO) and is designated strain IFO 4177 (synonym A1560).

The taxonomic characteristics of the host strain of *A. oryzae* A1560 are the following:

Name:	*Aspergillus oryzae*
Class:	Blastodeuteromycetes (Hyphomycetes)
Order:	Phialidales (Moniliales)
Genus:	*Aspergillus*
Species:	*Oryzae*

This classification has been confirmed by the Centraalbureau voor Schimmelculutres, Baarn, Holland (Ref. 16).

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2.5 Construction of the Recombinant Production Organism

The recombinant DNA molecule was introduced into the recipient *A. oryzae* How B711 by incubating protoplasts with pRaMB17.WT (laccase expression vector) and pToC90 (selectable marker). The selection of transformants was achieved by growing on a medium with acetamide as the sole nitrogen source and screening for co-expression of the *M. thermophila* laccase. A single colony of transformant HowB711/RaMB17.WT was selected.

The production strain is obtained from transformant HowB711/RaMB17.WT following mutation with nitrosoguanidine (NTG) and screening for high yield of the *Myceliophthora thermophila* laccase.

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Pages 000135-000148 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

CENTRAALBUREAU VOOR SCHIMMELCULTURES

Appendix 5

Postal address:
P.O. Box 273,
3740 AG BAARN
The Netherlands

Visiting address:
Oosterstraat 1,
3742 SK BAARN

Telephone:
02154-11841

Uw/Your Ref:
Onze/Our Ref: RAS/as

F-9501135

Dr. R.I. Nielsen
Novo BioKontrol

NOVO Industri A/S
Novo Alle
DK-2880 BAGSVAERD

BAARN, 07.01.1988

Dear Dr. Nielsen,

Enclosed you find our identification report of the Aspergillus strain (A 1560).
We did only observe sparse sclerotium development on our Czapek Yeast medium.
Enclosed you will find the invoice for this service.
I am happy to use this opportunity to show the great importance of taxonomic investigations and its implication for the applied mycology like your own experiments in NOVO. However particularly the genera Penicillium ans Aspergillus are still problematic and a general consensus is strongly needed. When I organized the First International Penicillium and Aspergillus Workshop in May 1985 a first step was made to start the international dialogue and some progress could be made (see also R.A. Samson & J.I Pitt, (1985). Advances in Penicillium and Aspergillus Systematics Plenum Publishers, New York). At present I am planning to organize a second workshop in May 1989 and I hope the same successful output could be reached. Since the results of our taxonomic consensus have a direct implication to your research at NOVO, I wonder if your company would be interested to support the second workshop with (your) sponsorship.
I would very much appreciate to hear your response. If necessary I can supply more detailed data about the meeting, the participants and the support needed.

My personal wishes for the New Year.

Yours sincerely,

Dr. R.A. Samson

CENTRAALBUREAU VOOR SCHIMMELCULTURES - P.O. BOX 273 - BAARN

Identification report

Strain A1560 sent by NOVO Industri A/S
Novo Alle, DK-2880 Bagsvaerd, Denmark

Aspergillus oryzae (Ahlburg) Cohn

Colonies on Czapek agar attaining a diameter of 4.0-4.5 cm within 7 days at 24°C, greenish yellow near Olive Yellow (Ridgway, Pl. XXX), Dark Olive Buff (Pl. XL) or Yellowish Citrine (Pl. XVI). Somewhat floccose near centre often with long conidiophores intermixed with some aerial mycelium. Reverse not coloured. Conidial heads radiate, 100-300 µm diam. Conidiophores arising from the substrate, mostly 3-4 mm long and 8-20 µm in diam., finely roughened. Vesicles mostly subglobose to slightly clavate, variable in size but mostly 50-70 µm in diam. Phialides flask shaped, directly borne on vesicle or sometimes on metulae. Abortive or atypical phialidic development often present. Conidia globose to subglobose, often ellipsoidal to pyriform when young, variable in size, but mostly 3-6 µm in diam., when ellipsoidal up to 7-8 µm in long axis, at first smooth-walled, becoming slightly to finely roughened with age.
Colonies on 2% Maltextractagar growing faster (6-7 cm in diam) but thinner, yellow-green near Vetiver-green (Pl. XLVII). Morphology similar as on Czapek. Sclerotia were not observed in 14 day-old cultures (only sparsely sclerotium development observed on Czapek Yeast Agar).

Note: The taxonomy used is in accordance to Raper & Fennell (1965) - The genus Aspergillus, Williams & Wilkens Co, Baltimore. The species concept in the Aspergillus flavus group is still problematic, because of the great variability. The strain is classified in Aspergillus oryzae based on colony colour, floccose growth, uni- and biseriate conidial heads, size and ornamentation of the conidia. Identification was only based on morphological criteria and not on biochemical properties (e.g. mycotoxin-production.

Legends to figures.

Fig 1-5 Conidial heads 1-2 Young heads showing uniseriate and biseriate pattern 3-4 Atypical phialidic development, some phialides not producing conidia but with elongated growth. 5. Abortive conidial head with sparse conidiation. 6. Conidia Micrographics taken at 7 days, all 1000x.

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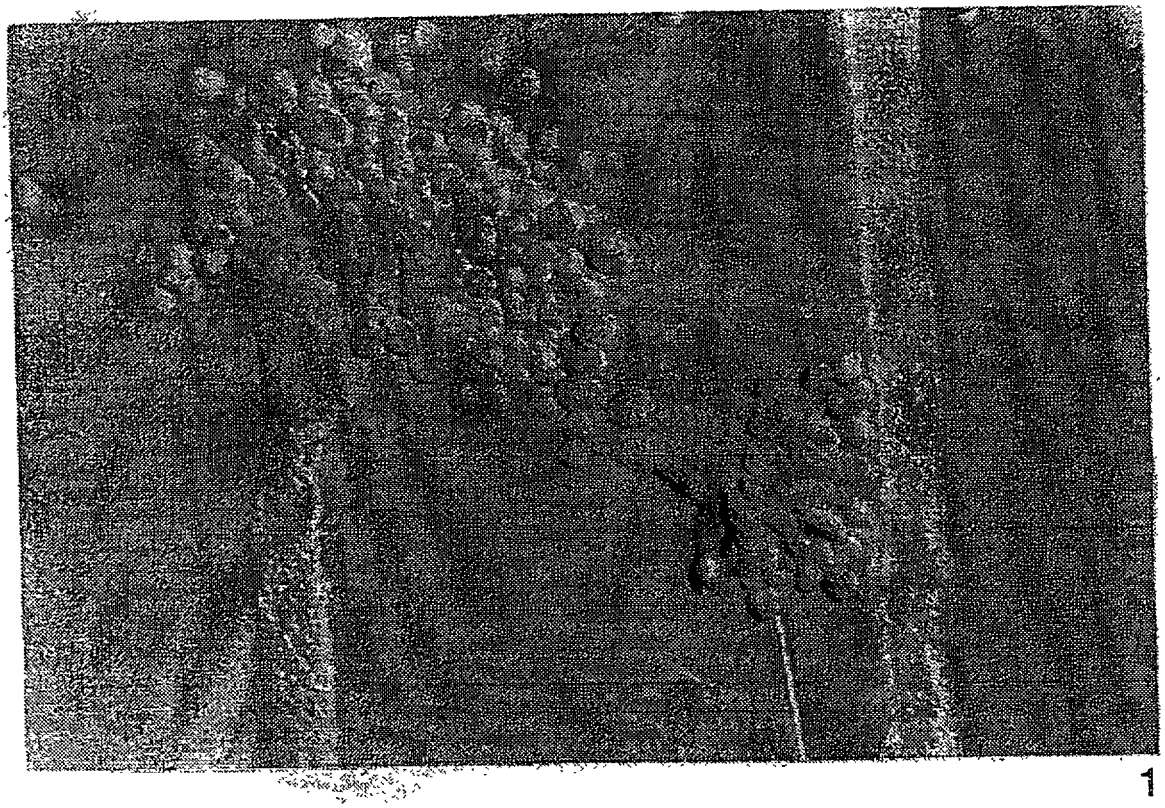

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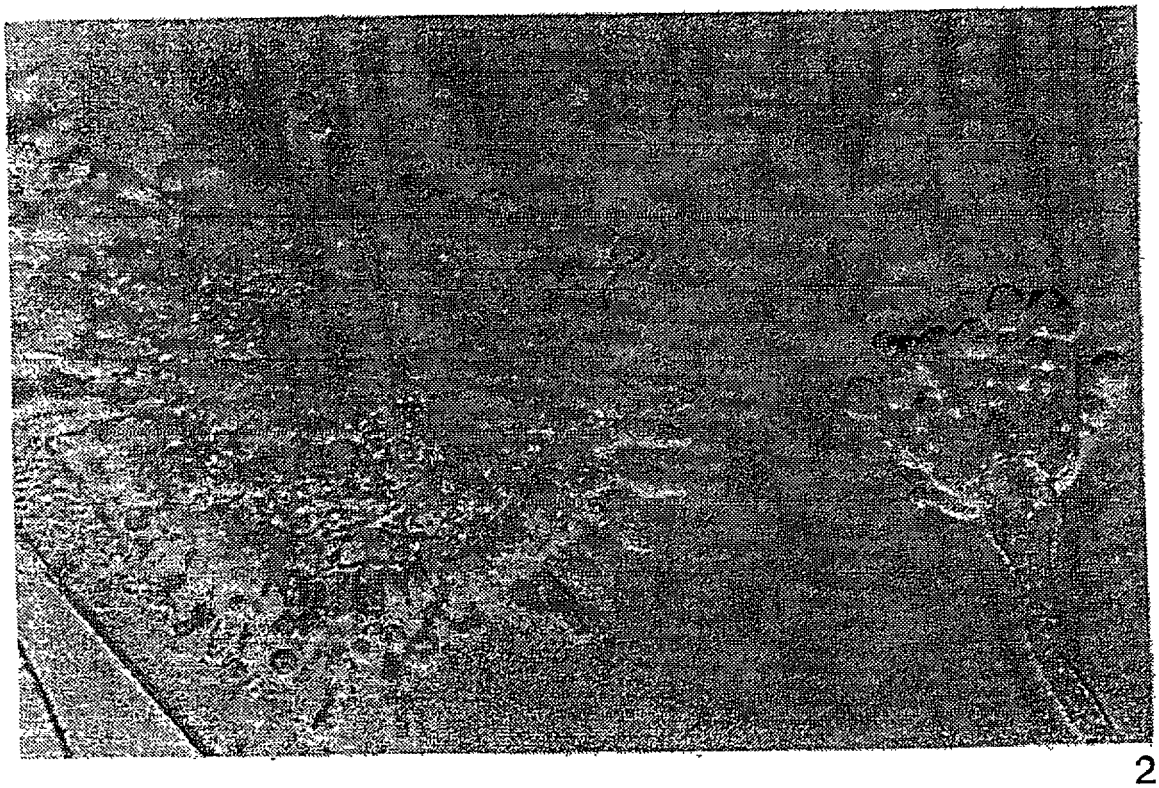

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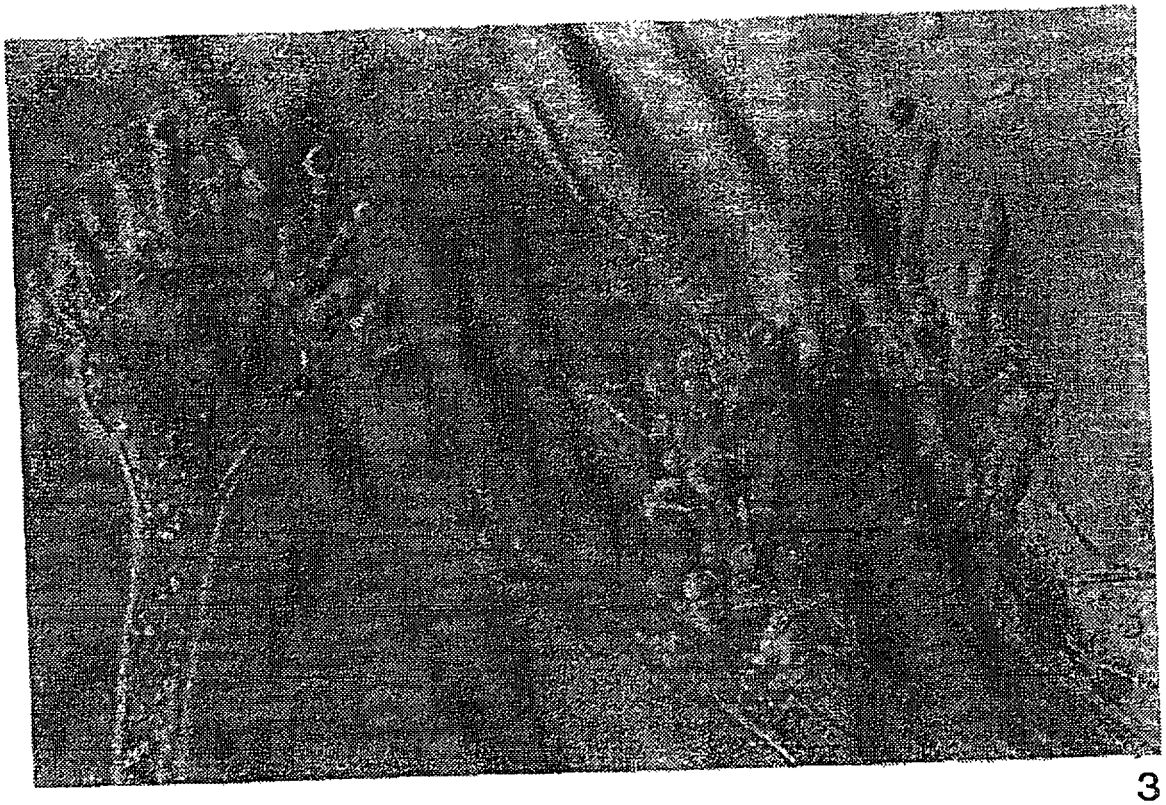

