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April 11, 2001

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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' lipase enzyme preparation produced by *Aspergillus oryzae* expressing the gene encoding a lipase from *Fusarium oxysporum*. The lipase enzyme preparation is intended for use in the fats and oils industry, the baking industry, the hydrolysis of lecithin, and the modification of egg yolks.

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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April 11, 2001

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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 oryzae* expressing the gene encoding a lipase from *Fusarium oxysporum* 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 oryzae* expressing the gene encoding a lipase from *Fusarium oxysporum.*

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

The lipase is intended for use in the fats and oils industry, the baking industry, the hydrolysis of lecithin, and the modification of egg yolks. 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 *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.

11 April, 2001
Date

John Carroll
Director, Regulatory Affairs

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A lipase preparation produced by *Aspergillus oryzae* expressing the gene encoding a lipase from *Fusarium oxysporum*

Lori Gregg, Regulatory Affairs, Novozymes North America, Inc., USA
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TABLE OF CONTENTS

		PAGE
1.	**GENERAL INTRODUCTION**	3
2.	**PRODUCTION MICROORGANISM**	4
2.1	Production Strain	4
2.2	Recipient Organism	4
2.3	Lipase Expression Fragment	5
2.4	Stability of the Introduced Genetic Sequences	6
2.5	Antibiotic Resistance Gene	6
2.6	Absence of the Production Organism in Product	6
3.	**MANUFACTURING PROCESS**	6
3.1	Raw Materials	6
3.2	Fermentation Process	7
3.3	Recovery Process	8
3.4	Quality Control of Finished Product	9
4.	**ENZYME IDENTITY**	9
5.	**COMPOSITION AND SPECIFICATIONS**	9
5.1	Quantitative Composition	9
5.2	Specifications	10
6.	**APPLICATION**	11
6.1	Mode of Action	11
6.2	Use Levels	12
6.3	Enzyme Residues in the Final Food	12
7.	**SAFETY EVALUATION**	13
7.1	Safety of the Production Strain	13
7.2	Safety of the Lipase Enzyme	14
7.3	Safety of the Manufacturing Process	17
7.4	Safety Studies	17
7.5	Estimates of Human Consumption and Safety Margin	21
7.6	Results and Conclusion	26
8.	**LIST OF APPENDICES**	27
9.	**LIST OF REFERENCES**	28

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1. GENERAL INTRODUCTION

The subject of this notification is a lipase preparation produced by submerged fermentation of an *Aspergillus oryzae* microorganism carrying the gene coding for lipase from *Fusarium oxysporum.*

There are two main formulations of this lipase preparation. One formula, Lecitase® Novo, is to be used as a processing aid primarily in the fats and oils industry. The other formula, Lipopan™ F, is to be used as a processing aid mainly in the baking industry. Other potential applications are: hydrolysis of lecithin, and modification of egg yolks. For simplicity, the name Lipase will be used in this document to describe the lipase preparation produced by submerged fermentation of an *A. oryzae* microorganism carrying the gene coding for lipase from *F. oxysporum.*

The enzyme is a lipase (EC 3.1.1.3), which specifically acts on the fatty acid in position 1 in both triglyceride substrates and, like other known lipases[1], in phospholipid substrates. Therefore, the enzyme hydrolyzes ester bonds in triglycerides, resulting in formation of diglycerides, monoglycerides and free fatty acids, and catalyzes the hydrolysis of the sn-1 ester bond of diacylphospholipids to form 2-acyl-1-lysophospholipid and a free fatty acid.

In the baking applications, both the activity towards triglyceride and phospholipid substrates is utilized to obtain improved dough properties and improved bread-making quality in terms of larger volume and improved crumb structure. In the other applications (oil degumming, lecithin, and egg yolk modification), the activity towards the phospholipids (phosphatides, lecithins) will predominate due to higher substrate availability.

