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October 5, 2004

Linda S. Kahl, Ph.D.
Office of Food Additive Safety, HFS-255
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

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' lipase enzyme preparation produced by *Aspergillus niger* expressing the gene encoding a lipase from *Candida antarctica*. The lipase enzyme preparation is intended for use in the fats and oils industry.

Please contact me by direct telephone at 919 494-3151 or direct fax at 919 494-3420 if you have any questions or require additional information.

Sincerely,

Lori Gregg
Regulatory Specialist

Enclosures (3 binders)

Novozymes North America, Inc.
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Tel: 919-494-3000 *Fax: 919-494-3450* *Internet: www.novozymes.com*

October 4, 2004

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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 lipase preparations produced by submerged fermentation of *Aspergillus niger* expressing a gene encoding a lipase from *Candida antarctica* 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.*

Lipase enzyme preparation from *Aspergillus niger* expressing a gene encoding a lipase from *Candida antarctica.*

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

The lipase is intended for use in the fats and oils industry as a processing aid in the production of triglyceride products with improved physical and/or nutritional properties. 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. 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.

John Carroll — Date
Director, Regulatory Affairs

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LUNA No. 2004-39073-01

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Lipozyme® CalB L

A lipase preparation produced by *Aspergillus niger* expressing a gene encoding a lipase from *Candida antarctica*

Lori Gregg, Regulatory Affairs, Novozymes North America, Inc., USA
Peter Hvass, Regulatory Affairs, Novozymes A/S, Denmark

October 2004

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1. GENERAL INTRODUCTION

Lipozyme® CalB L is the Novozymes A/S trade name used for a lipase preparation produced by submerged fermentation of *Aspergillus niger* carrying a gene coding for a lipase from *Candida antarctica*.

The enzyme preparation is to be used as a processing aid in the fats and oils industry.

The enzyme is a lipase (EC 3.1.1.3), which catalyzes esterification and interesterification reactions of triglycerides. This is utilized in applications within the oils and fats industry to improve the physical and/or the nutritional properties of triglyceride products.

The information provided in the following sections is the basis for our determination of general recognition of safety of a lipase enzyme preparation produced by *A. niger* expressing a gene encoding a lipase from *Candida antarctica*. Our safety evaluation in Section 7 includes an evaluation of the production strain, the enzyme, and the manufacturing process, as well as an evaluation of dietary exposure to the preparation.

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[1,2]. The production organism for this Lipase, *A. niger*, is discussed in Sections 2 and 7. An essential aspect of the safety evaluation of food components derived from genetically modified organisms is the identification and characterization of the inserted genetic material[3-8]. The genetic modifications used to construct the production microorganism are well defined and are described in Section 2. The safety studies performed and described in Section 7 show no evidence to indicate that any of the cloned DNA sequences and incorporated DNA encode or express a harmful or toxic substance.

It should be noted that in some reports, the enzyme product is designated "Candida B Lipase", "CALB" or batch "PPW 22633". Candida B Lipase or CALB are names used internally during development. Batch PPW 22633 is the internal code of the test batch used for safety testing.

2. PRODUCTION MICROORGANISM

2.1 Production Strain

The *A. niger* strain, designated pCaHj600/MBin118#11, was constructed by transformation of the recipient strain, designated MBin118 (see Section 2.2). The selection of transformants was achieved by growing on a medium with acetamide as the sole nitrogen source and subsequent screening for *Candida antarctica* lipase. A tranformant pCAHj600/MBin118#11 was selected and used for enzyme production. This genetically modified production organism complies with the OECD (Organization for Economic Co-operation and Development) criteria for GILSP (Good Industrial Large

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Scale Practice) microorganisms[9]. It also meets the criteria for a safe production microorganism as described by Pariza and Foster and several expert groups[3-8].

The *Candida antarctica* lipase B expression plasmid, pCaHj600 used in the strain construction contains strictly defined fungal chromosomal DNA fragments and synthetic DNA linker sequences. The specific DNA sequences include: a gene encoding a *Candida antarctica* lipase enzyme; an *A. nidulans* selectable marker gene, *amd*S (acetamidase)[10]; well-characterized noncoding regulatory sequences including the *A. niger* terminator[11], the *A. niger* NA2 promoter[12], the 5' nontranslated leader of the *A. nidulans* triose phospate isomerase gene[13]; a 1163 bp fragment from the *Escherichia coli* cloning vector pUC19[14], and the *Saccharomyces cerevisiae* URA3 gene.

2.2 Recipient Organism

The host strain is an *Aspergillus niger*, derived from *A. niger* strain C-40 which was isolated from a soil sample.

Aspergillus niger C-40 was genetically modified by means of classical mutagenesis to remove the α-1,6 transglucosidase activity and to increase the yield of glucoamylase. Site-directed gene disruption resulted in the deletion of the glucoamylase gene and the interruption of the acid stable amylase gene, the neutral amylase I gene, the neutral amylase II gene and the general protease regulator *prtT* gene.

The host, *A. niger* strain MBin118 was constructed through the steps outlined below. Only *A. niger* DNA was transformed into strain C-40 during the development of the host, *A. niger* strain MBin118.

1) An α-1,6 transglucosidase deficient mutant and amyloglycosidase yield mutant was isolated from C-40 by means of several rounds of mutagenesis, resulting in the strain BO1.

2) A *pyrG* minus isolate was obtained by transformation of BO1 with a fragment of the *Aspergillus niger pyrG* gene (only containing the *pyrG* flanking sequences) and subsequent selection on agar plates containing 5-flouro-orotic acid (5-FOA). The resulting strain, JRoy3, contains a large deletion in the *pyrG* gene.

3) Transformation of JRoy3 with linear DNA containing the glucoamylase gene interrupted with the *A. niger pyrG* gene resulted in the strain SMO110.