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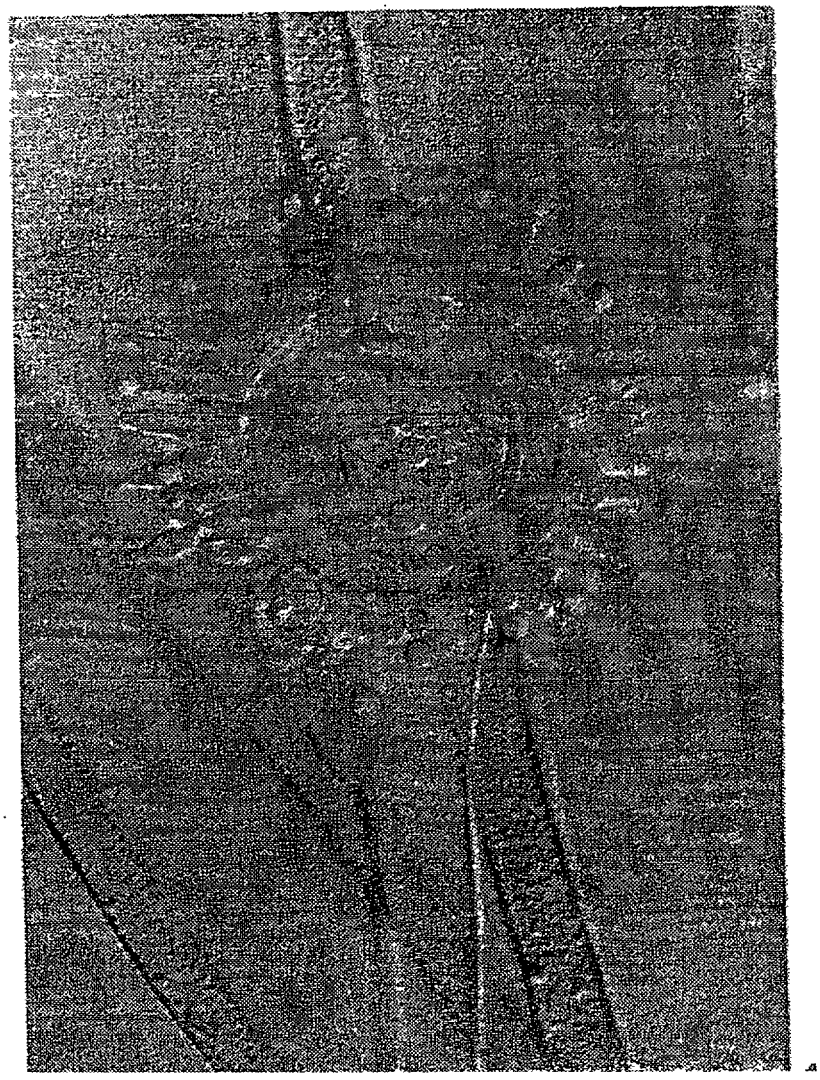

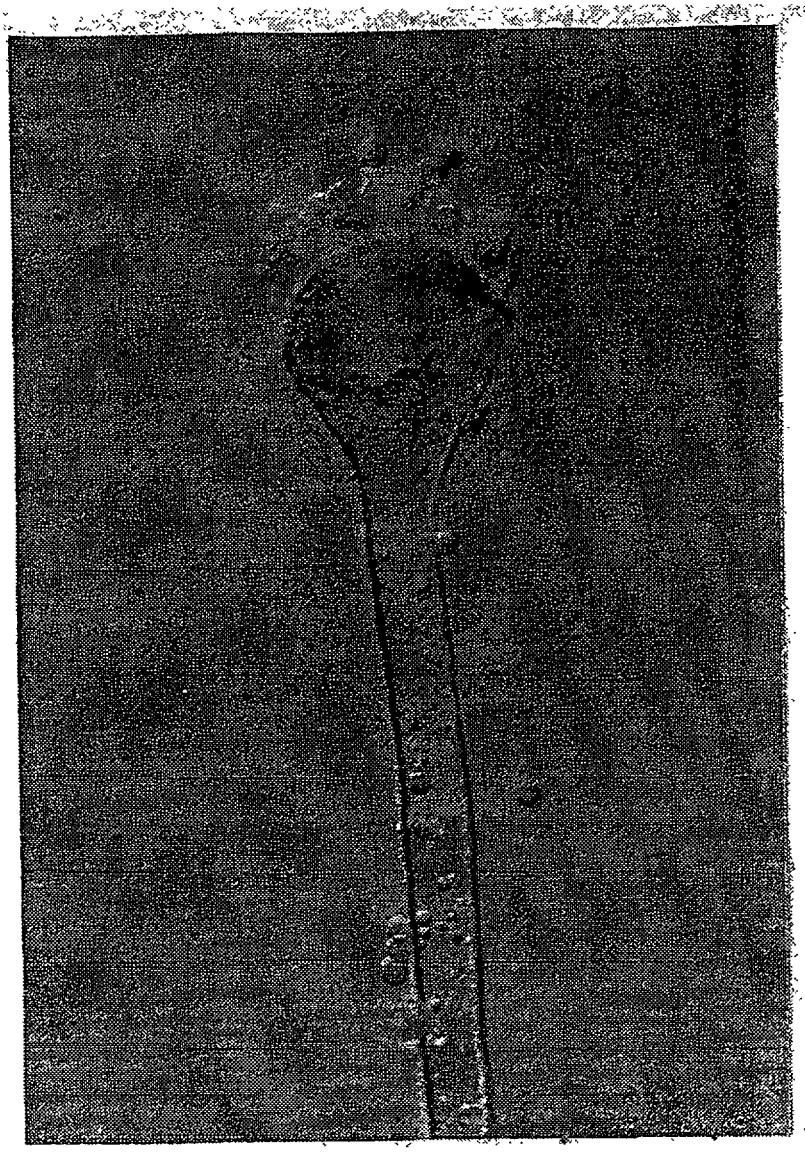

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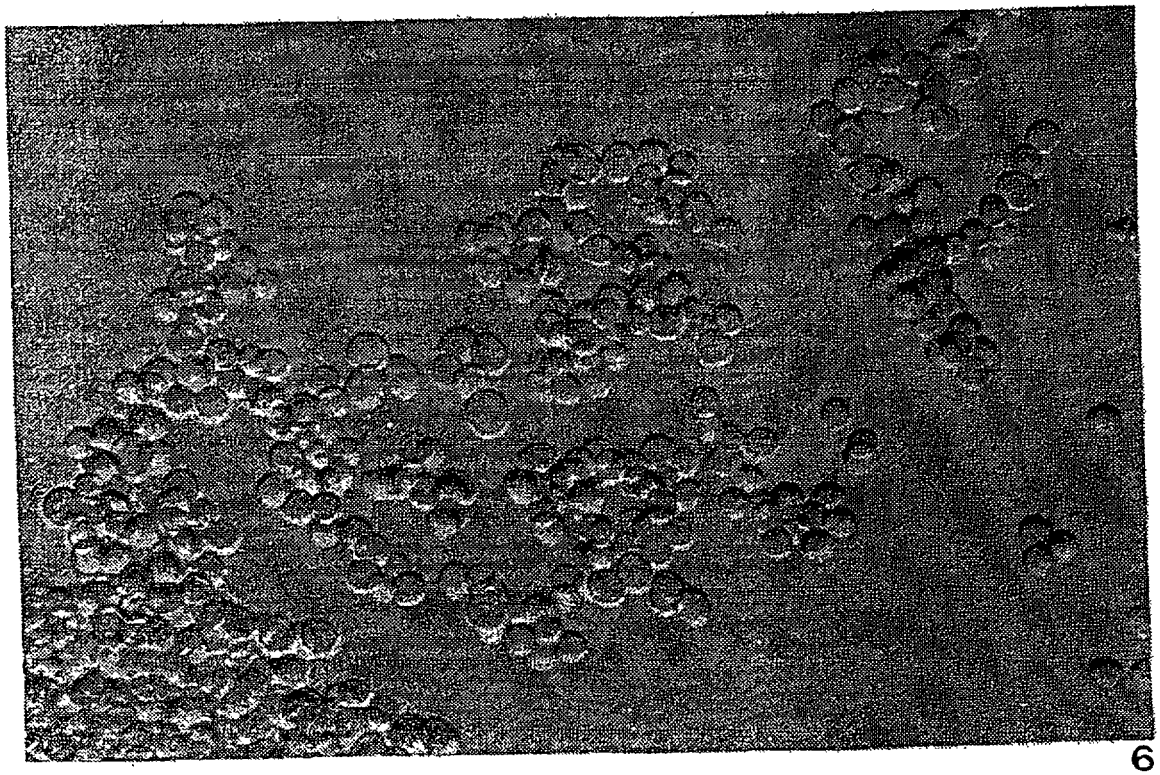

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Appendix 7

Novo Nordisk

Enzyme Business Toxicology

Date : 1997-09-08
File: ED-9713942
Ref.: ASB/DSc

REPORT

Pathogenicity of *Aspergillus oryzae* to rats after oral administration

Novo Nordisk Study No: 1288

This report is a translation of the original report on the study 1288.
Tables and appendices are not included.

000158

Author:
Anne Sietske de Boer

Issued by:
Novo Nordisk A/S
Novo Allé
2880 Bagsvaerd
Denmark

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1. GENERAL INFORMATION

TEST FACILITY	Novo Nordisk A/S Novo Allé DK - 2880 Bagsvaerd
DEPARTMENTS INVOLVED	EB Toxicology Animal Unit, Bagsvaerd
ARCHIVE	Enzyme Business Information & Documentation (EB INFODOC) Novo Nordisk A/S Novo Allé DK - 2880 Bagsvaerd

PERSONNEL INVOLVED IN THE STUDY

STUDY DIRECTOR	Anne Sietske de Boer, D.V.M. Hans Peter Heldt-Hansen
LABORATORY ASSISTANT	Jens Bak

DATE OF FINAL REPORT

Date : 1997-09-09

Anne Sietske de Boer
D.V.M.
Study Director

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2. SUMMARY

Study design:
The aim of the study was to evaluate the pathogenicity of the production strain *Aspergillus oryzae* A1560 and to investigate the possibility of this organism to colonise in the gastrointestinal tract of rats.

The test strain was grown in a nutrient broth. The nutrient broth and the fungi was homogenised prior to dosing.

The fungi was dosed orally to a group of five male and five female Wistar rats. The animals were observed for any clinical reactions to treatment for 10 days. All rats were subjected to macroscopic pathological investigation after the terminal kill at day 10. During the ten day observation period faeces from each animal was collected daily and examined for the presence of the dosed micro-organism.

Result:
The oral administration of *Aspergillus oryzae* to rats did not cause any clinical reaction to the test animals during the ten day observation period. The micro-organism was recovered the day after dosing in only one animal.

Conclusion:
It is concluded that *Aspergillus oryzae* A1560 is non pathogenic to rats after oral application and that the micro-organism is unable to colonise in the gastrao-intestinal tract of the rat.

3. AIM OF THE STUDY

The aim of this study was to investigate the pathogenic potential of *Aspergillus oryzae* to rats when dosed orally and to investigate the capability of the micro-organism to colonise in the gastro-intestinal tract.

4. MATERIAL

4.1 Test material

Aspergillus oryzae was cultured in nutrient broth for 3 day at 30° C. At the termination of the growth period the broth was blended to achieve a homogenous suspension of the test material.

The viable count of the broth before administration of the test substance to the rats was 3×10^6 colony forming units/ml.

4.2 Animals

Male and female Wistar rats were received from Møllegaard Breeding Centre. Lille Skensved, Denmark.

The animals were acclimatised for four days in cages holding five animals of same sex. Thereafter the rats were caged singly for three days in cages having grid floors. The rats were fed standard rat maintenance diet (Brood Stock Feed for Rats and Mice - R3 - Ewos).

During the acclimatisation period and the test period the animals were weighed at the arrival and at termination of the acclimatisation and there after once each fifth day.

4.3 Dosing

The rats were dosed orally a single dose of the micro-organism after depriving of food for 20 hours. All animals were dosed either concentrated test material or the control substance (0.9% NaCl) according to the dosing scheme:

Test substance	Dosage Viable counts/kg b.w.	Animal number Males	Females
Sodium chloride	-	1 - 5	6 - 10
A. oryzae A 1560	3×10^7	21 - 25	26 - 30

4.4 Observation
The rats were observed daily during the acclimatisation period and the test period. The animals observed 30 minutes and 2 hours after dosing and at least once daily the following 10 days.

4.5 Termination of the study
All animals were killed by CO_2+O_2 anaesthesia and bleeding and autopsy.

5. RESULTS FROM THE ANIMAL EXPERIMENTS

5.1 Mortality and clinical symptoms
No animals died or showed clinical symptoms during the study.

5.2 Body weight
The body weight development was not affected by treatment.

5.3 Gross pathology
No macroscopic alterations were observed at the autopsy.

6. MICROBILOGICAL RECOVERY. METHOD AND RESULTS

6.1 Method
The faeces of the rats was collected daily and examined immediately after.

The faecal sample was diluted 5 times by sterile water and mixed until a complete suspension was present.

From each suspension 1 ml was transferred to each of 4 different Petri dishes (ø=13 cm) and was incubated for 30°C for 4-6 days.

At the evaluation the number of colonies of filamentous fungi and the number of these which have morphological similarities with the test organism. The final identification is performed by RIE (rocket immunoelectroforesis).

6.2 Results
The micro-organism was recovered for faeces from one animal one day after dosing, only.

7. DISCUSSION AND CONCLUSION

Based upon the experimental procedures it can be concluded that *A. oryzae* A1560 is non pathogenic to rats after oral administration. Furthermore it is concluded that the micro-organism is unable to colonise in the gastro-intestinal tract of the rat.

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Appendix 8

U.S. Environmental Protection Agency

Biotechnology Program Under Toxic Substances Control Act (TSCA)

Aspergillus oryzae TSCA Section 5(h)(4) Exemption: Final Decision Document

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Fax-On-Demand:

Telephone: (202) 401-0527

Item No.: 3172

FINAL DECISION DOCUMENT:

TSCA SECTION 5(H)(4) EXEMPTION FOR

ASPERGILLUS ORYZAE

I. BACKGROUND

In the September 1, 1994, Federal Register (59 FR 45526), the Environmental Protection Agency (EPA) proposed at 40 CFR Part 700 under section 5(h)(4) of the Toxic Substances Control Act (TSCA), Tier I and Tier II exemptions. These exemptions, which would be found at õ 725.400, are exemptions from EPA review and expedited EPA review, respectively, for certain microorganisms under certain use conditions. EPA proposed to include Aspergillus oryzae at õ 725.420 as a candidate recipient microorganism for the tiered exemptions. Aspergillus oryzae is an asexual, ascomycetous fungus used for hundreds of years in the production of soy sauce, miso and sake without recorded incidents. It has also been used in the fermentation industry for production of enzymes and other organic compounds.

This final decision document describes the basis for EPA's decision to include Aspergillus oryzae as a recipient microorganism at õ 725.420.

II. CONDITIONS OF EXEMPTION

EPA recognizes that some microorganisms present a low risk when used under specific conditions at general commercial use. Therefore, EPA proposed to institute expedited regulatory processes for certain microorganisms under these specific conditions at the general commercial use stage. Microorganism uses that are exempt would meet criteria addressing: (1) performance based standards for minimizing the numbers of microorganisms emitted from the manufacturing facility; (2) the introduced genetic material; and (3) the recipient microorganism. Microorganisms that qualify for these exemptions, termed Tier I and Tier II, must meet a standard of no unreasonable risk in the exempted use.