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. oryzae* expressing the gene encoding a lipase from *F. oxysporum.* 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[2]. The production organism for this Lipase, *A. oryzae*, 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

000006

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.

2. PRODUCTION MICROORGANISM

2.1 Production Strain

The *A. oryzae* strain, designated MStr110, was constructed by transformation of the recipient strain, designated Jal228 (see Section 2.2), with a purified DNA fragment. Subsequently, a spontaneous mutant of MStr110 with improved production performance was identified, named MStr115, and is 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 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 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 gene encoding a *F. oxysporum* lipase enzyme; an *Aspergillus nidulans* selectable marker gene, *amd*S (acetamidase)[10]; well-characterized noncoding regulatory sequences including the *Aspergillus niger* terminator[11], the *A. niger* NA2 promoter[12], and *A. nidulans* triose phospate isomerase gene[13]; and a fragment from a known sequence from *Escherichia coli* plasmid pUC19[14].

2.2 Recipient Organism

The recipient microorganism, designated Jal228, used in the construction of the lipase production strain is an amylase negative, alkaline protease (alp) negative, neutral metalloprotease I (NpI) negative 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). This classification of A1560 as *A. oryzae* has been confirmed by the Centraalbureau voor Schimmelcultures, Baarn, Holland[15].

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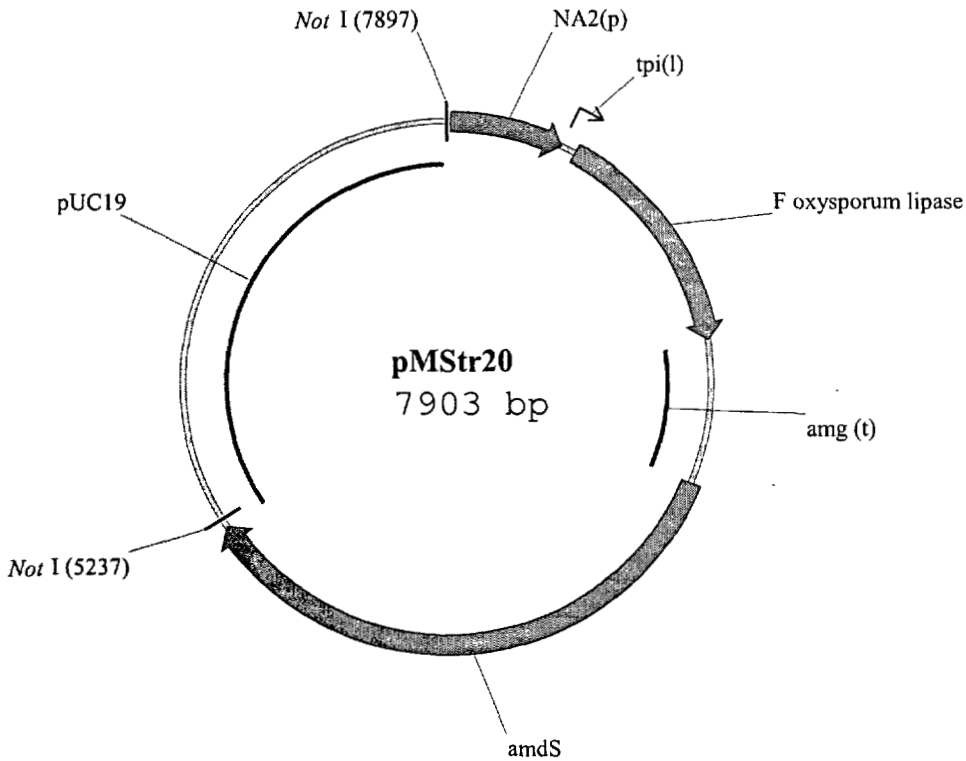
2.3 Lipase Expression Fragment

The plasmid pMStr20 was digested with NotI as indicated in the map below, and the 5247 kb fragment containing the lipase gene was isolated.