4) MBin111, a *pyrG* deleted strain of SMO110, was isolated by transforming with linear DNA containing the *A. niger* glucoamylase promoter and terminator.

5) Transformation of MBin111 with linear DNA containing the acid stable amylase gene interrupted with the *A. niger pyrG* gene resulted in the strain MBin112.

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6) MBin113, a spontaneous *pyrG* loop-out of MBin112, was isolated by plating of spores on agar plates containing 5-flouro-orotic acid (5-FOA).

7) Transformation of MBin113 with linear DNA containing the general protease regulator *prtT* gene interrupted with the *A. niger pyrG* gene resulted in the strain MBin114.

8) MBin115, a spontaneous *pyrG* loop-out of MBin114, was isolated by plating of spores on agar plates containing 5-flouro-orotic acid (5-FOA).

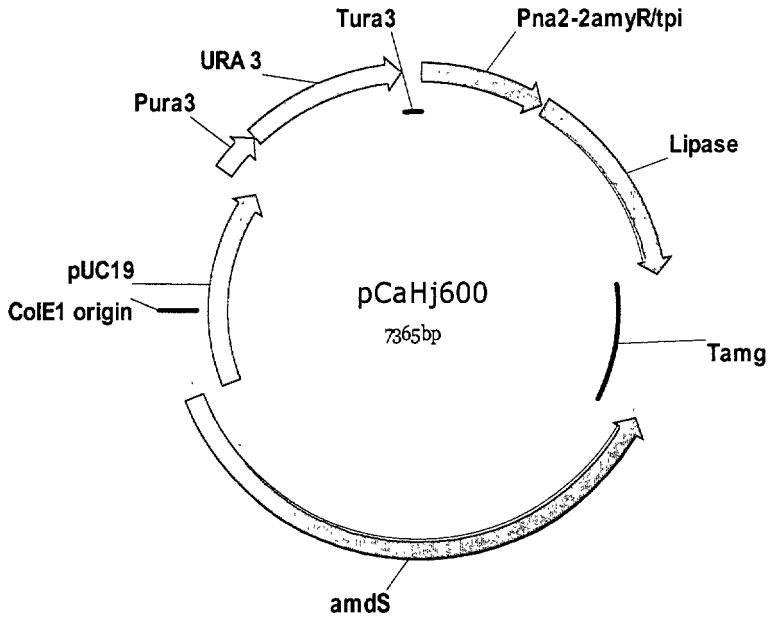
9) Transformation of MBin115 with linear DNA containing the neutral amylase II gene interrupted with the *A. niger pyrG* gene resulted in the strain MBin116.

10) MBin117, a spontaneous *pyrG* loop-out of MBin116, was isolated by plating of spores on agar plates containing 5-flouro-orotic acid (5-FOA).

11) Transformation of MBin117 with linear DNA containing the neutral amylase I gene interrupted with the *A. niger pyrG* gene resulted in the strain MBin118.

2.3 Lipase Expression Plasmid

The7365 bp lipase expression plasmid pCaHj600 consists of the following elements:



Plasmid map of pCaHj600

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Position (bp)	Size (bp)	Element	Origin
1-616	616	Pna2/TPI	*A. niger* BO1
617-627	11	Linker	Synthetic
628-1653	1026	lipase	*C. antarctica*
1654-1671	18	Linker	Synthetic
1672-2369	698	Tamg	*A. niger* BO1
2370-5094	2725	amdS	*A. nidulans*
5095-6257	1163	pUC19	*E. coli*
6258-6480	223	Pura3	*S. cerevisiae*
6481-7281	801	URA3	*S. cerevisiae*
7282-7365	84	Tura3	*S. cerevisiae*

Pna2/tpi is the neutral amylase II promoter from *Aspergillus niger*. The 5' nontranslated part of this promoter has been replaced with the 5' nontranslated part of the *Aspergillus nidulans* triose phosphate isomerase (TPI) promoter (position 550-616).
Lipase is the *C. Antarctica* lipase genomic gene.
Tamg is the amyloglycosidase terminator of *Aspergillus niger*.
amdS is the acetamide gene (including promoter and terminator) from *A. nidulans*.
pUC19 is a fragment of the pUC19 vector including the origin of replication. The origin of replication initiation site (colE1 origin) is in position 5529.
Pura3, URA3 and Tura 3 are the *S. cerevisiae* URA3 promoter, coding sequence and terminator respectively.

2.4 Stability of the Introduced Genetic Sequences

The presence of the introduced DNA sequences was 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[3-8]. The transforming DNA is stably integrated into the *A. niger* chromosome and, as such, is poorly mobilizable for genetic transfer to other organisms and is mitotically stable[9].

2.5 Antibiotic Resistance Gene

The DNA used for transforming the *A. niger* host strain does not contain any antibiotic resistance genes.

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2.6 Absence of Production Organism in Product

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

3. ENZYME IDENTITY

The primary enzyme activity is lipase. According to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUB), "Enzyme Nomenclature 1992" and the Chemical Abstracts Service, the lipase activity is classified as:

Classification	Lipase (generic name)
IUB nomenclature:	Triacylglycerol lipase
IUB No.:	3.1.1.3
CAS No.:	9001-62-1

The enzyme catalyzes the rearrangement of fatty acids in triglycerides, resulting in triglyceride products with improved physical and/or nutritional properties.

4. MANUFACTURING PROCESS

This section describes the manufacturing process for Lipozyme CalB L which follows standard industry practices[15-17]. The quality management system used in the manufacturing process complies with the requirements of ISO 9001. It is also manufactured in accordance with current good manufacturing practices.

4.1 Raw Materials

The raw materials used in the fermentation and recovery process for the lipase enzyme concentrate are standard ingredients used in the enzyme industry[15-17]. 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 antifoams and flocculants 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 and flocculants in the Lipase product is less than 1%.