To evaluate the potential for unreasonable risk to human health or the environment in developing these exemptions, EPA focuses primarily on the characteristics of the recipient microorganisms. If the recipient is shown to have little or no potential for adverse effects, introduced genetic material meeting the specified criteria would not likely significantly increase potential for adverse effects. As further assurance that risks would be low, EPA is also specifying procedures forminimizing numbers of organisms emitted from the facility. When balanced against resource savings for society and expected product benefits, these exemptions will not present unreasonable risks.

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A. Criteria for Minimizing Release from Manufacturing Facilities

The standards proposed for the Tier I exemption were the following: (1) the structure(s) be designed and operated to contain the microorganism, (2) access to the structure should be limited to essential personnel, (3) inactivation procedures shown to be effective in reducing the number of viable microorganisms in liquid and solid wastes should be followed prior to disposal of the wastes, (4) features to reduce microbial concentrations in aerosols and exhaust gases released from the structure should be in place, and (5) general worker hygiene and protection practices should be followed.

1. Definition of structure. EPA considers the term "structure" to refer to the building or vessel which effectively surrounds and encloses the microorganism. Vessels may have a variety of forms, e.g., cubic, ovoid, cylindrical, or spherical, and may be the fermentation vessel proper or part of the downstream product separation and purification line. All would perform the function of enclosing the microorganism. In general, the material used in the construction of such structure(s) would be impermeable, resistant to corrosion and easy to clean/sterilize. Seams, joints, fittings, associated process piping, fasteners and other similar elements would be sealed.

2. Standards to minimize microbial release. EPA is taking, for several reasons, a somewhat cautious approach in prescribing standards for minimizing the number of microorganisms emitted through the disposal of waste and the venting of gases. First, a wide range of behaviors can be displayed by microorganisms modified consistent with EPA's standards for the introduced genetic material. Second, EPA will not conduct any review whatsoever for Tier I exemptions. EPA believes the requirement to minimize emissions will provide a measure of risk reduction necessary for making a finding of no unreasonable risk. Taken together, EPA's standards ensure that the number of microorganisms emitted from the structure is minimized.

EPA's standards for minimizing emission specify that liquid and solid waste containing the microorganisms be treated to give a validated decrease in viable microbial populations so that at least 99.9999 percent of the organisms resulting from the fermentation will be killed. Since the microorganisms used in fermentation processes are usually debilitated, eitherintentionally or through acclimation to industrial fermentation, the small fraction of microorganisms remaining viable after inactivation treatments will likely have a reduced ability to survive during disposal or in the environment. Moreover, industrial companies, in an attempt to keep their proprietary microorganisms from competitors and to reduce the microbial numbers to those permitted by local sanitation authorities, modify the microorganisms to increase the ability of their microorganisms to survive and perform their assigned tasks in the fermentor but decrease their ability to survive in the environment external to the fermentor.

EPA requirements also address microorganisms in the exhaust from the fermentor and along the production line. To address exhaust from fermentors, EPA is requiring that the number of microorganisms in fermentor gases be minimized by the use of standard industry equipment prior to the gases being exhausted from the fermentor. EPA selected this standard based on an estimate of the numbers of microorganisms likely to be in the exhaust from an uncontrolled fermentor and common industry practice. Moreover, microorganisms that are physiologically acclimated to the growth conditions within the fermentor are likely to be compromised in their ability to survive aerosolization. EPA anticipates, therefore, that few microorganisms will survive the stresses of aerosolization associated with being exhausted with the gases from the fermentor. The provision requiring reduction of microorganisms in fermentor exhaust gases contributes to minimizing the number of viable microorganisms emitted from the facility.

EPA is also requiring that other systems be in place to control dissemination of microorganisms by other routes. This would include programs to control pests such as insects or rats, since these might serve as vectors for carrying microorganisms out of the fermentation facilities.

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3. Worker protection. The requirement to minimize microbial emissions, in conjunction with the requirement for general worker safety and hygiene procedures, also affords a measure of protection for workers. Potential effects on workers that exist with microorganisms in general (e.g., allergenicity) will be present with the microorganisms qualifying for this exemption. As with other substances that humans may react to (e.g., pollen, chemicals, dust), the type and degree of allergenic response is determined by the biology of the exposed individual. It is unlikely that a microorganism modified in keeping with EPA's specifications for the introduced genetic material would induce a heightened response. The general worker hygiene

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procedures specified by EPA should protect most individuals from the allergenic responses associated with microorganisms exhausted from fermentors and/or other substances emitted along the production line. The EPA requirement thataccess to the structure be controlled also addresses this consideration by reducing to a minimum the number of individuals exposed.

4. Effect of containment criteria. As further assurance that risks would be low, EPA is specifying procedures for minimizing the number of organisms emitted from the facility for the Tier I exemption. EPA is not specifying standards for minimizing the number of microorganisms emitted from the facility for microorganisms qualifying for Tier II exemption. Rather, the Agency requests that submitters utilize as guidance the standards set forth for Tier I procedures. The procedures proposed by the submitter in a Tier II exemption request will be reviewed by the Agency. EPA will have the opportunity to evaluate whether the procedures the submitter intends to implement for reducing the number of organisms emitted from the facility are appropriate for that microorganism.

B. Introduced Genetic Material Criteria

In order to qualify for either the Tier I or Tier II exemption, any introduced genetic material must be limited in size, well characterized, free of certain nucleotide sequences, and poorly mobilizable.

1. Limited in size. Introduced genetic material must be limited in size to consist only of the following: (1) the structural gene(s) of interest; (2) the regulatory sequences permitting the expression of solely the gene(s) of interest; (3) the associated nucleotide sequences needed to move genetic material, including linkers, homopolymers, adaptors, transposons, insertion sequences, and restriction enzyme sites; (4) the nucleotide sequences needed for vector transfer; and (5) the nucleotide sequences needed for vector maintenance.

The limited in size criterion reduces risk by excluding the introduction into a recipient of extraneous and potentially uncharacterized genetic material. The requirement that the regulatory sequences permit the expression solely of the structural gene(s) of interest reduces risk by preventing expression of genes downstream of the inserted genetic material. The limitation on the vector sequences that are components of the introduced genetic material prevents the introduction of novel traits beyond those associated with the gene(s) of interest. The overall result of the limited in size criterion is improved ability to predict the behavior of the resulting microorganism.

2. Well characterized. For introduced genetic material, well characterized means that the following have been determined: (1) the function of all of the products expressed from the structural gene(s); (2) the function of sequences that participate in the regulation of expression of the structuralgene(s); and (3) the presence or absence of associated nucleotide sequences.

Well characterized includes knowledge of the function of the introduced sequences and the phenotypic expression associated with the introduced genetic material. Genetic material which has been examined at the restriction map or sequence level, but for which a function or phenotypic trait has not yet been ascribed, is not considered well characterized. Well characterized would include knowing whether multiple reading frames exist within the operon. This relates to whether more than one biological product might be encoded by a single sequence, and addresses the possibility that a modified microorganism could display unpredicted behavior should such multiple reading frames exist and their action not be anticipated.

3. Free of certain sequences. In addition to improving the ability to predict the behavior of the modified microorganism, the well characterized requirement ensures that segments encoding for either part or the whole of the toxins listed in the proposed regulatory text for the TSCA biotechnology rule would not inadvertently be introduced into the recipient microorganism.

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These toxins are polypeptides of relatively high potency. Other types of toxins (e.g., modified amino acids, heterocyclic compounds, complex polysaccharides, glycoproteins, and peptides) are not listed for two reasons. First, their toxicity falls within the range of moderate to low. Second, these types of toxins generally arise from the activity of a number of genes in several metabolic pathways (multigenic).

In order for a microorganism to produce toxins of multigenic origin, a large number of different sequences

would have to be introduced and appropriately expressed. It is unlikely that all of the genetic material necessary for producing multigenic toxins would be inadvertently introduced into a recipient microorganism when requirements that the genetic material be limited in size and well characterized are followed.

Similarly, other properties that might present risk concerns result from the interactive expression of a large number of genes. For example, pathogenic behavior is the result of a large number of genes being appropriately expressed. Because of the complex nature of behaviors such as pathogenicity, the probability is low that an insert consisting of well characterized, limited in size genetic material could transform the microorganisms listed for exemption into microorganisms which display pathogenic behavior.

4. Poorly mobilizable. Poorly mobilizable means the ability of the introduced genetic material to be transferred andmobilized is inactivated, with a resulting frequency of transfer of less than 10^{-8} transfer events per recipient. The requirement that the introduced genetic material be poorly mobilizable reduces potential for transfer of introduced genetic sequences to other microorganisms in the environment. Such transfers would occur through the interaction of the introduced microorganism with indigenous microorganisms through conjugation, transduction, or transformation. Through such transfers, the introduced genetic material could be transferred to and propagated within different populations of microorganisms, including microorganisms which may never previously have been exposed to this genetic material. It is not possible to predict how the behavior of these potential recipient microorganisms will be affected after uptake and expression of the genetic material.

Since EPA is not limiting the type of organism that can serve as the source for the introduced genetic material, some limitation is placed on the ability of the introduced genetic material to be transferred. This limitation mitigates risk by significantly reducing the probability that the introduced genetic material would be transferred to and expressed by other microorganisms.

The 10^{-8} frequency is attainable given current techniques. Plasmids with transfer rates of 10^{-8} exist or are easily constructed. Some of the plasmids most commonly employed as vectors in genetic engineering (e.g., pBR325, pBR322) have mobilization/transfer frequencies of 10^{-8} or less.

The criteria set for "poorly mobilizable" for transduction and transformation should be readily met since the majority of transfer frequencies reported for transduction and natural transformation are less than 10^{-8}. Higher frequencies are likely only under special circumstances, such as when the introduced genetic material has been altered or selected to enhance frequency. Because the risk concern EPA addresses with the 10^{-8} criterion is spread of the introduced genetic material broadly through microbial populations, exchanges between very closely related microorganisms, even if occurring at high frequency, is not a concern so long as the spread through populations does not occur at high frequency.

Fungal gene transfer has also been considered in development of the poorly mobilizable criterion. Although mobile genetic elements such as transposons, plasmids and double stranded RNA exist in fungi and can be readily transferred, this transfer usually is only possible between members of the same species during anastomosis, a process specific to fungi. Since anastomosis only occurs between members of the same species, the introduced genetic material would not be transferred to distantly related fungi as may occur with bacteria.

5. Effect of introduced genetic material criteria. The requirements placed on the introduced genetic material, in concert with the level of safety associated with Aspergillus oryzae, ensure that the resulting microorganisms present low or negligible risk. The probability is low that the insertion of genetic material meeting EPA's criteria into strains of A. oryzae will change their behavior so that they would acquire the potential for causing adverse effects. Risks would be mitigated by the four criteria placed on the introduced genetic material, the relative safety of A. oryzae, and the inactivation criteria specified for the Tier I exemption. In the case of Tier II exemption, risks would be mitigated in light of the four criteria placed on introduced genetic material, the relative safety of A. oryzae, and EPA's review of the containment conditions selected.

C. Recipient Microorganism Criteria

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Six criteria were used by EPA to determine eligibility of recipient microorganisms for the tiered exemption.

All of the criteria were used together to determine whether, on balance, the microorganisms would not present an unreasonable risk to human health or the environment. Microorganisms which EPA finds meet these criteria are listed as eligible recipients. The first criteria would require that it be possible to clearly identify and classify the microorganism. Available genotypic and phenotypic information should allow the microorganism to be assigned without confusion to an existing taxon which is easily recognized. Second, information should be available to evaluate the relationship of the microorganism to any other closely related microorganisms which have a potential for adverse effects on human health or the environment. Third, there should be a history of safe commercial use for the microorganism. Fourth, the commercial uses should indicate that the microorganism products might be subject to TSCA jurisdiction. Fifth, studies are available which indicate the potential for the microorganism to cause adverse effects on human health and the environment. Sixth, studies are available which indicate the survival characteristics of the microorganism in the environment.

After each microorganism was reviewed using the six evaluation criteria, a decision was made as to whether to place the microorganism on the list. The Agency's specific determination for Aspergillus oryzae is discussed in the next unit.

III. EVALUATION OF ASPERGILLUS ORYZAE

A. History of Use

1. History of safe commercial use. A. oryzae has been used for centuries in the production of many differentoriental foods such as soy sauce, sake and miso. As a "koji" mold, A. oryzae has been used safely in the food industry for several hundred years. It is also used to produce livestock probiotic feed supplements. The koji mold enzymes were among the first to be isolated and commercialized nearly 100 years ago. A. oryzae is considered a Class 1 Containment Agent under the NIH Guidelines for Research Involving Recombinant DNA Molecules. In Europe, Aspergillus species are considered category 2 under the European Federation of Biotechnology guidelines and category 1 under the OECD containment scale.

2. Products subject to TSCA jurisdiction. A. oryzae is currently used in the production of organic compounds such as glutamic acid, and several enzymes that are of potential use commercially, for example, amylase, protease, b-galactosidase, lipase, and cellulase. While these enzymes could be used as TSCA products, several of them have been more often used in food processing. In 1989, EPA reviewed a premanufacture notice (PMN) for a strain of A. oryzae modified for enhanced production of a lipase enzyme to be used primarily in detergent formulations for the removal of fat-containing stains. In 1994, EPA reviewed a PMN for a similar strain of A. oryzae modified for enhanced production of a cellulase gene for use in detergents as a color brightening agent.

B. Identification of Microorganism

1. Classification. The genus Aspergillus represents a grouping of a very large number of asexual fungi whose taxonomy is based on morphological features. The genus has been divided into groups based on attributes of the spores, conidiophores, and sclerotia. Because this separation of individual species into groups is based on morphological or physiological characteristics, it has resulted in somewhat tenuous and overlapping classification. While it has been hypothesized that A. oryzae is a domesticated version of A. flavus, a species known to produce potent aflatoxins, this theory has not been fully accepted by the scientific community. Distinguishing between these two species can be difficult.

2. Related species of concern. A. oryzae is a member of the A. flavus group of Aspergillus species. Most of the members of this group are known to produce potent mycotoxins, including aflatoxins. Chromosomal DNA homology and other techniques have shown strains of A. oryzae and A. flavus to be essentially indistinguishable. It has been assumed that A. oryzae is a domesticated version of A. flavus that has been selected for use in foods because of its loss of ability for aflatoxin production.

C. Risk Summary

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1. Studies regarding potential for adverse effects. A. oryzae strains can produce a variety of mycotoxins after extended fermentation; however, only a few strains are known to produce the more potent toxins. A.

oryzae does not appear to be a significant human pathogen, nor has it been reported as a plant or animal pathogen. Although A. oryzae strains appear stable under cultivation, in theory, there remains a low probability that reversion to the A. flavus phenotype could occur, if rearrangement rather than deletion is the mechanism by which the A. flavus phenotype is lost. However, through centuries of use in the food industry, A. oryzae has not been shown to produce aflatoxins.

2. Studies regarding survival in the environment. There are conflicting opinions about whether A. oryzae can be isolated from the environment. A. oryzae seems to be a species created by domestication of the A. flavus wild type, and therefore, may have lost certain features important to survival in the environment. Although soil is a possible natural habitat for A. oryzae, the intrinsic features of domesticated strains are expected to limit their ability to survive in a natural environment.