This purified fragment was used to transform the *A. oryzae* host strain, Jal 228, and contains the following genetic material:

Position (bp)	Size (bp)	Element	Origin
1-549	549	NA2 (p)	*A. niger* BO1
550-613	64	tpi (l)	*A. nidulans*
614-618	5	Linker	synthetic
619-1762	1144	Lipase	*F. oxysporum*
1763-1776	4	Linker	synthetic
1777-2464	688	AMG (t)	*A. niger* BO1
2465-2466	2	Linker	synthetic
2467-5192	2726	*amdS*	*A. nidulans*
5193-5247	55	pUC19	*E. coli*

NA2 (p) is the promoter from *Aspergillus niger* neutral amylase II gene.
tpi (l) is the 5' nontranslated leader of the *Aspergillus nidulans* triose phosphate isomerase gene.
Lipase is a cDNA of the *Fusarium oxysporum* lipase gene.
AMG (t) is the terminator of *Aspergillus niger* amyloglycosidase gene.
amdS is the complete acetamidase encoding gene from *Aspergillus nidulans*.
pUC19 is a plasmid vector from *E. coli*.

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2.4 Stability of the Introduced Genetic Sequences

The presence and configuration 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. oryzae* chromosome and, as such, is poorly mobilizable for genetic transfer to other organisms and is mitotically stable[9].

2.5 Antibiotic Resistance Gene

The transforming DNA does not contain antibiotic resistance genes.

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. MANUFACTURING PROCESS

This section describes the manufacturing process for the Lipase which follows standard industry practices[16-18]. The quality management system used in the manufacturing process for the Lipase complies with the requirements of ISO 9001. It is also manufactured in accordance with current good manufacturing practices.

3.1 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[16-18]. 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 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 Lipase product is less than 1%.

3.2 Fermentation Process

The Lipase is manufactured by submerged fed-batch pure culture fermentation of the genetically modified strain of *A. 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 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.

3.2.1 Production Organism

Each batch of the fermentation process is initiated with a lyophilized stock culture of the production organism, *A. oryzae,* 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.

3.2.2 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.

000010

3.3 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.

3.3.1 Purification Process

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

1. Pretreatment - pH adjustment
2. Primary Separation - vacuum drum filtration
3. Concentration - ultrafiltration and 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 chloride/sugar addition
6. Final concentration – evaporation if enzyme concentration is too low to reach target yield

3.3.2 Formulation and Standardization Processes

Lipase formula for fats and oils applications (Lecitase Novo)

The stabilized concentrate is blended with water, sodium chloride and sucrose. The product is standardized according to the product specification.

Lipase formula for baking applications (Lipopan F)

The liquid concentrate is mixed with granulation aids (stabilizers/binders) such as dextrin and spray dried by means of atomization into a fluidized spray dryer. The powder from the primary drying zone is directed into an integrated fluid bed for agglomeration and further drying. The product is discharged continuously after sieving. The product is standardized to the declared enzyme activity by addition of wheat flour.

3.4 Quality Control of Finished Product

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

4. ENZYME IDENTITY

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

Classification	Lipase (generic name)
IUB nomenclature:	Triacylglycerol lipase
IUB No.:	3.1.1.3
CAS No.:	9001-62-1
EINECS No.:	232-619-9
Specificity	1,3-position ester bonds in triglycerides; specificity towards fatty acid in position 1 in both triglycerides and phospholipids.
Molecular weight	29 kDa
Amino acid sequence	the total nucleotide and amino acid sequences have been determined

5. COMPOSITION AND SPECIFICATIONS

The lipase enzyme preparation is presently available in formulas for use in fats and oils applications and baking applications.