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4.2 Fermentation Process

The lipase is manufactured by submerged fed-batch pure culture fermentation of the genetically modified strain of *A. niger* described in Section 2. All equipment is carefully designed, constructed, operated, cleaned, and maintained so as to prevent contamination by foreign microorganisms. During all 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

Each batch of the fermentation process is initiated with a lyophilized stock culture of the production organism, *A. niger,* described in section 2. Each new batch of the stock culture is thoroughly controlled 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 are fulfilled:

1. Infection is observed in 2 or more samples by microscopy
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 which starts immediately after the fermentation process and consists of both the purification and the formulation processes.

4.3.1 Purification Process

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

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1. Pretreatment - pH adjustment
2. Primary Separation - vacuum drum filtration
3. Concentration - ultrafiltration and/or evaporation
4. Pre- and Germ Filtration - for removal of residual production strain organisms and as a general precaution against microbial degradation
5. Preservation and Stabilization – sodium benzoate and potassium sorbate addition
6. Final concentration – evaporation and/or ultrafiltration if enzyme concentration is too low to reach target yield

4.3.2 Formulation and Standardization Process

Lipozyme CalB L

The enzyme concentrate is standardized with sorbitol and glycerol according to the product specification.

4.4 Quality Control of Finished Product

The final products are analyzed according to the specifications given in section 5.

5. COMPOSITION AND SPECIFICATIONS

5.1 Composition

Lipozyme CalB L is typically composed as follows:

Enzyme solids (TOS*)	approx.	4 %
Glycerol	approx.	25 %
Sorbitol	approx.	25 %
Water	approx.	46 %
Sodium benzoate		0.2 %
Potassium sorbate		0.1 %

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

5.2 Specifications

The Lipase conforms to the general and additional requirements for enzyme preparations as described in Food Chemicals Codex, 5th edition, 2003[18]. In addition, the Lipase 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[19].

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The following Novozymes' specifications have been established for the Lipase:

Enzyme activity, KLU/g*	according to declaration
Heavy metals	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

Antibiotic activity, and mycotoxins are analyzed at regular intervals.

*The activity of this enzyme is measured in KLU/g. The LU method is based on the speed at which the enzyme hydrolyzes tributyrin at pH 7.0. The butyric acid formed is titrated with sodium hydroxide and the consumption of the latter recorded as a function of time. 1 LU is the amount of enzyme, which releases 1 µmol butyric acid per minute under the given standard conditions. 1 KLU = 1000 LU.

6. APPLICATION

6.1 Mode of Action

The enzyme in Lipozyme CalB L is a lipase (EC 3.1.1.3). Lipases hydrolyze esters in aqueous solutions, and they catalyze esterification of fatty acids and alcohol or rearrange fatty acids in glycerides under certain conditions i.e. low water content.

Lipozyme CalB L is used for several applications in the oils and fats industry. Typical substrate reaction mixtures comprise triglycerides with or without free fatty acids and a small amount of water. Under such conditions, the product catalyzes the rearrangement of fatty acids in triglycerides, resulting in triglyceride products with improved physical and/or nutritional properties.

The product sheet for Lipozyme CalB L is included in Appendix 1.

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6.2 Use Levels

The enzyme preparation is used at minimum levels necessary to achieve the desired effect.

In most cases, the enzyme will be loaded to and immobilized on a carrier in the reactor prior to the enzyme catalyzed reaction. The optimum dosage is dependent on the specific substrate, reactor configuration and desired conversion etc. However, the recommended dosage of Lipozyme CalB L would be up to 100 KLU per kg of triglyceride, corresponding to 20 kg of Lipozyme CalB L to produce 1 ton of modified triglyceride.

6.3 Enzyme Residues in the Final Food

As described in section 6.2, in most cases, the enzyme is immobilized on a carrier in the reactor prior to the enzyme catalyzed reaction. Therefore, carry-over of enzyme into the final food preparation is negligible.

7. SAFETY EVALUATION

7.1 Safety of the Production Strain

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[1,2]. If the organism is nontoxigenic and nonpathogenic, then it is assumed that food or food ingredients produced from the organism, using current Good Manufacturing Practices, is safe to consume[3]. Pariza and Foster (1983) define a nontoxigenic organism as "one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure" and a nonpathogenic organism as "one that is very unlikely to produce disease under ordinary circumstances". *A. niger* meets these criteria for nontoxigenicity and nonpathogenicity. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) evaluated enzyme preparations derived from *A. niger* and determined that enzymes from this source do not constitute a toxicological hazard[20].

Schuster et al. (Appendix 2) reviewed the safety of *A. niger* and describe it as having a long history of safe industrial use, being widely distributed in nature, and being commonly used for production of food grade enzymes and citric acid[21]. This was also the conclusion in a risk assessment document for *Aspergillus niger* prepared by the US Environmental Protection Agency to exempt *A. niger* strains from its TSCA Biotechnology Program[22].

Aspergillus niger has been used industrially since 1919, for the production of citric acid[21]. The US Food and Drug Administration (FDA) has listed *A. niger* as a source of citric acid (21 CFR §173.280). Carbohydrase, pectinase, protease, glucose oxidase,

catalase, lipase and lactase enzyme preparations from *Aspergillus niger* are the subjects of GRAS Notices GRN 000089, GRN 000111, GRN 000132 (originally part of GRAS petition 3G0016). *A. niger* has a documented history of safe use in food and is considered to be a safe organism for the production of enzymes or ingredients for use in food[2,3].

An evaluation of the genetically modified production microorganism for the Lipase, embodying the concepts initially outlined by Pariza and Foster, 1983 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, 2001 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 plasmid used, the sources and 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 and 3.