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IV. PUBLIC COMMENTS RELEVANT TO THE RISK ASSESSMENT

A request for comments was made in the Proposed Risk Assessment and the Proposed Decision Document for A. oryzae pertaining to (1) the conclusion that genetic modification of A. oryzae cannot inadvertently produce an aflatoxigenic strain, and (2) whether there is a need to differentiate between strains of A. oryzae having a long history of safe use and recently isolated strains. However, no comments were received on this specific microorganism either in general or addressing these two requests for comments. There were a number of comments received on the tiered exemption, however, and some of these comments are relevant to the criteria discussed at Section II.A. of this document considered in listing this microorganism as an eligible recipient microorganism at õ 725.420.

Some of the general comments received on the exemptions addressed the six criteria EPA used to select candidate recipient microorganisms to include at õ 725.420. None of these comments questioned the eligibility of A. oryzae for inclusion on the list at õ 725.420. EPA's responses to comments on how it used the six criteria to select candidate microorganisms at õ 725.420 are detailed in the Response to Comments document to be found in the docket for this rulemaking.

The other comments pertained to the proposed containment conditions at õ 725.422. Commenters questioned two criteria, the first of which is at õ 725.422(b): "limit entry only to those persons whose presence is critical to the reliability or safety of the activity". Commenters pointed out that under the requirement as proposed, managers may be precluded from allowing administrative personnel, customers, and school and other educational tours into the facility. EPA had not intended to constrain facility managers to this extent and reconsidered the standard. EPA has revised õ 725.422(b) to read "Control access to the structure". Additional explanation for this revision is given in the Response to Comments document (Section III.C.4.a.) and the Preamble of the Final Rule. Both of these documents can be found in the docket for this rulemaking.

The other criterion in the proposed rule which commenters questioned was at õ 725.422(e): "provide and document effectiveness of features to reduce microbial concentrations by at least two logs in aerosols and exhaust gases released from the structure". Commenters argued that the requirement as written would require retrofitting of equipment in order to permit measurement within the fermentor headspace of microbial concentrations in aerosols. EPA had not intended that manufacturers be required to retrofit their fermentation equipment in order to qualify for this exemption. Therefore, EPA re-examined the basis for this criterion by reviewing information submitted on physical containment and control technologies in the PMNs it had received for intergeneric microorganisms between 1986 and 1995. Examination of these PMNs revealed that the number of microorganisms potentially released through fermentor exhaust gases is negligible compared to the number contained in the liquid and solid waste streams. Even under a worst case scenario of an uncontrolled release, as evaluated in the accompanying risk assessment, the number of viable microorganisms aerosolized with the fermentor exhaust gases would still be low, and therefore, the risk would remain low. Moreover, the use of a criterion requiring controls to minimize microbial numbers released through aerosolization at õ 725.422, as compared to the worst case scenario of an uncontrolled release, would result in lesser exposure, and therefore, lower risk than under the uncontrolled release scenario. Uncontrolled releases are not standard industry practice because there are a number of economic considerations driving the control of exhaust gases such as maintaining proper molality of the fermentation broth by the use of a vapor recovery system, maintaining sterility, and preventing release of microorganisms for proprietary reasons. Therefore, upon re-evaluation, the Agency decided that language requiring minimization of microbial concentrations in aerosols could be substituted for the requirement of

the 2-log reduction performance criterion without affecting the no unreasonable risk finding necessary for a 5(h)(4) exemption under TSCA. The potentially increased exposure to this organism from themodification of the containment criteria from the proposed 2-log reduction to minimizing microbial numbers in exhaust gases does not change the risk of using this microorganism for fermentation. Therefore, EPA has revised ó 725.422(e) to read: "Use features known to be effective in minimizing viable microbial populations in aerosols and exhaust gases released from the structure, and document use of such features". The Response to Comments document (Section III.C.4.b.) and the Preamble of the Final Rule provide a thorough explanation for the change in requirements for microbial releases through exhaust gases.

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V. BENEFITS SUMMARY

Substantial benefits are associated with this exemption. Aspergillus oryzae is already widely employed in general commercial use. Many of these uses, such as the production of soy sauce, miso, and sake, and the production of enzymes such as a-amylase, lactase, and b-galactosidase are food uses and not subject to TSCA. However, some enzymes such as a-amylase and protease, both of which are produced by A. orzyae, have wide applications in the detergent industry. The Agency has already reviewed two PMNs involving the use of A. oryzae for production of two enzymes, a lipase and a cellulase, which were for use in commercial detergents.

The Agency believes this exemption will result in resource savings both to EPA and industry without compromising the level of risk management afforded by the full 90 day review. The exemption will result in reduced reporting costs and a decrease in delay associated with reporting requirements. The savings in Agency resources can be directed to reviewing activities and microorganisms which present greater uncertainty. This exemption should also facilitate development and manufacturing of new products and the accumulation of useful information.

VI. FINAL ANALYSIS

1. 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.

A. oryzae does not appear to produce aflatoxins. Additionally, in the industrial setting, the use of proper safety precautions, good laboratory practices, and proper protective clothing, allay concerns for exposure of workers to mycotoxins potentially produced by this microorganism. A. oryzae appears to lack many survival features necessary for establishment in theenvironment. Potential hazards to the public and the environment are also mitigated by limitations to exposure brought about by the conditions of contained use which are designed to limit release of the microorganisms to the environment.

2. Use of strains of A. oryzae which are eligible for the TSCA section 5(h)(4) exemption present no unreasonable risk. Concern has been expressed that genetic modification of A. oryzae could inadvertently produce an aflatoxigenic strain, although this is of low probability. Naturally occurring strains of A. oryzae are not known to produce aflatoxins; however, some scientists have suggested that A. oryzae is a domesticated version of A. flavus and may possess dormant genes for aflatoxin production. This exemption is for strains of A. oryzae and does not include A. flavus. As part of their eligibility for this TSCA section 5(h)(4) exemption, companies are required to certify that they are using A. oryzae. It is therefore expected that companies will have information in their files which documents the correct identification of their strains. Additionally, it is expected that companies will choose well-characterized industrial strains for further development through genetic modification. These expectations in combination with the use of Good Laboratory Practices should ensure the use of the correct species.

The limitations placed by the section 5(h)(4) exemption on the introduced genetic material, in particular the well-characterized and limited in size restrictions, should reduce the likelihood that any sequences relating to aflatoxin production could be introduced. The containment requirements would limit exposure to any mycotoxins produced.

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Because the recipient microorganism was found to have little potential for adverse effects, introduced

genetic material meeting the specified criteria would not likely significantly increase potential for adverse effects. As further assurance that risks would be low, EPA is specifying procedures for minimizing numbers of organisms emitted from the facility for the Tier I exemption and will be reviewing the conditions selected for the Tier II exemption.

Modification of the language of the two proposed containment requirements õ 725.422 does not affect EPA's original determination that microorganisms that are eligible for and used under the conditions of the Tier I exemption will not present an unreasonable risk of injury to human health or the environment. Increased exposure to the microorganisms within or outside the facility resulting from these revisions will be minimal. The risk of using this microorganism in fermentation under the final conditions of this exemption is still low.

When balanced against resource savings for society and expected product benefits, this exemption will not present unreasonable risks.

VII. ACTION

Aspergillus oryzae is included as a recipient microorganism at õ 725.420 for the tiered exemption.

Attachment I - Final Risk Assessment of Aspergillus oryzae

Note: For Attachment I to this Final Decision Document, see "Final Risk Assessment of Aspergillus oryzae" appearing elsewhere in the list of "Support Documents."

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June 13 , 2003

03-06-18P03:30 RCVD

Susan J. Carlson, Ph.D.
Division of Biotechnology and
GRAS Notice Review, HFS-255
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

Re: GRAS Notice No. GRN 000122

Dear Dr. Carlson:

In response to your recent emails of April 16 and 17th, thank you for the opportunity to clarify the lineage of the production strain Mt-3; and to clarify and provide additional information on the nontoxigenicity and nonpathogenicitiy of *Aspergillus oryzae*, particularly *Aspergillus oryzae* A1560.

Lineage of the production strain Mt-3

The production strain is designated Mt-3. The *pyrG* marker is a bidirectional marker, i.e., it is possible to screen for both the presence and absence of a functional *pyrG* gene. Screening for the presence of the *pyrG* gene is done simply by growth on a minimal medium, e.g., without uridine. As a *pyrG* mutant is an auxotrophic mutant, strains not having a functional *pyrG* gene will fail to grow whereas strains having a functional *pyrG* gene will grow. These *pyrG* mutants require uridine for growth and survival. Resistance to 5-fluoro-orotic acid (5-FOA) is a phenotypic characteristic of *pyrG* mutants. So selection for the absence of the *pyrG* gene is based on growth on a minimal medium supplemented with uridine, which complements the lack of the *pyrG* gene and the presence of the compound 5-FOA. 5-FOA is converted to a toxic compound by the *pyrG* gene and so strains having a functional *pyrG* gene are killed whereas strains not having a functional *pyrG* gene are not affected and grow. This means that the *pyrG* gene is a suitable marker for doing sequential genetic manipulations. A *pyrG* mutant is isolated as the starting point. The desired DNA is then transformed into the *pyrG* mutant using the *pyrG* marker for the selection. When the genetic manipulation has been validated (e.g., by Southern blot analysis), a *pyrG* mutant is isolated, and the next genetic manipulation can then be done using the *pyrG* marker again.

A flow chart of the lineage of the production strain is attached as suggested in your email (**Appendix 1**). For your convenience, an updated version of Sections 2.1, 2.2, and 2.5 is enclosed (**Appendix 2**).

Novozymes North America, Inc.
77 Perry Chapel Church Road
P.O. Box 576
Franklinton, North Carolina 27525

Tel. 919-494-3000 *Fax: 919-494-3450* *Internet: www.novozymes.com*

novozymes®

Nontoxigenicity & nonpathogenicity of Aspergillus oryzae, including strain A1560
The information presented below addresses the nontoxigenicity and nonpathogenicity of *A. oryzae*, including *A. oryzae* A1560.

Section 7.1 of the original submission sets forth the definitions of nontoxigenicity and nonpathogenicity - a nontoxigenic organism was defined by Pariza and Foster (1983) (**Appendix 3**) as "one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure" and a nonpathogenic organism as "one that is very unlikely to produce disease under ordinary circumstances." According to the definitions set forth by Pariza and Foster, *A. oryzae*, including *A. oryzae* A1560, meets these criteria for nontoxigenicity and nonpathogenicity.

According to a second review article (Pariza and Johnson, 2001) (**Appendix 4**), "the safety of the production strain should be the primary consideration in evaluating enzyme safety" and is the basis for establishing the "Safe Strain Lineage" concept - "[t]horoughly characterized nonpathogenic, nontoxigenic microbial strains, particularly those with a history of safe use in food enzyme manufacture, are logical candidates for generating a *safe strain lineage...*" *A. oryzae* has a long history of safe use and is considered a safe strain as set forth by the original submission and by further information provided in this response.

1. The taxonomic characteristics of the host strain *A. oryzae* A1560 as set forth in Section 2.2 of the original submission was confirmed by the Centraalbureau voor Schimmelcultures. Reference 16 of the original submission, "Identification Report on Strain A1560 Centraalbureau voor Schimmelcultures, Baarn, Holland, 1998," is enclosed to support that this is indeed *A. oryzae* (**Appendix 5**).

2. The laccase is manufactured in accordance with current Good Manufacturing Practices for food (Section 4 of the original submission). Further, the quality management system used in the manufacturing process complies with ISO 9001. ISO is an acronym for International Organization for Standardization (www.iso.ch/), which is a network of national standards institutes from 145 countries working in partnership with international organizations, governments, industry, business and consumer representatives. ISO serves as a bridge between public and private sectors.

3. *A. oryzae* has a history of safe use in the food industry. *A. oryzae* has been used for hundreds of years in the production of food products such as soy sauce, miso, and sake "without recorded incidents" (*Aspergillus oryzae*, TSCA Section 5(h)(4) Exemption: Final Decision Document, U.S. Environmental Protection Agency (EPA), Biotechnology Program Under Toxic Substances Control Act (TSCA), Section I, (**Appendix 6**). More specifically, *A. oryzae* has been used to produce soy sauce in the United

States since before 1958 and meets the criterion of '"common use in foods in the US before 1958" and, as a result, [products derived from *A. oryzae*] can be considered "generally recognized as safe"' – GRAS (**Appendices 7 & 8**, page S116). Further, according to a 1987 report by JECFA (Joint FAO/WHO Expert Committee on Food Additives), "different varieties of *A. oryzae* are used in certain parts of the world in the preparation of foods" and enzymes derived from *A. oryzae* "should be considered as normal constituents of food" (**Appendix 9**, pages 16 &17).

4. Section 7.1, page 21, of the original submission discusses the review article of Barbesgaard et al. regarding the safety of *A. oryzae* (**Appendix 7**). The authors conclude that "*A. oryzae* does not produce aflatoxins or any other cancerogenic metabolites. The absence of significant levels of mycotoxins in industrial products is regularly checked." This reference strongly supports the safety of *A. oryzae*, namely its nonpathogenic and nontoxigenic nature.

5. The Summary of Toxicity Data (**Appendix C of the original submission**) and the published safety paper (**Appendix B of the original submission**) document the safety of laccase by conducting a series of toxicological tests including that it was not found to be mutagenic in the *Salmonella typhimurium* reverse mutation assay, nor did it cause chromosomal aberrations in cultured human lymphocytes.

6. The pathogenic potential of *A. oryzae* strain A1560 was evaluated. A pathogenicity study entitled "Report - Pathogenicity of *Aspergillus oryzae* to rats after oral administration, Novo Nordisk Study No. 1288" concluded that "*A. oryzae* A1560 is nonpathogenic to rats after oral administration" (**Appendix 10**). In addition, Section 2.8 of the original submission states that "the production organism does not end up in food", which is "an established specification for the commercial product as specified in Section 5.2."

7. Four batches of laccase have been analyzed for toxins according to the former JECFA specifications to document the absence of these toxins. The results are shown below in **Table 1**.

Table 1: Mycotoxin analyses of four laccase batches produced with *A. oryzae* Mt-3

	OMF 0005	OMF 0006	OMF 0008	OMF 0009
Aflatoxin B_1	ND	ND	ND	ND
Ochratoxin A	ND	ND	ND	ND
Sterigmatocystein	ND	ND	ND	ND
T2-toxin	ND	ND	ND	ND
Zearalenon	ND	ND	ND	ND

ND = Not Detected

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According to the results shown in **Table 1**, the production strain *A. oryzae* Mt-3 does not produce aflatoxin, ochratoxin A, sterigmatocystein, T2-toxin and zearalenon. *A. oryzae* has been used in the food industry for centuries and has not been shown to produce aflatoxins as evaluated by U.S. EPA (**Appendix 6**).