5.1 Quantitative Composition

Lipase for fats and oils applications (Lecitase Novo) has the following typical composition:

Water	approx. 53%
Sucrose	approx. 31.5%
Sodium chloride	approx. 14%
Enzyme solids (TOS)	approx. 1.5%

Lipase for baking applications (Lipopan F) has the following typical composition:

Wheat flour solids	approx. 76%
Sodium chloride	approx. 9%
Water	approx. 8%
Enzyme solids (TOS)	approx. 4%
Dextrin	approx. 3%

000012

5.2 Specifications

The Lipase conforms to the general and additional requirements for enzyme preparations as described in Food Chemicals Codex, 4th edition, 1996[19]. 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[20].

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

Enzyme activity, LENU/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 5×10^4
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, lead, arsenic, antibiotic activity, and mycotoxins are analyzed at regular intervals.

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 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 Lecitase Novo Units (LENU/g) using the Novozymes Phospholipase Activity Method. The enzyme hydrolyzes the thioester bond in the A1 position of the substrate which releases a free thiol group which can be detected spectrophotometrically using the reagent DTNB. A Lecitase Novo Unit (LENU) is defined as the unit that under standard conditions releases 2

000013

micromole 5-mercapto-2-nitrobenzoesyre/minute by digestion of the substrate 1-(S-decanoyl)-2-decanoyl-1-thio-sn-glycero-3-phosphocholin and subsequent reaction with DTNB.

6. APPLICATION

6.1 Mode of Action

The enzyme is a lipase (EC 3.1.1.3). This product hydrolyzes ester bonds of triacylglycerol to release free fatty acids. It also catalyzes the hydrolysis of the sn-1 ester bond of diacylphospholipids and forms 2-acyl-1lysophospholipid and a free fatty acid.

Lipopan F will be used for the baking applications, whereas Lecitase Novo will be used in the fats and oils industry for de-gumming of vegetable oil. Lecitase Novo may also be used for the hydrolysis of lecithin for altered emulsifying properties and modification of egg yolk. In the baking applications, both the activity towards triglyceride and phospholipid substrates is utilized, while in the fats and oils applications, the activity towards the phospholipids (phosphatides, lecithins) will predominate due to higher substrate availability.

In the "degumming" of oil, phospholipids (phosphatides, lecithins) are removed from oil during the oil purification process in order to improve the taste and quality of the oil. Removal of phospholipids can also improve the storage stability of the oil and facilitate downstream processing. The Lipase is applied in an aqueous phase in oil. This aqueous phase is typically relatively low e.g. around 5 % in oil. Phospholipids hydrolyzed with the Lipase migrate into the aqueous phase, and can be removed as a water based sludge.

The Lipase can also be used to hydrolyze lecithin. Lysolecithin, 2-acyl-1-lysophophospholipid, as a product of enzymatic hydrolysis of lecithin, has excellent emulsifying properties. The lipase can also hydrolyze the lecithin in egg yolks improving the emulsifying properties of the egg yolks. More information on the use of Lecitase Novo in fats and oils applications can be found in the product sheet in Appendix 1.

In the baking application, the Lipase shows activity towards both triglyceride and phospholipid substrates. The Lipase is used to obtain improved dough properties and improved bread making quality in terms of larger volume and improved crumb structure.

6.2 Use Levels

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

In the fats and oils applications, the optimum use level is dependent on the specific substrate, reactor configuration and desired conversion. However, the recommended use level of the Lecitase Novo is:

a) up to 400 LENU per kg of triglycerides for de-gumming, corresponding to 1 kg of Lecitase Novo to produce 10 ton of de-gummed oil
b) up to 10000 LENU per kg of crude lecithin for lecithin hydrolysis, corresponding to 1 kg of Lecitase Novo to produce 400 kg of modified lecithin
c) up to 8000 LENU per kg of egg yolk for egg yolk modification, corresponding to 1 kg of Lecitase Novo to produce 500 kg of modified egg yolk

In the baking application, the recommended use level for the Lipase is up to 500 LENU per kg flour, corresponding to 5g of Lipase per 100 kg flour.