Because the genetic modifications are well characterized and specific, and the incorporated DNA does not encode and express any known harmful or toxic substances, the lipase enzyme preparation derived from the genetically modified *A.niger* is considered safe[3,23]. To confirm the safety of the enzyme, safety studies were performed on the enzyme preparation and are described in Section 7.5.

7.1.1 Secondary Metabolites

Some *A. niger* strains may produce ochratoxin A[21]. Thorough investigations of the metabolic potential revealed that the recipient *A. niger* strain used for the Candida antarctica B lipase production strain, when grown under optimized conditions for the production of secondary metabolites, was not capable of producing ochratoxin A or any other known mycotoxin associated with the genus *Aspergillus*. Further, Candida B Lipase PPW 22633 was analyzed for the presence of the secondary metabolites shown in the table below:

Secondary Metabolite Analyses

	Unit	PPW 22633
Aflatoxin B1	mg/kg	ND (<0.0002)
Ochratoxin A	mg/kg	ND (<0.001)
Sterigmatocystin	mg/kg	ND (<0.02)
T-2 toxin	mg/kg	ND (<0.02)
Zearalenon	mg/kg	ND (<0.02)

ND = Not Detected (limit of detection is given in the brackets).

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7.2 Safety of the donor organism

The donor organism for the enzyme gene is a strain of *Candida antarctica*. The strain was isolated from a soil sample from Japan and identified by DSM (Deutsche Sammlung von Mikroorganismen). *Candida antarctica* meets the criteria for a classification in Risk Group 1 according to the NIH guidelines. *Candida antarctica* is not regarded to be pathogenic or known as a toxin producer[24, 25].

7.3 Safety of the Lipase Enzyme

Enzyme proteins themselves do not generally raise safety concerns[2,26,27]. As indicated in section 3, the Lipase is a triacylglycerol lipase, IUB EC 3.1.1.3, which hydrolyzes the primary ester bonds in triglycerides. Most of the lipases commonly used in food processing belong to this group[26].

Microbial lipases have been reported to be used in food production since 1952[28,29]. Animal lipase is affirmed as GRAS (21 CFR 184.1415) based on its common use in food prior to 1958. Esterase-lipase from *Mucor miehei* (now known as *Rhizomucor miehei*) is approved for use as a food additive (21 CFR 173.140). Lipase enzyme from *Rhizopus niveus* is affirmed as GRAS based on scientific procedures (21 CFR 184.1420). Also, Novo Nordisk filed a petition in 1989 proposing to affirm that insoluble esterase-lipase enzyme preparation derived from *Mucor miehei* which has been fixed by immobilization with a substance that is generally recognized as safe or an approved food additive is GRAS for use as a direct human food ingredient (54 FR 9565). Esterase lipase enzyme preparations from *Mucor miehei* have been marketed by Novozymes as GRAS since that time. A lipase preparation produced by *A. oryzae* expressing the gene encoding a lipase from *T. lanuginosus* is the subject of GRAS notification GRN #43. Also, a lipase preparation produced by *A. oryzae* expressing the gene encoding a lipase from *F. oxysporum* is the subject of GRAS notification GRN #75.

7.3.1 Consideration of the Allergenic Potential of the Enzyme

Enzymes are proteins and proteins can be potential allergens. However, only a small percentage of all dietary proteins are allergens. Novozymes is not aware of any allergic reactions caused by the ingestion of lipase. Novozymes' believes that, in general, the risk of allergenicity due to ingestion of enzymes is negligible based on the following considerations:

- the long history of safe use of a wide variety of enzymes in food and food processing
- the ubiquitous nature of enzymes
- the extremely low exposure potential
- the purpose

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As discussed in Section 7.3, enzyme proteins do not generally raise safety concerns. A wide variety of enzymes have been used safely for centuries in food processing. Enzymes, including lipase enzymes, are found in many cells and tissues of plants and animals including those that are consumed by man. They are ubiquitous and in this respect, common and ordinary. They are a normal part of the human diet.

Enzymes are a special type of protein with a highly specific catalytic function. They are essential to many biochemical reactions in microorganisms, plants, animals and humans. Due to the specific nature of enzymes, only very small amounts are required to achieve the desired effect in food. In this case the exposure to the lipase enzyme will be negligible due to the nature of fats and oils processing.

Based on the fact that:

- there are no known cases of allergic responses to lipase in food,
- known cases of allergic responses to food enzymes are very rare, and
- exposure to this enzyme protein is extremely low,

the allergy risk due to the ingestion of this lipase is negligible.

7.4 Safety of the Manufacturing Process

The Lipase 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 lipase preparation is produced in accordance with current good manufacturing practices, using ingredients that are acceptable for general use in foods, and under conditions that ensure a controlled fermentation. These methods are based on generally available and accepted methods used for production of microbial enzymes[15-17].

7.5 Safety studies

This section describes the studies and analysis performed to evaluate the safety of the use of the Lipase.

7.5.1 Description of Test Material

The safety studies described below were conducted on a liquid lipase enzyme concentrate that was prepared according to the description given in Section 4 except that stabilization and standardization were omitted.

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7.5.2 Studies

The following studies were performed:

-13 weeks Oral Toxicity in rats
-Test for mutagenic activity (Ames test)
-Human lymphocyte cytogenetic assay

A summary of the safety studies performed on the lipase is included in Appendix 3.

7.6 Estimates of Human Consumption and Safety Margin

As stated in section 6.3, any carry-over of enzyme into the final food is expected to be negligible.

However, in order to illustrate a "worst case" situation the following calculation is made assuming that the entire enzyme dosage is carried over to the modified oil and fat products.

Lipozyme CalB L has an activity of 5 KLU/g and an approximate content of 4% TOS (Total Organic Substances from the fermentation, mainly protein and carbohydrate components).