8. Low levels of the mycotoxins/secondary metabolites that may be potentially produced by *A. oryzae* are kojic acid, cyclopiazonic acid and β-nitropropionic acid (**Appendix 7**). Four batches of laccase have been analyzed for these secondary metabolites. The results are shown below in **Table 2**.

Table 2: Additional Mycotoxin analyses of four laccase batches produced with *A. oryzae* Mt-3

	OMF 0005	OMF 0006	OMF 0008	OMF 0009
Kojic acid	<6ppm	<6ppm	<6ppm	<6ppm
Cyclopiazonic acid	<5ppm	<7ppm	<6ppm	<5ppm
β-nitropropionic acid	<10ppm	<10ppm	<10ppm	<10ppm

Based on the test results presented in **Table 2** and literature on safety assessments (Burdock, et al., **Appendices 11-13**), it is concluded that the production strain *A. oryzae* Mt-3 does not produce secondary metabolites of toxicological concern to humans.

9. Novozymes has received Agency Response Letters for enzymes derived from the well-known industrial production strain *A. oryzae* A1560 (e.g., GRAS Notice Nos. GRN 000008, 000034, 000043, 000075, 000103 and 000106). Thus, it was a surprise to receive a request for clarification of the nonpathogenic and nontoxigenic nature of *A. oryzae* A1560.

Conclusion

Based upon full consideration of the laccase GRN Notification and the information presented above, including the definitions set forth by Pariza and Foster, we conclude that *Aspergillus oryzae* A1560 is nonpathogenic and nontoxigenic.

GRAS Notice No. GRN 000122

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We would like to take this opportunity to thank you for reviewing our submission. We hope this package addresses your outstanding concerns. Should you have additional questions or comments, please contact me directly at (b) (6)

Sincerely yours,

Denise Bernstein
Sr. Regulatory Specialist

Enclosures: List of Appendices
13 Appendices

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List of Appendices

Appendix 1 Flow Chart - Lineage of A. oryzae production strain Mt-3.

Appendix 2 Laccase GRAS Notification - Updated Version of Sections 2.1, 2.2 & 2.5.

Appendix 3 Pariza, M.W. and Foster, E.M. Determining the Safety of Enzymes Used in Food Processing, *Journal of Food Protection*, 46:5:453-468, 1983.

Appendix 4 Pariza, M.W. and Johnson, E.A. Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing: Update for a New Century, *Regulatory Toxicology and Pharmacology*, 33:173-186, 2001.

Appendix 5 Report on Strain A1560, Centraalbureau voor Schimmelcultures, Baarn, The Netherlands, 1988.

Appendix 6 *Aspergillus oryzae*, TSCA Section 5(h)(4) Exemption: Final Decision Document, U.S. Environmental Protection Agency, Biotechnology Program Under Toxic Substances Control Act (TSCA), Section IIIC, http://www.epa.gov/opptintr/ biotech/fra/ fd007.htm).

Appendix 7 Barbesgaard, Peder, Heldt-Hansen, Hans Peter and Diderichsen, Børge. On the Safety of *Aspergillus oryzae:* a Review, *Appl. Microbiol. Biotechnol.*, 36:569-572, 1992.

Appendix 8 IFBC (International Food Biotechnology Council). Chapter 4: Safety Evaluation of Foods and Food Ingredients Derived from Microorganisms in Biotechnologies and Food: Assuring the Safety of Foods Produced by Genetic Modification. *Regulatory Toxicology and Pharmacology*, 12:S1-S196, 1990.

Appendix 9 JEFCA, Evaluation of certain food additives and contaminants, Thirty-first report of the Joint FAO/WHO Expert Committee on Food Additives. Technical Report Series 759. World Health Organization, Geneva, 1987.

Appendix 10 Report - Pathogenicity of *Aspergillus oryzae* to rats after oral administration. Novo Nordisk Study No. 1288, 1997.

Appendix 11 Burdock, G.A., Soni, M.G., and Carabin, I.G. Evaluation of Health Aspects of Kojic Acid in Food, *Regulatory Toxicology and Pharmacology*, 33:80-101, 2001.

Appendix 12 Burdock, G.A. and Flamm, W.G. Review Article: Safety Assessment of the Mycotoxin Cyclopiazonic Acid., *International Journal of Toxicology*, 19:195-218, 2000.

Appendix 13 Burdock, G.A., Carabin, I. And G., Soni, M.G. Safety assessment of ß-Nitropropionic acid: a monograph in support of an acceptable daily intake in humans, *Food Chemistry*, 75:1-27, 2001.

Appendix

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Appendix 1

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Lineage of *A. oryzae* production strain Mt-3

A1560 (IFO 4177)	Host
↓	Delete *pyrG* gene - A1560 undergoes transformation with a fragment of a *pyrG* disruption plasmid containing the *A. oryzae pyrG* gene with an internal deletion. A transformant resistant to 5-fluoro-orotic acid (5-FOA) (HowB101) was selected while cells with intact *pyrG* gene copy were killed by 5-FOA
HowB101 (pyrG mutant)	
↓	Disrupt TAKA amylase gene using a disruption plasmid based on the *pyrG* selection marker (the *pyrG* gene is inserted into theTAKA-amylase gene) and selecting for prototrophs; resulted in one transformant; #18); which decreased amylase activity. Southern blot analysis demonstrates that two of the three resident TAKA amylase genes of the pyrimidine prototroph transformants have been disrupted.
#18 (reduced TAKA amylase activity, pyrG+)	
↓	Selection of spontaneous *pyrG* mutant by plating of spores on agar plates containing 5-FOA - cells having an intact pyrG gene copy are killed by FOA while the selected *pyrG* mutant (#18P1) is resistant to FOA.
#18P1 (reduced TAKA activity, pyrG mutant)	
↓	Disrupt the final resident TAKA amylase gene using a disruption plasmid based on the pyrG selection marker (the *pyrG* gene is inserted into theTAKA-amylase gene) and select for prototrophs that do not secrete TAKA amylase; resulted in one transformant (HowB711) that did not secrete TAKA amylase.
HowB711 (TAKA amylase negative, pyrG+)	
↓	The plasmids pRaMB17.WT (laccase expression vector) and pToC90 are used to transform the host strain HowB711 using selection on acetamide (Plasmid pToC90 contains the selectable marker gene *amdS* from *A. nidulans* encoding acetamidase that makes it possible for *A. oryzae* to metabolize acetamide. So *A. oryzae* cells containing acetamidase can be selected when acetamidase is provided as a sole Nitrogen source).
HowB711/RaMB17.WT	
↓	Grow transformants on medium with acetamide as the sole nitrogen source and screening for co-expression of the laccase. A single colony is selected.
Mt-3	The resultant production strain from HowB711/RaMB17.WT following mutation with nitrosoguanidine and screening for high yield of laccase to produce an amylase negative, *M. thermophila* laccase expressing strain designated as Mt-3.

Appendix 2

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Appendix 2

Laccase GRAS Notification - Updated Version of Sections 2.1, 2.2 & 2.5

2. PRODUCTION MICROORGANISM

2.1 Production Strain

The *Aspergillus oryzae* strain, designated Mt-3, was constructed by transformation of the recipient strain, designated HowB711, by incubating protoplasts with pRaMB17.WT (laccase expression vector) and pToC90 (selectable marker). This genetically modified production organism complies with the OECD (Organization for Economic Cooperation and Development) criteria for GILSP (Good Industrial Large Scale Practice) microorganisms (Ref. 1). It also meets the criteria for a safe production microorganism as described by Pariza and Foster, Pariza and Johnson, and several expert groups (Ref. 2-9).

The DNA fragment used in the strain construction contains strictly defined fungal chromosomal DNA fragments and synthetic DNA linker sequences. The specific DNA sequences include a laccase enzyme gene from *Myceliopthora thermophila* strain E421 (CBS 117.65); an *Aspergillus nidulans* selectable marker gene, *amd*S (acetamidase) (Ref. 10); well-characterized non-coding regulatory sequences including the *Aspergillus niger strain* BO-1 terminator (Ref. 11), the TAKA-amylase promoter from *Aspergillus oryzae* strain HW325 (Ref. 12), and a fragment from a well known sequence from *Escherichia coli* plasmids pUC19 and pUC18 (Ref. 13).

2.2 Recipient Organism

The recipient microorganism, designated How B711, used in the construction of the laccase production strain is a genetically modified derivative of *A. oryzae* A1560 by means of site-directed gene disruption to cause interruption of the three resident TAKA amylase genes. The obtained strain *A. oryzae* How B711 is a derivative of the fully characterized, well-known industrial production strain of *A. oryzae* (Ahlburg) Cohn. The strain was obtained from the Institute for Fermentation, Osaka, Japan (IFO) and is designated strain IFO 4177 (synonym A1560).

The taxonomic characteristics of the host strain of *A. oryzae* A1560 are the following:

Name:	*Aspergillus oryzae*
Class:	Blastodeuteromycetes (Hyphomycetes)
Order:	Phialidales (Moniliales)
Genus:	*Aspergillus*
Species:	*Oryzae*

This classification has been confirmed by the Centraalbureau voor Schimmelculutres, Baarn, Holland (Ref. 16).

2.5 Construction of the Recombinant Production Organism

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The recombinant DNA molecule was introduced into the recipient *A. oryzae* How B711 by incubating protoplasts with pRaMB17.WT (laccase expression vector) and pToC90 (selectable

novozymes®

marker). The selection of transformants was achieved by growing on a medium with acetamide as the sole nitrogen source and screening for co-expression of the *M. thermophila* laccase. A single colony of transformant HowB711/RaMB17.WT was selected.

The production strain is obtained from transformant HowB711/RaMB17.WT following mutation with nitrosoguanidine (NTG) and screening for high yield of the *Myceliophthora thermophila* laccase.

Appendix 3

Pages 000213-000229 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

Appendix
4

Pages 000231-000244 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

Appendix 5

CENTRAALBUREAU VOOR SCHIMMELCULTURES

Postal address:
P.O. Box 273,
3740 AG BAARN
The Netherlands

Visiting address:
Oosterstraat 1,
3742 SK BAARN

Telephone:
02154-11841

Appendix 5

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Uw/Your Ref:
Onze/Our Ref: RAS/as

F-9501135

Dr. R.I. Nielsen
Novo BioKontrol

NOVO Industri A/S
Novo Alle
DK-2880 BAGSVAERD

BAARN, 07.01.1988

Dear Dr. Nielsen,

Enclosed you find our identification report of the Aspergillus strain (A 1560).
We did only observe sparse sclerotium development on our Czapek Yeast medium.
Enclosed you will find the invoice for this service.
I am happy to use this opportunity to show the great importance of taxonomic investigations and its implication for the applied mycology like your own experiments in NOVO. However particularly the genera Penicillium ans Aspergillus are still problematic and a general consensus is strongly needed. When I organized the First International Penicillium and Aspergillus Workshop in May 1985 a first step was made to start the international dialogue and some progress could be made (see also R.A. Samson & J.I Pitt, (1985). Advances in Penicillium and Aspergillus Systematics Plenum Publishers, New York). At present I am planning to organize a second workshop in May 1989 and I hope the same successful output could be reached. Since the results of our taxonomic consensus have a direct implication to your research at NOVO, I wonder if your company would be interested to support the second workshop with (your) sponsorship.
I would very much appreciate to hear your response. If necessary I can supply more detailed data about the meeting, the participants and the support needed.

My personal wishes for the New Year.

Yours sincerely,

Dr. R.A. Samson

CENTRAALBUREAU VOOR SCHIMMELCULTURES - P.O. BOX 273 - BAARN

Identification report

Strain A1560 sent by NOVO Industri A/S
Novo Alle, DK-2880 Bagsvaerd, Denmark

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Aspergillus oryzae (Ahlburg) Cohn

Colonies on Czapek agar attaining a diameter of 4.0-4.5 cm within 7 days at 24°C, greenish yellow near Olive Yellow (Ridgway, Pl. XXX), Dark Olive Buff (Pl. XL) or Yellowish Citrine (Pl. XVI). Somewhat floccose near centre often with long conidiophores intermixed with some aerial mycelium. Reverse not coloured. Conidial heads radiate, 100-300 µm diam. Conidiophores arising from the substrate, mostly 3-4 mm long and 8-20 µm in diam., finely roughened. Vesicles mostly subglobose to slightly clavate, variable in size but mostly 50-70 µm in diam. Phialides flask shaped, directly borne on vesicle or sometimes on metulae. Abortive or atypical phialidic development often present. Conidia globose to subglobose, often ellipsoidal to pyriform when young, variable in size, but mostly 3-6 µm in diam., when ellipsoidal up to 7-8 µm in long axis, at first smooth-walled, becoming slightly to finely roughened with age.
Colonies on 2% Maltextractagar growing faster (6-7 cm in diam) but thinner, yellow-green near Vetiver-green (Pl. XLVII). Morphology similar as on Czapek. Sclerotia were not observed in 14 day-old cultures (only sparsely sclerotium development observed on Czapek Yeast Agar).

Note: The taxonomy used is in accordance to Raper & Fennell (1965) - The genus Aspergillus, Williams & Wilkens Co, Baltimore. The species concept in the Aspergillus flavus group is still problematic, because of the great variability. The strain is classified in Aspergillus oryzae based on colony colour, floccose growth, uni- and biseriate conidial heads, size and ornamentation of the conidia. Identification was only based on morphological criteria and not on biochemical properties (e.g. mycotoxin-production.

Legends to figures.

Fig 1-5 Conidial heads 1-2 Young heads showing uniseriate and biseriate pattern 3-4 Atypical phialidic development, some phialides not producing conidia but with elongated growth. 5. Abortive conidial head with sparse conidiation. 6. Conidia Micrographics taken at 7 days, all 1000x.