6.3 Enzyme Residues in the Final Food

In the oil degumming application, the enzyme is applied in a 5% water phase in oil. The Lipase is hydrophobic. It is not likely to migrate into the oil phase. After the reaction has taken place, the water phase containing the enzyme and the phospholipids will be removed. Furthermore, after coming into contact with the enzyme preparation, the oil will be subjected to oil refining methods such as filtration, distillation, deacidification, bleaching and deodorization which would remove any potential residues.

In the lecithin hydrolysis and egg yolk modification applications, the enzyme is inactivated by a heat treatment.

In the baking application, Lipase is active during the mixing, fermentation and proofing stages of bread production but is inactivated during baking.

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[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. oryzae* meets these criteria for nontoxigenicity and nonpathogenicity. In addition, *A. oryzae* is not considered pathogenic by JECFA[21].

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[22] (Appendix 2). *A. oryzae* is accepted as a constituent of foods[21]. *A. oryzae* has been used to produce soy sauce in the United States since before 1958[3,22]. Therefore, *A. oryzae* meets the criterion of "common use in foods in the US before 1958" and can be considered "generally recognized as safe", GRAS[3]. A GRAS petition, 3G0016, proposing affirmation that enzyme preparations from *A. oryzae* are GRAS for use in food was submitted to FDA and accepted for filing in 1973[23]. Enzyme preparations from *A. oryzae* have been marketed in the US as GRAS by Novozymes and other companies since that time. Therefore, enzyme preparations from *A. oryzae* are also considered GRAS[2,3,21].

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, and JECFA in 1998 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 incorporated DNA fragment, 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.

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.oryzae* is considered safe[3,24]. To confirm the safety of the enzyme, safety studies were performed on the enzyme preparation and are described in Section 7.4.

7.2 Safety of the Lipase Enzyme

Enzyme proteins themselves do not generally raise safety concerns[2,25,26]. As indicated in section 4, 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[25]. Like other known lipases[1], this lipase also catalyzes the hydrolysis of the sn-1 ester bond of diacylphospholipids and forms 2-acyl-1-lysophospholipid and free fatty acid.

7.2.1 Lipases

Microbial lipases have been reported to be used in food production since 1952[27,28]. 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 *Aspergillus oryzae* expressing the gene encoding a lipase from *Thermomyces lanuginosus* is the subject of GRAS notification GRN #43.

7.2.2 Substantial Equivalence

Several expert groups, as well as FDA and FDA scientists have discussed the concept of substantial equivalence relative to food safety assessment[5-8,24,29-31]. Essentially all these groups conclude that if a food ingredient is substantially equivalent to an existing food ingredient known to be safe, then no further safety considerations other than those for the existing ingredient are necessary.

In addition, FDA has applied this concept in the determination that several enzyme preparations are safe for use in food[29,32,33]. In particular, differences in glycosylation between enzyme proteins was considered. FDA has also stated that enzyme proteins demonstrated to be substantially equivalent to enzymes known to be safely consumed but having differences in specific properties due to changes in the enzyme amino acid sequence by natural selection, chemical modification, or site-directed mutagenesis would not raise safety concerns[24,30].

The lipase enzyme from the genetically modified *A. oryzae* is a lipase from *F. oxysporum* . The *F. oxysporum* lipase coding sequence incorporated into the *A. oryzae* production strain has not been altered. The genus *Fusarium* belongs to the class of imperfect fungi Hyphomycetes (Moniliales). *Fusarium* species are very common saprophytes on plant debris and in soil and world wide in distribution. Hyphomycetes, like species of the genus *Fusarium*, are generally not regarded as primary human pathogens.