The average human intake of oils and fats is estimated using well-established statistics from various countries.

United Kingdom
The Ministry of Agriculture, Fisheries and Food: 1987 Annual Report of the National Food Survey Committee, Household Food Consumption and Expenditure: Consumption of oil & fat excluding butter per person per day is 32 g.

Denmark
"Levnedsmiddelstyrelsen": Development of Food Consumption in Denmark, 1955-1990, Description of the Danish Diet based on food statistics and nutrition calculated data: Consumption of oil and fat per day is 144 g.

USA
The Economics Research Service, USDA: Consumption of vegetable oil per person per day is 69 g.

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

a) The calculation is made assuming that all TOS is carried over to the oil, although it would not be the case in reality. Lipozyme CalB L contains 4 % TOS.

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b) It is assumed that all oils and fats are produced using Lipozyme CalB L as a processing aid, used at the highest recommended dosage.

The maximum recommended dosage is 100 KLU per kg of triglyceride, corresponding to 20 kg of Lipozyme CalB L to produce 1 ton of modified triglyceride as described in Section 6.2. Lipozyme CalB L contains 4 % TOS. Therefore, the triglycerides would contain 0.8 g TOS/kg triglyceride.

Based on the highest average daily intake of oils and fats (144 g), the daily intake per person of Lipozyme CalB L corresponds to 0.8 x 0.144 = 0.12 g TOS per day.

For an average person weighing 60 kg this corresponds to 1.9×10^{-3} g TOS/ kg body weight per day.

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 1.97 g TOS/kg/day.

The estimated human consumption is 1.9×10^{-3} g TOS/kg/day

The safety margin can thus be calculated to be:

$1.97 / 1.9 \times 10^{-3} = 1030$

7.7 Results and Conclusion

The results of the tests described in section 7.5.2 show that the Lipase enzyme preparation does not exhibit any mutagenic activity, clastogenic activity, or toxic effect under the conditions of each specific test. On the basis of the evaluation contained in Sections 7.1-7.5, a review of the published literature, the history of use of *A. niger,* and the limited and well defined nature of the genetic modifications, the Lipase enzyme preparation can be safely manufactured and used as a processing aid in the fats and oils industry as well as in other food or non-food applications.

8. LIST OF APPENDICES

1. Product Sheet for Lipozyme CalB L
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Appendix 1

Product Sheet

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Lipozyme® CALB L

Description

Lipozyme CALB L is a lipase (B lipase) from *Candida antarctica* produced by submerged fermentation of a genetically modified *Aspergillus niger* micro organism.

Product properties

Product type

Lipozyme CALB L is a yellow to light brown liquid. The colour may vary from batch to batch. The colour intensity is not an indication of product strength. Lipozyme CALB L is a food-grade product intended for food and technical applications.

Activity

Lipozyme CALB L contains 5 KLU/g (Kilo Lipase Units per gram).
A detailed description of the analytical method (Lipase Units – ref. 2001-07792) is available on request.

Packaging

Lipozyme CALB L is available in 25 kg Jerry cans.

Application

The enzyme is a thermostable, triacylglycerol hydrolase (E.C. no. 3.1.1.3) and also an effective carboxylesterase. The positional specificity of Lipozyme CALB L depends on the reactants. In some reactions Lipozyme CALB L shows 1.3 positional specificity, whereas in other reactions the lipase functions as a non-positional specific lipase. The lipase has a broad substrate specificity, i.e. it promotes the reaction between a broad range of primary and secondary alcohols and organic acid. Lipozyme CALB L can be used at pH 5-9 but has its optimum at pH 7-8. Lipozyme CALB L can be used at temperatures between 60 and 75 °C (140-167 °F)

Reaction parameters

Lipozyme CALB L is a heat-tolerant product Due to inactivation when running the reaction at elevated temperatures it is recommended to operate in the range of 40-60 °C (104-140 °F)

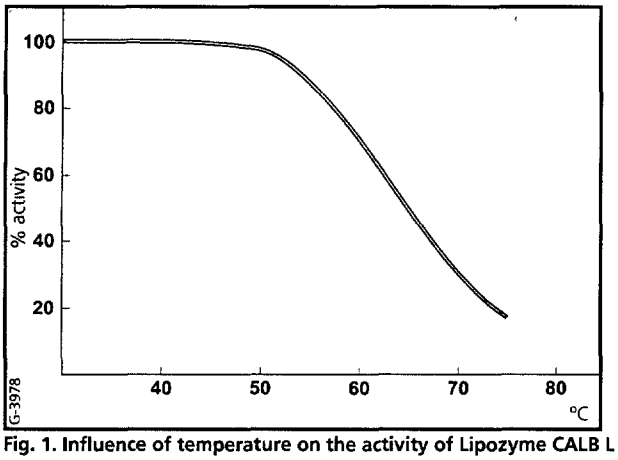

Fig. 1. Influence of temperature on the activity of Lipozyme CALB L

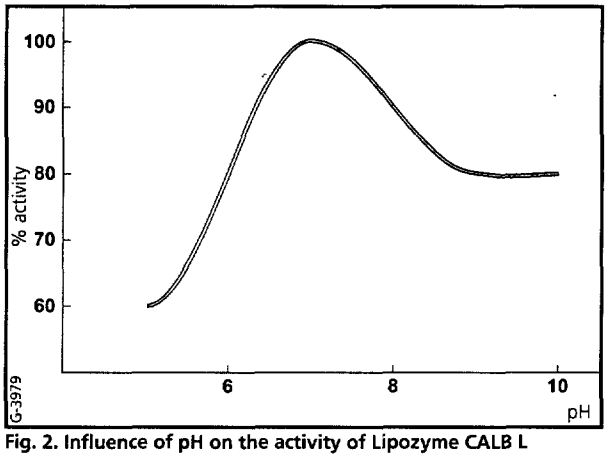

Fig. 2. Influence of pH on the activity of Lipozyme CALB L

Safety

Enzymes are proteins and inhalation of dust or aerosols may induce sensitization and may cause allergic reactions in sensitized individuals. Some enzymes may irritate the skin, eyes and mucous membranes upon prolonged contact.