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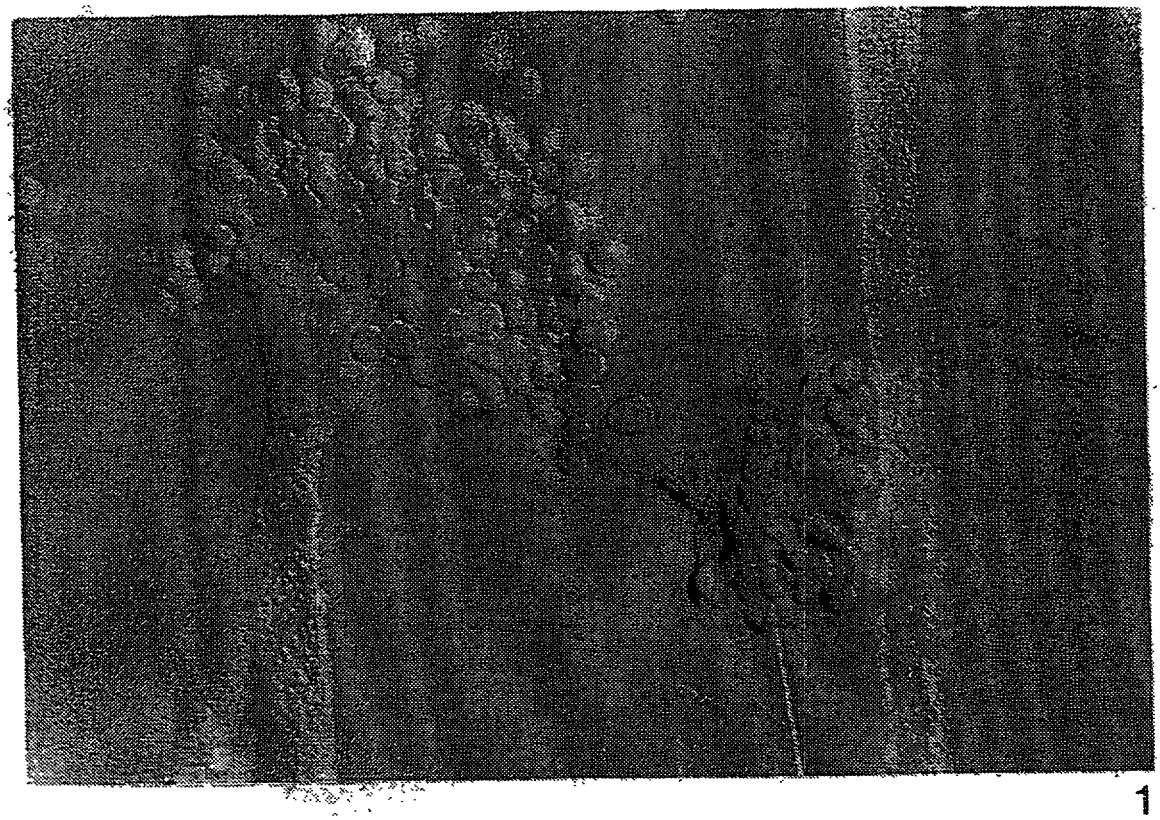

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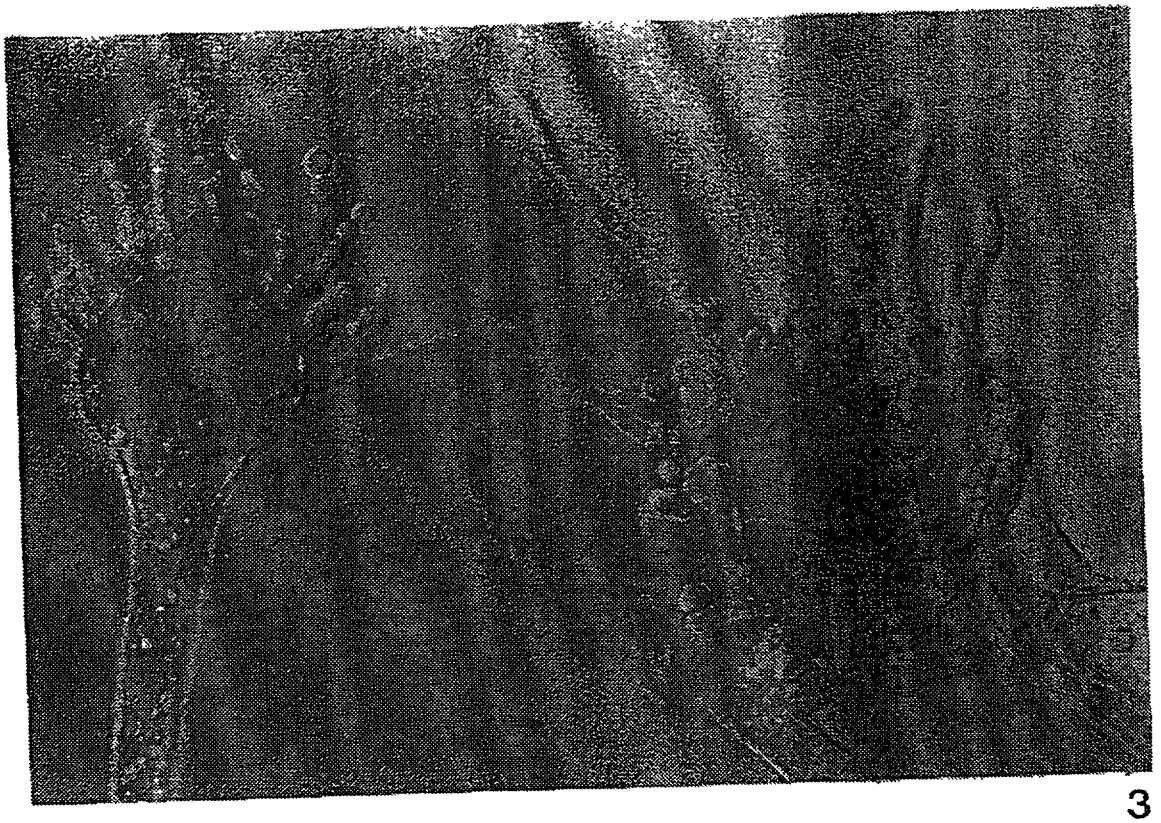

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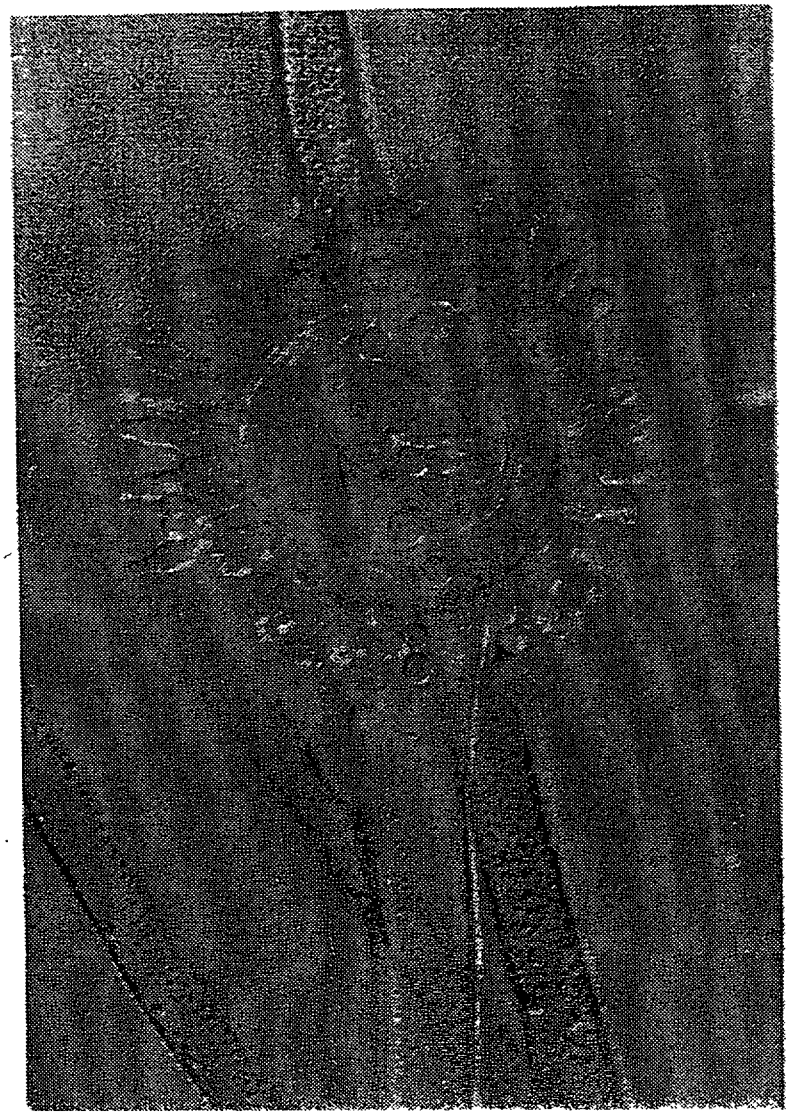

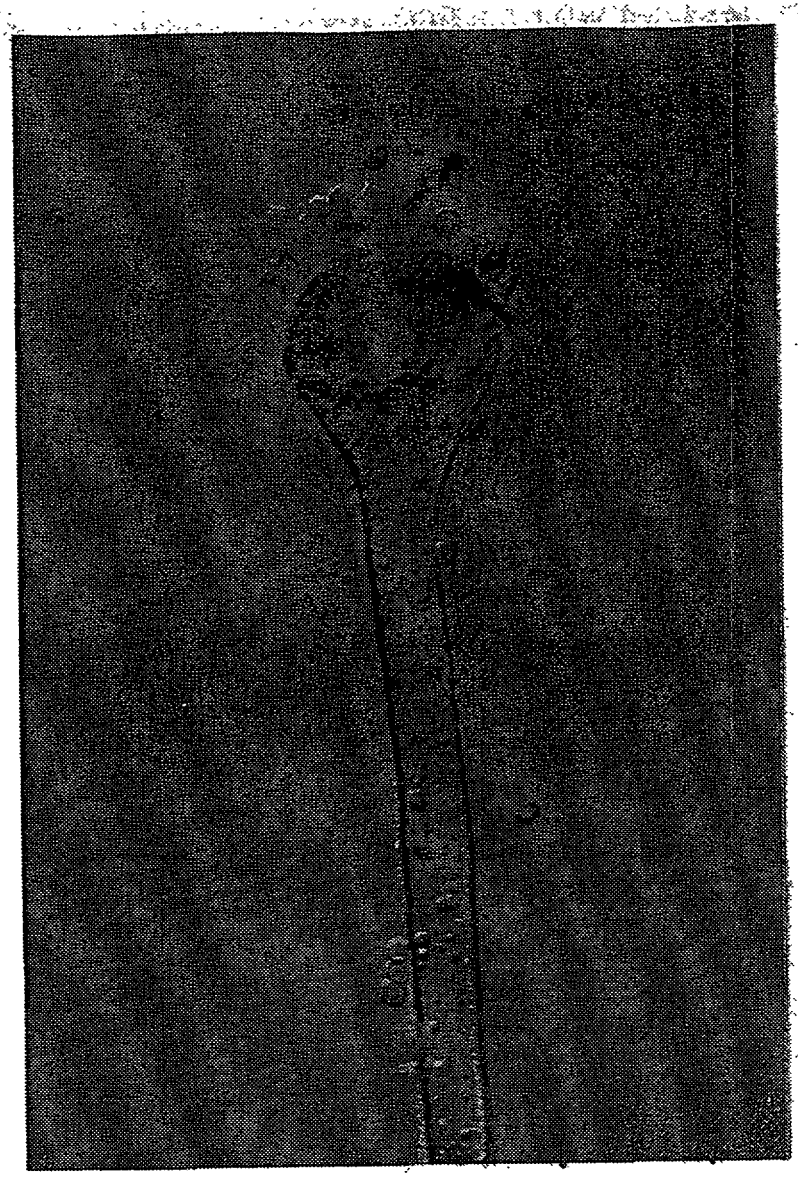

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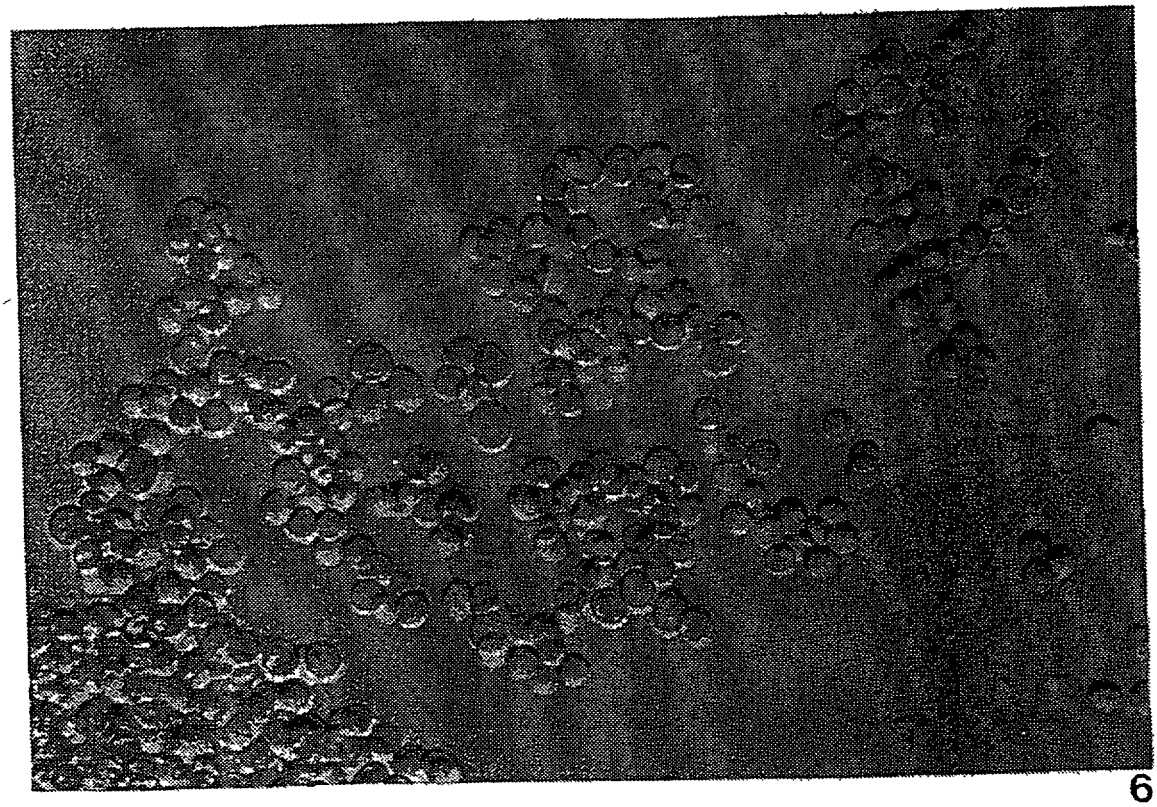

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Appendix 6

Aspergillus oryzae TSCA Section 5(h)(4) Exemption: Final Decision Document

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Fax-On-Demand:

Telephone: (202) 401-0527

Item No.: 3172

FINAL DECISION DOCUMENT:

TSCA SECTION 5(H)(4) EXEMPTION FOR

ASPERGILLUS ORYZAE

I. BACKGROUND

In the September 1, 1994, Federal Register (59 FR 45526), the Environmental Protection Agency (EPA) proposed at 40 CFR Part 700 under section 5(h)(4) of the Toxic Substances Control Act (TSCA), Tier I and Tier II exemptions. These exemptions, which would be found at õ 725.400, are exemptions from EPA review and expedited EPA review, respectively, for certain microorganisms under certain use conditions. EPA proposed to include Aspergillus oryzae at õ 725.420 as a candidate recipient microorganism for the tiered exemptions. Aspergillus oryzae is an asexual, ascomycetous fungus used for hundreds of years in the production of soy sauce, miso and sake without recorded incidents. It has also been used in the fermentation industry for production of enzymes and other organic compounds.