The general structure of known lipases is characterized by four major structural, functional or sequence homologies: 1) the consensus sequence Glycine-X-Serine-X-Glycine around the active-site serine; 2) the strand-helix motif around the active serine residue; 3) a buried active site covered by a lid or lids; 4) the active site catalytic residues found in a triad in the order Serine...Asparagine/Glutamine...Histidine, lying 50 residues from one another in the sequence[34]. *F. oxysporum* lipase (FoL) is structurally homologous to the *Rhizomucor miehei* lipase (Rml) and the *Thermomyces lanuginosus* lipase (Tll). The amino acid sequences of the FoL, Rml and Tll lipases have been compared[35]. FoL is 59% homologous to Tll, 49% to Rml and 53% to the *Rhizopus delemar* lipase (Rdl) (see Table 1 below). Generally a sequence homology below 50% would indicate possible differences in the structural organisation, and above 50% would indicate a possible relationship in structure. In the present lipase family with Tll, Rml, Rdl and FoL it is clearly seen that the same structural patterns are present (see sequence alignment below). The sequence alignment shows that the pentapeptide consensus sequence and the active site catalytic residues are the same in all four lipases. There are also conserved residues in the lid and hinge regions.

Table 1. The homology of the lipases

		1	2	3	4
1	*Fusarium oxysporum*	100	49	53	59
2	*Rhizomucor miehei*	.....	100	65	56
3	*Rhizopus delemar*	.....	.....	100	53
4	*Thermomyces lanuginosus*	.....	.....	.....	100

000018

Sequence alignment

```
      1                                                  50
RdL   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~SD GGKVVAATTA QIQEFTKYAG
RML   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~SI DGGIRAATSQ EINELTYYTT
FOL   ~~~                            L EERAVGVTTT DFSNFKFYIQ
TlL   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~EVSQD LFNQFNLFAQ

      51                                                 100
RdL   IAATAYCRSV VPGNKWDCVQ C......QKW VPDGKIITTF T.SLLSDTNG
RML   LSANSYCRTV IPGATWDCIH C......DA. TEDLKIIKTW S.TLIYDTNA
FOL   HGAAAYC..N SEAAAGSKIT CSNNGCPTVQ GNGATIVTSF VGSK.TGIGG
TlL   YSAAAYCGKN NDAPAGTNIT CTGNACPEVE KADATFLYSF EDSGVGDVTG

      101                                                150
RdL   YVLRSDKQKT IYLVFRGTNS FRSAITDIVF NFSDYKPV.K GAKVHAGFLS
RML   MVARGDSEKT IYIVFRGSSS IRNWIADLTF VPVSYPPV.S GTKVHKGFLD
FOL   YVATDSARKE IVVSFRGSIN IRNWLTNLDF GQEDC.SLVS GCGVHSGFQR
TlL   FLALDNTNKL IVLSFRGSRS IENWIGNLNF DLKEINDICS GCRGHDGFTS
                        <----Lid------->

      151                                                200
RdL   SYEQVVNDYF PVVQEQLTAH PTYKVIVTGH SLGGAQALLA GMDLYQREPR
RML   SYGEVQNELV ATVLDQFKQY PSYKVAVTGH SLGGATALLC ALGLYQREEG
FOL   AWNEISSQAT AAVASARKAN PSFNVISTGH SLGGAVAVLA AANL....RV
TlL   SWRSVADTLR QKVEDAVREH PDYRVVFTGH SLGGALATVA GADL....RG
                                     <-Cons->
                                        *

      201                                                250
RdL   LSPKNLSIFT VGGPRVGNPT FAYYVES.TG IPFQRTVHKR DIVPHVPPQS
RML   LSSSNLFLYT QGQPRVGDPA FANYVVS.TG IPYRRTVNER DIVPHLPPAA
FOL   GGTP.VDIYT YGSPRVGNAQ LSAFVSNQAG GE.YRVTHAD DPVPRLPPLI
TlL   NGY.DIDVFS YGAPRVGNRA FAEFLTVQTG GTLYRITHTN DIVPRLPPRE
                                                  *

      251                                                300
RdL   FGFLHPGVES WI.KSGTSNV QICTSEIETK DCSNSIVPFT SILD......
RML   FGFLHAGEEY WITDNSPETV QVCTSDLETS DCSNSIVPFT SVLD......
FOL   FGYRHTTPEF WLSGGGGDKV DYTISDVKVC EGAANLGCNG GTLGLDIAA.
TlL   FGYSHSSPEY WIKSG..TLV PVTRNDIVKI EGIDATGGNN QPNIPDIPA.

      301
RdL   HLSYFDINEGSCL~~~ ~
RML   HLSYFGINTGLCS~~~ ~
FOL   HLHYFQ ATDACNAGGFS
TlL   HLWYFG LIGTCL~~~~
      *
```