The product may create easily inhaled aerosols if splashed or vigorously stirred. Spilled product may dry out and create dust. Spilled material should be flushed away with water. Avoid splashing. Left-over material may dry out and create dust.

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Wear suitable protective clothing, gloves and eye/face protection as prescribed on the warning label. Wash contaminated clothes.
Material Safety Data Sheets are supplied with all products. Further information describing how to handle the product safely is available upon request.

Storage

Recommended storage conditions are 0-10°C (32-50°F) in unbroken packaging protected from the sun. The product has been formulated for optimal stability. However, enzymes gradually lose activity over time. Extended storage or adverse conditions such as higher temperature, may lead to a higher dosage requirement.

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Appendix 2

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Appendix 3

Safety & Toxicology

Date: March 2004
Ref.: DSBr/PBjP
File: 2004-12560-01

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SUMMARY OF TOXICITY DATA

Candida B Lipase

Authors:
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1. ABSTRACT

Candida B Lipase, PPW 22633, is a liquid enzyme concentrate. It is produced by submerged fermentation of a strain of *Aspergillus niger*, containing the gene code of a lipase originating from *Candida antarctica*.

In the following, toxicity studies carried out with Candida B Lipase are summarised.

All 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 Scantox and Novozymes, Denmark and Covance Laboratories, England during the period June 2003 to January 2004.

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

Daily oral administration (by gavage) of Candida B Lipase, PPW 22633 to rats at dosages of up to 10.0 mL/kg bw/day (equivalent to 18601 LU-CA/kg bw/day or 1967 mg TOS/kg bw/day) for two weeks resulted in no treatment related effects. In this study the no observed adverse effect level (NOAEL) was considered to be 10 mL/kg/day and dosages up to this are therefore considered suitable for use in a 13 week toxicity study in this species.

Daily oral administration (by gavage) of Candida B Lipase, PPW 22633 to rats at dosages of up to 10.0 mL/kg bw/day (equivalent to 18601 LU-CA/kg bw/day or 1967 mg TOS/kg bw/day) for thirteen weeks resulted in no treatment related effects. Consequently, the no observed adverse effect level (NOAEL) was considered to be 10 mL/kg/day (equivalent to 18601 LU-CA/kg bw/day or 1967 mg TOS/kg bw/day).

Candida B Lipase has shown no mutagenic activity in either a bacterial reverse mutation assay or an investigation for chromosome aberrations in human lymphocytes *in vitro*.

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2. TEST SUBSTANCE

2.1 Production of test substance

Candida B Lipase, PPW 22633, is a liquid enzyme concentrate. The principal enzyme activity is a lipase B classified as triacylglycerol acylhydrolase (EC 3.1.1.3). The enzyme originates from a strain of Candida antarctica and is expressed in a strain of Aspergillus niger. The product is produced by submerged fermentation and recovered by purification/ concentration of the fermented culture broth according to the same procedures as are used for the production of commercial Candida B Lipase preparations, except that formulation/standardisation is omitted.

The Candida B Lipase batch, PPW 22633, used for the present toxicological programme, was a mixture of 3 identically produced sub-batches.

2.2 Characterisation

Batch PPW 22633, a dark brown liquid was used for all the studies.

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Table 1. Characterisation of Candida B Lipase, PPW 22633

	Batch No. PPW 22633
Activity, LU-CA/g	1740
Water (KF)	81.0%
Dry matter	19.0%
Ash (600°C)	0.6%
Total Organic Solids (TOS[1])	18.4%
Specific gravity (g/mL)	1.069

[1] %TOS = 100%- % water- % ash- % diluents

2.3 Production Microorganism

Candida B Lipase is produced by a strain of *Aspergillus niger* containing the gene code for a lipase B originating from *Candida antarctica*.
This genetically modified production strain meets the criteria for a safe production micro-organism. It is constructed by common transformation procedures using well-known plasmid vectors with strictly defined and well-characterized DNA sequences that are not known to encode or express any harmful or toxic substances. The strain is free of any antibiotic resistance marker The development of the production strain was evaluated at every step to assess incorporation of the desired functional genetic information and to ensure no unintended sequences were incorporated.

In general industrial strains of *A. niger* are non-pathogenic and non-toxigenic and the potential risk associated with the use of this bacterium in fermentation facilities is low. *A. niger* has a long history of safe use and it has been used for decades in the production of enzymes and in the last decade as recombinant organism for production of a variety of bio-industrial products. It is accepted as well as the enzymes it produces as a constituent of food (JECFA, 1987).
A few percent of *A.niger* wild type strains may produce ochratoxin A, a mycotoxin of major concern regarding risk to human health. It is a highly toxic and carcinogenic. Therefore it is importance to demonstrate that industrial production strain don't have this toxigenic potential.
Thorough investigations of the metabolic potential revealed that the recipient strain of the Candida B Lipase production strain, when grown under optimized conditions for production of secondary metabolites, was not capable of producing ochrotoxin A or any other known mycotoxins associated with the genus *Aspergillus*. Further Candida B Lipase, PPW 22633 was analysed for the presence of ochratoxin A as well as aflatoxin B_1, sterigmatocystin, T2 toxin and zearalenone (JECFA, 1992). None of these mycotoxins were detected.

The test substance does not contain the production strain and its absence is part of the complete specification of the product.