This final decision document describes the basis for EPA's decision to include Aspergillus oryzae as a recipient microorganism at õ 725.420.

II. CONDITIONS OF EXEMPTION

EPA recognizes that some microorganisms present a low risk when used under specific conditions at general commercial use. Therefore, EPA proposed to institute expedited regulatory processes for certain microorganisms under these specific conditions at the general commercial use stage. Microorganism uses that are exempt would meet criteria addressing: (1) performance based standards for minimizing the numbers of microorganisms emitted from the manufacturing facility; (2) the introduced genetic material; and (3) the recipient microorganism. Microorganisms that qualify for these exemptions, termed Tier I and Tier II, must meet a standard of no unreasonable risk in the exempted use.

To evaluate the potential for unreasonable risk to human health or the environment in developing these exemptions, EPA focuses primarily on the characteristics of the recipient microorganisms. If the recipient is shown to have little or no potential for adverse effects, introduced genetic material meeting the specified criteria would not likely significantly increase potential for adverse effects. As further assurance that risks would be low, EPA is also specifying procedures forminimizing numbers of organisms emitted from the facility. When balanced against resource savings for society and expected product benefits, these exemptions will not present unreasonable risks.

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A. Criteria for Minimizing Release from Manufacturing Facilities

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The standards proposed for the Tier I exemption were the following: (1) the structure(s) be designed and operated to contain the microorganism, (2) access to the structure should be limited to essential personnel, (3) inactivation procedures shown to be effective in reducing the number of viable microorganisms in liquid and solid wastes should be followed prior to disposal of the wastes, (4) features to reduce microbial concentrations in aerosols and exhaust gases released from the structure should be in place, and (5) general worker hygiene and protection practices should be followed.

1. Definition of structure. EPA considers the term "structure" to refer to the building or vessel which effectively surrounds and encloses the microorganism. Vessels may have a variety of forms, e.g., cubic, ovoid, cylindrical, or spherical, and may be the fermentation vessel proper or part of the downstream product separation and purification line. All would perform the function of enclosing the microorganism. In general, the material used in the construction of such structure(s) would be impermeable, resistant to corrosion and easy to clean/sterilize. Seams, joints, fittings, associated process piping, fasteners and other similar elements would be sealed.

2. Standards to minimize microbial release. EPA is taking, for several reasons, a somewhat cautious approach in prescribing standards for minimizing the number of microorganisms emitted through the disposal of waste and the venting of gases. First, a wide range of behaviors can be displayed by microorganisms modified consistent with EPA's standards for the introduced genetic material. Second, EPA will not conduct any review whatsoever for Tier I exemptions. EPA believes the requirement to minimize emissions will provide a measure of risk reduction necessary for making a finding of no unreasonable risk. Taken together, EPA's standards ensure that the number of microorganisms emitted from the structure is minimized.

EPA's standards for minimizing emission specify that liquid and solid waste containing the microorganisms be treated to give a validated decrease in viable microbial populations so that at least 99.9999 percent of the organisms resulting from the fermentation will be killed. Since the microorganisms used in fermentation processes are usually debilitated, eitherintentionally or through acclimation to industrial fermentation, the small fraction of microorganisms remaining viable after inactivation treatments will likely have a reduced ability to survive during disposal or in the environment. Moreover, industrial companies, in an attempt to keep their proprietary microorganisms from competitors and to reduce the microbial numbers to those permitted by local sanitation authorities, modify the microorganisms to increase the ability of their microorganisms to survive and perform their assigned tasks in the fermentor but decrease their ability to survive in the environment external to the fermentor.

EPA requirements also address microorganisms in the exhaust from the fermentor and along the production line. To address exhaust from fermentors, EPA is requiring that the number of microorganisms in fermentor gases be minimized by the use of standard industry equipment prior to the gases being exhausted from the fermentor. EPA selected this standard based on an estimate of the numbers of microorganisms likely to be in the exhaust from an uncontrolled fermentor and common industry practice. Moreover, microorganisms that are physiologically acclimated to the growth conditions within the fermentor are likely to be compromised in their ability to survive aerosolization. EPA anticipates, therefore, that few microorganisms will survive the stresses of aerosolization associated with being exhausted with the gases from the fermentor. The provision requiring reduction of microorganisms in fermentor exhaust gases contributes to minimizing the number of viable microorganisms emitted from the facility.

EPA is also requiring that other systems be in place to control dissemination of microorganisms by other routes. This would include programs to control pests such as insects or rats, since these might serve as vectors for carrying microorganisms out of the fermentation facilities.

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3. Worker protection. The requirement to minimize microbial emissions, in conjunction with the requirement for general worker safety and hygiene procedures, also affords a measure of protection for workers. Potential effects on workers that exist with microorganisms in general (e.g., allergenicity) will be present with the microorganisms qualifying for this exemption. As with other substances that humans may react to (e.g., pollen, chemicals, dust), the type and degree of allergenic response is determined by the biology of the exposed individual. It is unlikely that a microorganism modified in keeping with EPA's specifications for the introduced genetic material would induce a heightened response. The general worker hygiene

procedures specified by EPA should protect most individuals from the allergenic responses associated with microorganisms exhausted from fermentors and/or other substances emitted along the production line. The EPA requirement thataccess to the structure be controlled also addresses this consideration by reducing to a minimum the number of individuals exposed.

4. Effect of containment criteria. As further assurance that risks would be low, EPA is specifying procedures for minimizing the number of organisms emitted from the facility for the Tier I exemption. EPA is not specifying standards for minimizing the number of microorganisms emitted from the facility for microorganisms qualifying for Tier II exemption. Rather, the Agency requests that submitters utilize as guidance the standards set forth for Tier I procedures. The procedures proposed by the submitter in a Tier II exemption request will be reviewed by the Agency. EPA will have the opportunity to evaluate whether the procedures the submitter intends to implement for reducing the number of organisms emitted from the facility are appropriate for that microorganism.

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B. Introduced Genetic Material Criteria

In order to qualify for either the Tier I or Tier II exemption, any introduced genetic material must be limited in size, well characterized, free of certain nucleotide sequences, and poorly mobilizable.

1. Limited in size. Introduced genetic material must be limited in size to consist only of the following: (1) the structural gene(s) of interest; (2) the regulatory sequences permitting the expression of solely the gene(s) of interest; (3) the associated nucleotide sequences needed to move genetic material, including linkers, homopolymers, adaptors, transposons, insertion sequences, and restriction enzyme sites; (4) the nucleotide sequences needed for vector transfer; and (5) the nucleotide sequences needed for vector maintenance.

The limited in size criterion reduces risk by excluding the introduction into a recipient of extraneous and potentially uncharacterized genetic material. The requirement that the regulatory sequences permit the expression solely of the structural gene(s) of interest reduces risk by preventing expression of genes downstream of the inserted genetic material. The limitation on the vector sequences that are components of the introduced genetic material prevents the introduction of novel traits beyond those associated with the gene(s) of interest. The overall result of the limited in size criterion is improved ability to predict the behavior of the resulting microorganism.

2. Well characterized. For introduced genetic material, well characterized means that the following have been determined: (1) the function of all of the products expressed from the structural gene(s); (2) the function of sequences that participate in the regulation of expression of the structuralgene(s); and (3) the presence or absence of associated nucleotide sequences.

Well characterized includes knowledge of the function of the introduced sequences and the phenotypic expression associated with the introduced genetic material. Genetic material which has been examined at the restriction map or sequence level, but for which a function or phenotypic trait has not yet been ascribed, is not considered well characterized. Well characterized would include knowing whether multiple reading frames exist within the operon. This relates to whether more than one biological product might be encoded by a single sequence, and addresses the possibility that a modified microorganism could display unpredicted behavior should such multiple reading frames exist and their action not be anticipated.

3. Free of certain sequences. In addition to improving the ability to predict the behavior of the modified microorganism, the well characterized requirement ensures that segments encoding for either part or the whole of the toxins listed in the proposed regulatory text for the TSCA biotechnology rule would not inadvertently be introduced into the recipient microorganism.

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These toxins are polypeptides of relatively high potency. Other types of toxins (e.g., modified amino acids, heterocyclic compounds, complex polysaccharides, glycoproteins, and peptides) are not listed for two reasons. First, their toxicity falls within the range of moderate to low. Second, these types of toxins generally arise from the activity of a number of genes in several metabolic pathways (multigenic).

In order for a microorganism to produce toxins of multigenic origin, a large number of different sequences

would have to be introduced and appropriately expressed. It is unlikely that all of the genetic material necessary for producing multigenic toxins would be inadvertently introduced into a recipient microorganism when requirements that the genetic material be limited in size and well characterized are followed.

Similarly, other properties that might present risk concerns result from the interactive expression of a large number of genes. For example, pathogenic behavior is the result of a large number of genes being appropriately expressed. Because of the complex nature of behaviors such as pathogenicity, the probability is low that an insert consisting of well characterized, limited in size genetic material could transform the microorganisms listed for exemption into microorganisms which display pathogenic behavior.

4. Poorly mobilizable. Poorly mobilizable means the ability of the introduced genetic material to be transferred andmobilized is inactivated, with a resulting frequency of transfer of less than 10^{-8} transfer events per recipient. The requirement that the introduced genetic material be poorly mobilizable reduces potential for transfer of introduced genetic sequences to other microorganisms in the environment. Such transfers would occur through the interaction of the introduced microorganism with indigenous microorganisms through conjugation, transduction, or transformation. Through such transfers, the introduced genetic material could be transferred to and propagated within different populations of microorganisms, including microorganisms which may never previously have been exposed to this genetic material. It is not possible to predict how the behavior of these potential recipient microorganisms will be affected after uptake and expression of the genetic material.

Since EPA is not limiting the type of organism that can serve as the source for the introduced genetic material, some limitation is placed on the ability of the introduced genetic material to be transferred. This limitation mitigates risk by significantly reducing the probability that the introduced genetic material would be transferred to and expressed by other microorganisms.

The 10^{-8} frequency is attainable given current techniques. Plasmids with transfer rates of 10^{-8} exist or are easily constructed. Some of the plasmids most commonly employed as vectors in genetic engineering (e.g., pBR325, pBR322) have mobilization/transfer frequencies of 10^{-8} or less.

The criteria set for "poorly mobilizable" for transduction and transformation should be readily met since the majority of transfer frequencies reported for transduction and natural transformation are less than 10^{-8}. Higher frequencies are likely only under special circumstances, such as when the introduced genetic material has been altered or selected to enhance frequency. Because the risk concern EPA addresses with the 10^{-8} criterion is spread of the introduced genetic material broadly through microbial populations, exchanges between very closely related microorganisms, even if occurring at high frequency, is not a concern so long as the spread through populations does not occur at high frequency.

Fungal gene transfer has also been considered in development of the poorly mobilizable criterion. Although mobile genetic elements such as transposons, plasmids and double stranded RNA exist in fungi and can be readily transferred, this transfer usually is only possible between members of the same species during anastomosis, a process specific to fungi. Since anastomosis only occurs between members of the same species, the introduced genetic material would not be transferred to distantly related fungi as may occur with bacteria.

5. Effect of introduced genetic material criteria. The requirements placed on the introduced genetic material, in concert with the level of safety associated with Aspergillus oryzae, ensure that the resulting microorganisms present low or negligible risk. The probability is low that the insertion of genetic material meeting EPA's criteria into strains of A. oryzae will change their behavior so that they would acquire the potential for causing adverse effects. Risks would be mitigated by the four criteria placed on the introduced genetic material, the relative safety of A. oryzae, and the inactivation criteria specified for the Tier I exemption. In the case of Tier II exemption, risks would be mitigated in light of the four criteria placed on introduced genetic material, the relative safety of A. oryzae, and EPA's review of the containment conditions selected.

C. Recipient Microorganism Criteria

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Six criteria were used by EPA to determine eligibility of recipient microorganisms for the tiered exemption.

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All of the criteria were used together to determine whether, on balance, the microorganisms would not present an unreasonable risk to human health or the environment. Microorganisms which EPA finds meet these criteria are listed as eligible recipients. The first criteria would require that it be possible to clearly identify and classify the microorganism. Available genotypic and phenotypic information should allow the microorganism to be assigned without confusion to an existing taxon which is easily recognized. Second, information should be available to evaluate the relationship of the microorganism to any other closely related microorganisms which have a potential for adverse effects on human health or the environment. Third, there should be a history of safe commercial use for the microorganism. Fourth, the commercial uses should indicate that the microorganism products might be subject to TSCA jurisdiction. Fifth, studies are available which indicate the potential for the microorganism to cause adverse effects on human health and the environment. Sixth, studies are available which indicate the survival characteristics of the microorganism in the environment.

After each microorganism was reviewed using the six evaluation criteria, a decision was made as to whether to place the microorganism on the list. The Agency's specific determination for Aspergillus oryzae is discussed in the next unit.

III. EVALUATION OF ASPERGILLUS ORYZAE

A. History of Use

1. History of safe commercial use. A. oryzae has been used for centuries in the production of many differentoriental foods such as soy sauce, sake and miso. As a "koji" mold, A. oryzae has been used safely in the food industry for several hundred years. It is also used to produce livestock probiotic feed supplements. The koji mold enzymes were among the first to be isolated and commercialized nearly 100 years ago. A. oryzae is considered a Class 1 Containment Agent under the NIH Guidelines for Research Involving Recombinant DNA Molecules. In Europe, Aspergillus species are considered category 2 under the European Federation of Biotechnology guidelines and category 1 under the OECD containment scale.

2. Products subject to TSCA jurisdiction. A. oryzae is currently used in the production of organic compounds such as glutamic acid, and several enzymes that are of potential use commercially, for example, amylase, protease, b-galactosidase, lipase, and cellulase. While these enzymes could be used as TSCA products, several of them have been more often used in food processing. In 1989, EPA reviewed a premanufacture notice (PMN) for a strain of A. oryzae modified for enhanced production of a lipase enzyme to be used primarily in detergent formulations for the removal of fat-containing stains. In 1994, EPA reviewed a PMN for a similar strain of A. oryzae modified for enhanced production of a cellulase gene for use in detergents as a color brightening agent.

B. Identification of Microorganism

1. Classification. The genus Aspergillus represents a grouping of a very large number of asexual fungi whose taxonomy is based on morphological features. The genus has been divided into groups based on attributes of the spores, conidiophores, and sclerotia. Because this separation of individual species into groups is based on morphological or physiological characteristics, it has resulted in somewhat tenuous and overlapping classification. While it has been hypothesized that A. oryzae is a domesticated version of A. flavus, a species known to produce potent aflatoxins, this theory has not been fully accepted by the scientific community. Distinguishing between these two species can be difficult.