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3. TOXICITY DATA

3.1 Subacute Toxicity

3.1.1 A 14-Day Oral (Gavage) Dose-Range-Finder Study in Rats

The objective of this preliminary dose range finding (DRF) study was to assess the systemic toxic potential of the test substance in rats, when administered orally, and to aid selection of dosages for a subsequent 13-week toxicity study in this species.

Three groups each of five male and five female rats received Candida B Lipase, PPW 22633, orally by gavage, at dosages of 1.0, 3.3 or 10.0 mL/kg bw/day (equivalent to 1860.1, 6138.3 or 18601 LU-CA/kg bw/day and 196.7, 596.0 or 1967 mg TOS/kg bw/day) for two weeks. A similarly constituted group received the vehicle (tap water) at the same volume dosage and served as the negative control.

Clinical signs were recorded daily. Body weights and food consumption were recorded once weekly. At termination of the study, the animals were killed and subjected to a macroscopic examination.

No treatment related adverse signs were recorded at the clinical examinations, on food consumption and on body weight gain. Necropsy revealed no changes in any of the animals.

It was concluded that daily oral administration (by gavage) of Candida B Lipase, PPW 22633 to rats at dosages of up to 10.0 mL/kg bw/day (equivalent to 18601 LU-CA/kg bw/day or 1967 mg TOS/kg bw/day) for two weeks resulted in no treatment related effects. In this study the no observed adverse effect level (NOAEL) was considered to be 10 mL/kg/day and dosages up to this are therefore considered suitable for use in a 13 week toxicity study in this species.

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3.2. Subchronic Toxicity

3.2.1. A 13-week Oral (Gavage) Toxicity Study in Rats

The study was carried out in accordance with the OECD guideline 408 (adopted on September 1998). It was conducted in accordance with Good Laboratory Practice.

Three groups each of ten male and ten female rats received Candida B Lipase, PPW 22633, orally by gavage, at dosages of 1.0, 3.3 or 10.0 mL/kg bw/day (equivalent to 1860.1, 6138.3 or 18601 LU-CA/kg bw/day and 196.7, 596.0 or 1967 mg TOS/kg bw/day) for thirteen weeks. A similarly constituted group received the vehicle (tap water) at the same volume dosage and served as the negative control.

Clinical signs were recorded daily. Detailed clinical observations were performed once weekly. During week 11 of the study, the animals were examined for sensory reactivity, grip strength and motor activity. Body weights and food consumption were recorded once weekly. Ophthalmoscopy was performed on all animals before start of treatment, and on the animals of groups 1 and 4 during week 13 of the study. Before termination of treatment, blood samples were taken for haematology and clinical chemistry, and urine was collected for urinalysis. The animals were

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killed and subjected to a macroscopic necropsy. Specified organs and tissues were weighed, fixed and prepared for microscopic examination.

Analysis of achieved concentration was performed on samples taken once during weeks 1, 6 and 13. Achieved concentration was evaluated by measurement of enzyme activity, LU-CA/g.
No treatment related adverse signs were recorded at the clinical examinations, on food consumption, on body weight gain and at the opthalmoscopic examination.

No treatment related findings were observed on the parameters for clinical pathology and on organ weight.

Necropsy and the following microscopic examination revealed no treatment related effects.

The results from the content check analysis showed that in the samples from week 1, 6 and 13 there were no significant differences between the intended activity and the results.

It was concluded that daily oral administration (by gavage) of Candida B Lipase, PPW 22633 to rats at dosages of up to 10.0 mL/kg bw/day (equivalent to 18601 LU-CA/kg bw/day or 1967 mg TOS/kg bw/day) for thirteen weeks resulted in no treatment related effects. Consequently, the no observed adverse effect level (NOAEL) was considered to be 10 mL/kg/day (equivalent to 18601 LU-CA/kg bw/day or 1967 mg TOS/kg bw/day).

3.3. Mutagenic Potential

3.3.1. Bacterial Reverse Mutation Assay (Ames Test)

In order to determine the ability to induce gene mutations in bacteria Candida B Lipase, PPW 22633 was examined in the bacterial reverse mutation assay in accordance with the general principles of OECD Guideline for Testing of Chemicals No. 471 (1997).
Four histidine-requiring strains of *Salmonella typhimurium* (TA98, TA100, TA1535 and TA1537) and one tryptophan-requiring strain of *Escherichia coli* (WP2 uvrA) were applied in this study.

Crude enzyme preparations contain free amino acids such as histidine and tryptophan. Candida B Lipase significantly supports growth of the histidine-requiring *S.typhimurium* strains by direct incorporation of the test substance on selective agar media. In order to avoid the risk of artefacts due to growth stimulation, a "treat and plate" assay was applied.
S.typhimurium cultures were exposed to Candida B Lipase, solvent and positive controls in phosphate buffered nutrient broth for three hours at 37°C. After this period, all nutrients originating from the test substance and broth were removed by centrifugation of the bacterial suspensions.

The growth promotion of the tryptophan-requiring *E.coli* strain was only weak and insignificant. Therefore, this part of the study was conducted by direct plate incorporation.

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Two independent and identical experiments were performed. In both experiments, all bacterial strains were exposed to serial dilutions of Candida B

Lipase with 5.0, 2.5, 1.25, 0.625, 0.313, and 0.156 mg test substance per ml (*S.typhimurium*) or per plate (*E.coli*) as the final concentrations achieved. The study was carried out with and without the metabolic activation system S9. The sensitivity of the individual bacterial strains was confirmed by the induction of significant responses to positive control substances in the appropriate strains in similar conditions as the test article.

Candida B Lipase composes a rich growth medium to the test bacteria and significant growth stimulation was observed in several test series with the Salmonella strains in the "treat and plate" assay. This was demonstrated by dose-related increases in the viable counts of the treated cultures. This condition results in an increased number of spontaneous pre-existing revertants and therefore concurrent weak increases in the revertant colony counts per plate as observed in many of these test series.

We consider a test substance as positive, when it has induced at least a doubling in the mean number of revertants per plate compared to the appropriate solvent control if this response is dose related (at least 3 doses) and reproducible.
No treatments of any of the bacterial strains with Candida B Lipase resulted in any increases in revertant numbers that meets these criteria for a positive response.

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3.3.2 Chromosome Aberration Assay

The effects on chromosomal structure of exposure to Candida B Lipase were investigated in cultured human lymphocytes in accordance with the current guidelines of OECD (Guideline 473, July 1997).

Heparinized whole blood cultures, pooled from three female donors, were established, and division of the lymphocytes was stimulated by adding phytohaemagglutinin (PHA) to the cultures.
Two independent experiments were performed both in the absence and presence of metabolic activation by a rat liver post-mitochondrial fraction (S-9) from animals induced with Aroclor. Sets of duplicate cultures were treated with the solvent (sterile purified water), test chemical or positive controls (–S-9: 4-Nitroquinoline 1-oxide, +S-9: Cyclophosphamide).
Treatments with Candida B Lipase covered a broad range of doses, separated by narrow intervals, where the highest dose level used was 5000 µg/mL.
The lymphocyte cultures were exposed to the test substance for 3 hours and cells were harvested 17 hours later. The second experiment included a continuous exposure for 20 hours in the absence of S-9. The test article dose levels for chromosome analysis were selected by evaluating the effect of Candida B Lipase on mitotic index.
Chromosome aberrations were analysed at three consecutive dose levels. Cells were arrested in metaphase by colchicine and after centrifugation and hypotonic treatment metaphase spreads were prepared and stained with Giemsa. A total of 200 cells were scored per dose level (100 from each replicate culture) from Candida B Lipase treatments and negative controls. Slides were scored blind and aberrations recorded according to international classifications.

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The proportion of cells with structural aberrations in all cultures of the solvent controls (purified water) was within the limits of the historical ranges. The positive controls induced statistically significant increases in the proportion of cells with structural aberrations, thus demonstrating the sensitivity of the test procedure and the metabolic activity of the S-9 mix employed. In the first experiment the highest concentration of Candida B Lipase chosen for analysis, 5000 µg/mL, induced approximately 7% and 0% mitotic inhibition (reduction in mitotic index) in the absence and presence of S-9, respectively. In the second experiment the highest concentration chosen for analysis, 5000 µg/mL induced approximately 33% and 18% mitotic inhibition in the absence and presence of S-9, respectively.

Cells treated with Candida B Lipase, either in the absence and presence of S-9, had similar numbers of aberrations to those observed in concurrent vehicle controls. There were no reproducible increases in aberration frequency that were significantly higher than those observed in the negative controls or that fell outside the historical negative control ranges.
Normal frequencies of cells with numerical aberrations were seen under all treatment conditions.

Candida B Lipase (Batch No. PPW 22633), under the conditions of test, did not induce chromosome aberrations in cultured human blood lymphocytes when tested to a concentration of 5000 µg/mL in either the absence or presence of S-9.

4. CONCLUSION

The results of the toxicological studies outlined above did not reveal any adverse effect of Candida B Lipase, PPW 22633. Based on these toxicological data and the fact that the production strain reportedly has a history of safe use, it is our conclusion that there are no reasons for safety concerns when using this Candida B Lipase as a food additive. In our opinion Candida B Lipase can be considered generally recognized as safe.

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5. REFERENCES

5.1 Study Reports

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Candida B Lipase, PPW 22633: A 14-Day Oral (Gavage) Dose-Range-Finder Study in Rats. November 2003.

Scantox: Lab No No.: 52728. Novozymes Reference No.: 20036027.
Candida B Lipase, PPW 22633: A 13-week Oral (Gavage) Toxicity Study in Rats. January 2004.

Novozymes A/S Report No.: 2003-34207-02. NN Study No.: 20038046. Candida B Lipase (Batch No. PPW 22633): Test for mutagenic activity with strains of *Salmonella typhimurium* and *Escherichia coli.*

Covance Laboratories Limited Report No.1974/18. Novozymes Study No.20036024 Candida B Lipase: Induction of Chromosome Aberrations in Cultured Human Peripheral Blood Lymphocyte.

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Submission End

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Pages	*Author*	*Title*	*Publish Date*	*Publisher*	*BIB_Info*
000029 - 000038	Schuster, E.; Dunn-Coleman, N.; Frisvad, J.C.; van Dijck, P.W.M.	On the safety of Aspergillus niger - a review	2002	Appl Microbiol Biotechnol	Volume 59, pgs 426-435

NA- Not applicable

M

ker, Karin

From: LOBG (Lori Gregg) [LOBG@novozymes.com]
Sent: Thursday, March 03, 2005 8:49 AM
To: Kricker@cfsan.fda.gov
Subject: GRN 158

Dear Karin,

In our GRAS notification GRN 158 regarding a lipase preparation produced by *Aspergillus niger* expressing a gene encoding a lipase from *Candida antarctica* we stated on page 13 that the donor organism, *Candida antarctica* meets the criteria for a classification in Risk Group 1 according to NIH guidelines. The NIH guidelines we refer to are the:

NIH Guidelines For Research Involving Recombinant DNA Molecules (NIH Guidelines), Appendix B, Classification of Human Etiologic Agents On the Basis of Hazard, April 2002. You can find this on their website:

http://www4.od.nih.gov/oba/rac/guidelines/guidelines.html

There you can find a link to the full text of the document in pdf. If you open the document you can scroll down to the Table of Contents to Appendix B and click in the table of contents on Appendix B and it will take you directly to Appendix B.

Please let me know if you have any further questions or need additional information.

Best Regards,

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