2. Related species of concern. A. oryzae is a member of the A. flavus group of Aspergillus species. Most of the members of this group are known to produce potent mycotoxins, including aflatoxins. Chromosomal DNA homology and other techniques have shown strains of A. oryzae and A. flavus to be essentially indistinguishable. It has been assumed that A. oryzae is a domesticated version of A. flavus that has been selected for use in foods because of its loss of ability for aflatoxin production.

C. Risk Summary

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1. Studies regarding potential for adverse effects. A. oryzae strains can produce a variety of mycotoxins after extended fermentation; however, only a few strains are known to produce the more potent toxins. A.

oryzae does not appear to be a significant human pathogen, nor has it been reported as a plant or animal pathogen. Although A. oryzae strains appear stable under cultivation, in theory, there remains a low probability that reversion to the A. flavus phenotype could occur, if rearrangement rather than deletion is the mechanism by which the A. flavus phenotype is lost. However, through centuries of use in the food industry, A. oryzae has not been shown to produce aflatoxins.

2. Studies regarding survival in the environment. There are conflicting opinions about whether A. oryzae can be isolated from the environment. A. oryzae seems to be a species created by domestication of the A. flavus wild type, and therefore, may have lost certain features important to survival in the environment. Although soil is a possible natural habitat for A. oryzae, the intrinsic features of domesticated strains are expected to limit their ability to survive in a natural environment.

IV. PUBLIC COMMENTS RELEVANT TO THE RISK ASSESSMENT

A request for comments was made in the Proposed Risk Assessment and the Proposed Decision Document for A. oryzae pertaining to (1) the conclusion that genetic modification of A. oryzae cannot inadvertently produce an aflatoxigenic strain, and (2) whether there is a need to differentiate between strains of A. oryzae having a long history of safe use and recently isolated strains. However, no comments were received on this specific microorganism either in general or addressing these two requests for comments. There were a number of comments received on the tiered exemption, however, and some of these comments are relevant to the criteria discussed at Section II.A. of this document considered in listing this microorganism as an eligible recipient microorganism at õ 725.420.

Some of the general comments received on the exemptions addressed the six criteria EPA used to select candidate recipient microorganisms to include at õ 725.420. None of these comments questioned the eligibility of A. oryzae for inclusion on the list at õ 725.420. EPA's responses to comments on how it used the six criteria to select candidate microorganisms at õ 725.420 are detailed in the Response to Comments document to be found in the docket for this rulemaking.

The other comments pertained to the proposed containment conditions at õ 725.422. Commenters questioned two criteria, the first of which is at õ 725.422(b): "limit entry only to those persons whose presence is critical to the reliability or safety of the activity". Commenters pointed out that under the requirement as proposed, managers may be precluded from allowing administrative personnel, customers, and school and other educational tours into the facility. EPA had not intended to constrain facility managers to this extent and reconsidered the standard. EPA has revised õ 725.422(b) to read "Control access to the structure". Additional explanation for this revision is given in the Response to Comments document (Section III.C.4.a.) and the Preamble of the Final Rule. Both of these documents can be found in the docket for this rulemaking.

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The other criterion in the proposed rule which commenters questioned was at õ 725.422(e): "provide and document effectiveness of features to reduce microbial concentrations by at least two logs in aerosols and exhaust gases released from the structure". Commenters argued that the requirement as written would require retrofitting of equipment in order to permit measurement within the fermentor headspace of microbial concentrations in aerosols. EPA had not intended that manufacturers be required to retrofit their fermentation equipment in order to qualify for this exemption. Therefore, EPA re-examined the basis for this criterion by reviewing information submitted on physical containment and control technologies in the PMNs it had received for intergeneric microorganisms between 1986 and 1995. Examination of these PMNs revealed that the number of microorganisms potentially released through fermentor exhaust gases is negligible compared to the number contained in the liquid and solid waste streams. Even under a worst case scenario of an uncontrolled release, as evaluated in the accompanying risk assessment, the number of viable microorganisms aerosolized with the fermentor exhaust gases would still be low, and therefore, the risk would remain low. Moreover, the use of a criterion requiring controls to minimize microbial numbers released through aerosolization at õ 725.422, as compared to the worst case scenario of an uncontrolled release, would result in lesser exposure, and therefore, lower risk than under the uncontrolled release scenario. Uncontrolled releases are not standard industry practice because there are a number of economic considerations driving the control of exhaust gases such as maintaining proper molality of the fermentation broth by the use of a vapor recovery system, maintaining sterility, and preventing release of microorganisms for proprietary reasons. Therefore, upon re-evaluation, the Agency decided that language requiring minimization of microbial concentrations in aerosols could be substituted for the requirement of

the 2-log reduction performance criterion without affecting the no unreasonable risk finding necessary for a 5(h)(4) exemption under TSCA. The potentially increased exposure to this organism from themodification of the containment criteria from the proposed 2-log reduction to minimizing microbial numbers in exhaust gases does not change the risk of using this microorganism for fermentation. Therefore, EPA has revised ô 725.422(e) to read: "Use features known to be effective in minimizing viable microbial populations in aerosols and exhaust gases released from the structure, and document use of such features". The Response to Comments document (Section III.C.4.b.) and the Preamble of the Final Rule provide a thorough explanation for the change in requirements for microbial releases through exhaust gases.

V. BENEFITS SUMMARY

Substantial benefits are associated with this exemption. Aspergillus oryzae is already widely employed in general commercial use. Many of these uses, such as the production of soy sauce, miso, and sake, and the production of enzymes such as a-amylase, lactase, and b-galactosidase are food uses and not subject to TSCA. However, some enzymes such as a-amylase and protease, both of which are produced by A. orzyae, have wide applications in the detergent industry. The Agency has already reviewed two PMNs involving the use of A. oryzae for production of two enzymes, a lipase and a cellulase, which were for use in commercial detergents.

The Agency believes this exemption will result in resource savings both to EPA and industry without compromising the level of risk management afforded by the full 90 day review. The exemption will result in reduced reporting costs and a decrease in delay associated with reporting requirements. The savings in Agency resources can be directed to reviewing activities and microorganisms which present greater uncertainty. This exemption should also facilitate development and manufacturing of new products and the accumulation of useful information.

VI. FINAL ANALYSIS

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1. 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.

A. oryzae does not appear to produce aflatoxins. Additionally, in the industrial setting, the use of proper safety precautions, good laboratory practices, and proper protective clothing, allay concerns for exposure of workers to mycotoxins potentially produced by this microorganism. A. oryzae appears to lack many survival features necessary for establishment in theenvironment. Potential hazards to the public and the environment are also mitigated by limitations to exposure brought about by the conditions of contained use which are designed to limit release of the microorganisms to the environment.

2. Use of strains of A. oryzae which are eligible for the TSCA section 5(h)(4) exemption present no unreasonable risk. Concern has been expressed that genetic modification of A. oryzae could inadvertently produce an aflatoxigenic strain, although this is of low probability. Naturally occurring strains of A. oryzae are not known to produce aflatoxins; however, some scientists have suggested that A. oryzae is a domesticated version of A. flavus and may possess dormant genes for aflatoxin production. This exemption is for strains of A. oryzae and does not include A. flavus. As part of their eligibility for this TSCA section 5(h)(4) exemption, companies are required to certify that they are using A. oryzae. It is therefore expected that companies will have information in their files which documents the correct identification of their strains. Additionally, it is expected that companies will choose well-characterized industrial strains for further development through genetic modification. These expectations in combination with the use of Good Laboratory Practices should ensure the use of the correct species.

The limitations placed by the section 5(h)(4) exemption on the introduced genetic material, in particular the well-characterized and limited in size restrictions, should reduce the likelihood that any sequences relating to aflatoxin production could be introduced. The containment requirements would limit exposure to any mycotoxins produced.

000258

Because the recipient microorganism was found to have little potential for adverse effects, introduced

genetic material meeting the specified criteria would not likely significantly increase potential for adverse effects. As further assurance that risks would be low, EPA is specifying procedures for minimizing numbers of organisms emitted from the facility for the Tier I exemption and will be reviewing the conditions selected for the Tier II exemption.

Modification of the language of the two proposed containment requirements ô 725.422 does not affect EPA's original determination that microorganisms that are eligible for and used under the conditions of the Tier I exemption will not present an unreasonable risk of injury to human health or the environment. Increased exposure to the microorganisms within or outside the facility resulting from these revisions will be minimal. The risk of using this microorganism in fermentation under the final conditions of this exemption is still low.

When balanced against resource savings for society and expected product benefits, this exemption will not present unreasonable risks.

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VII. ACTION

Aspergillus oryzae is included as a recipient microorganism at ô 725.420 for the tiered exemption.

Attachment I - Final Risk Assessment of Aspergillus oryzae

Note: For Attachment I to this Final Decision Document, see "Final Risk Assessment of Aspergillus oryzae" appearing elsewhere in the list of "Support Documents."

Appendix
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Pages 000261-000264 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

Appendix 8

Pages 000266-000295 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

Appendix 10

Novo Nordisk

Enzyme Business Toxicology

Date : 1997-09-08
File: ED-9713942
Ref.: ASB/DSc

REPORT

Pathogenicity of *Aspergillus oryzae* to rats after oral administration

Novo Nordisk Study No: 1288

This report is a translation of the original report on the study 1288.
Tables and appendices are not included.

Author:
Anne Sietske de Boer

Issued by:
Novo Nordisk A/S
Novo Allé
2880 Bagsvaerd
Denmark

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1. GENERAL INFORMATION

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TEST FACILITY	Novo Nordisk A/S Novo Allé DK - 2880 Bagsvaerd
DEPARTMENTS INVOLVED	EB Toxicology Animal Unit, Bagsvaerd
ARCHIVE	Enzyme Business Information & Documentation (EB INFODOC) Novo Nordisk A/S Novo Allé DK - 2880 Bagsvaerd

PERSONNEL INVOLVED IN THE STUDY

STUDY DIRECTOR	Anne Sietske de Boer, D.V.M. Hans Peter Heldt-Hansen
LABORATORY ASSISTANT	Jens Bak

DATE OF FINAL REPORT

Date : 1997-09-09

Anne Sietske de Boer
D.V.M.
Study Director

000299

2. SUMMARY

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Study design:
The aim of the study was to evaluate the pathogenicity of the production strain *Aspergillus oryzae* A1560 and to investigate the possibility of this organism to colonise in the gastrointestinal tract of rats.

The test strain was grown in a nutrient broth. The nutrient broth and the fungi was homogenised prior to dosing.

The fungi was dosed orally to a group of five male and five female Wistar rats. The animals were observed for any clinical reactions to treatment for 10 days. All rats were subjected to macroscopic pathological investigation after the terminal kill at day 10. During the ten day observation period faeces from each animal was collected daily and examined for the presence of the dosed micro-organism.

Result:
The oral administration of *Aspergillus oryzae* to rats did not cause any clinical reaction to the test animals during the ten day observation period. The micro-organism was recovered the day after dosing in only one animal.

Conclusion:
It is concluded that *Aspergillus oryzae* A1560 is non pathogenic to rats after oral application and that the micro-organism is unable to colonise in the gastrao-intestinal tract of the rat.

3. AIM OF THE STUDY

The aim of this study was to investigate the pathogenic potential of *Aspergillus oryzae* to rats when dosed orally and to investigate the capability of the micro-organism to colonise in the gastro-intestinal tract.

4. MATERIAL

4.1 Test material

Aspergillus oryzae was cultured in nutrient broth for 3 day at 30° C. At the termination of the growth period the broth was blended to achieve a homogenous suspension of the test material.

The viable count of the broth before administration of the test substance to the rats was 3×10^6 colony forming units/ml.

4.2 Animals

Male and female Wistar rats were received from Møllegaard Breeding Centre. Lille Skensved, Denmark.

The animals were acclimatised for four days in cages holding five animals of same sex. Thereafter the rats were caged singly for three days in cages having grid floors. The rats were fed standard rat maintenance diet (Brood Stock Feed for Rats and Mice - R3 - Ewos).

During the acclimatisation period and the test period the animals were weighed at the arrival and at termination of the acclimatisation and there after once each fifth day.

4.3 Dosing

The rats were dosed orally a single dose of the micro-organism after depriving of food for 20 hours. All animals were dosed either concentrated test material or the control substance (0.9% NaCl) according to the dosing scheme:

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Substance	Dosage (cfu/cm²/kg bw)	Animal number Male	Female
Sodium chloride	-	1 - 5	6 - 10
A. oryzae A 1560	3×10^7	21 - 25	26 - 30

4.4 Observation
The rats were observed daily during the acclimatisation period and the test period. The animals observed 30 minutes and 2 hours after dosing and at least once daily the following 10 days.

4.5 Termination of the study
All animals were killed by CO_2+O_2 anaesthesia and bleeding and autopsy.

5. RESULTS FROM THE ANIMAL EXPERIMENTS

5.1 Mortality and clinical symptoms
No animals died or showed clinical symptoms during the study.

5.2 Body weight
The body weight development was not affected by treatment.

5.3 Gross pathology
No macroscopic alterations were observed at the autopsy.

6. MICROBILOGICAL RECOVERY. METHOD AND RESULTS

6.1 Method
The faeces of the rats was collected daily and examined immediately after.

The faecal sample was diluted 5 times by sterile water and mixed until a complete suspension was present.

From each suspension 1 ml was transferred to each of 4 different Petri dishes (ø=13 cm) and was incubated for 30°C for 4-6 days.

At the evaluation the number of colonies of filamentous fungi and the number of these which have morphological similarities with the test organism. The final identification is performed by RIE (rocket immunoelectroforesis).

6.2 Results
The micro-organism was recovered for faeces from one animal one day after dosing, only.

7. DISCUSSION AND CONCLUSION

Based upon the experimental procedures it can be concluded that *A. oryzae* A1560 is non pathogenic to rats after oral administration. Furthermore it is concluded that the micro-organism is unable to colonise in the gastro-intestinal tract of the rat.

Appendix 11

Pages 000303-000324 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

Pages 000303-000324 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

Pages 000326-000349 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

Appendix 13

Pages 000351-000377 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

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000093	Pariza, M.W.; Foster, E.M.	Determining the Safety of Enzymes Used in Food Processing	May 1983	Journal of Food Protection	Volume 46, Number 5, pgs 453-468
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000107	NA	Safety Evaluation of Foods and Food Ingredients Derived from Microorganisms	1990	Regulatory Toxicology and Pharmacology	Volume 12, pgs S114-S128
000108	Joint FAO/WHO Expert Committee on Food Additives	Evaluation of Certain food additives and contaminants	1987	Joint FAO/WHO	Series 759
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NA- Not applicable

Pages	Author	Title	Publish Date	Source	BIB_Info
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000186-000199	Joint FAO/WHO Expert Committee	Evaluation of certain food additives and contaminants	1987	Joint FAO/WHO Expert Committee	NA
000213-000229	Pariza, M.W.; Foster, E.M.	Determining the Safety of Enzymes Used in Food Processing	August 1983	Journal of Food Protection	Volume 46
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NA- Not applicable