* = Active site residues. Highlighted residues are identical between FOL and TlL.

The structural relation is also proved by 3D structural elucidation. The 3D structures of Rml, Tll, and Rdl are highly homologous[36] and thus are considered to be in the same lipase family, even though their sequence homology is only between 50-60 %. The 3D structure of the homologous *F. oxysporum* has been elucidated (unpublished results) in the same laboratory in which the Tll and the Rml were solved, and it has the same overall structure.

The *F. oxysporum* lipase expressed in *A. oryzae* is substantially equivalent to the lipase from *T. lanuginosus* and *R. miehei* and is safe for use in food.

7.3 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 3, 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[16-18].

7.4 Safety studies

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

7.4.1 Description of Test Material

The safety studies described below were conducted on a lipase batch that was prepared according to the description given in Section 3 except that stabilization and standardization were omitted.

7.4.2 Studies

The following studies were performed:

13 weeks Subchronic Oral Toxicity in rats
Scantox Lab. No. 35125, Novo Nordisk Study No. 20006004 : Lecitase Novo, batch PPW 6703. A 13-week oral (gavage toxicity study in rats.

The study was performed in accordance with the OECD Guideline for Testing of Chemicals no. 408 (adopted 1998).

Three groups of 10 male and 10 female Sprague Dawley Mol:SPRD rats were dosed Lecitase Novo, batch PPW 6703, by gavage at dosages of 10% w/v, 30% w/v and 100% v/v, using a constant dose volume of 10 ml/kg bw/day. This corresponds to 22,700 – 68,100 and 227,000 LEU (T)/kg/day or 0.083 – 0.249 and 0.830 g Total Organic Solids (TOS)/kg/day. A similar constituted group received the vehicle (tap water) and served to generate contemporaneous control data.

The rats were dosed for at least 91 days and until the day before necropsy.

There were no treatment related clinical observations. No dose-related deaths occurred. Bodyweight gain, food consumption and the efficiency of food utilization were considered to have been unaffected by treatment. There was no treatment-related ocular finding.

Males and females treated up to dosages of 10.0 ml/kg/day showed no evidence of neurotoxicity during the Functional Observation Battery procedures.

After 13 weeks of treatment, there was no treatment related alteration in the hematological or biochemical composition of the blood. Organ weights were unaffected by treatment and there was no treatment-related macroscopic or microscopic abnormality.

It is concluded that the oral administration to Sprague Dawley Mol:SPRD rats of Lecitase Novo batch PPW 6703 at dosages up to 10.0 ml/kg body weight/day (equivalent to 0.83 g TOS/kg/day for 13 weeks was not associated with any toxicologically significant change and that this dosage represents the No-Observed-Effect Level (NOEL) in this study.

Test for mutagenic activity (Ames test)
Novo Nordisk A/S Report No. 2000-06894-01. Novo Nordisk Study No: 20008020. Lecitase (Batch No. PPW 6703): Test for mutagenic activity with strains of Salmonella typhimurium and Escherichia coli.

Lecitase Novo, batch PPW 6703, was examined in order to determine the ability to induce gene mutations in strains of *Salmonella typhimurium and Escherichia coli.*

The study was conducted in accordance with OECD Guideline for Testing of Chemicals No. 471 (1997), the European Commission, Annex V, test method B 14, and the Japanese guideline: "Concrete Operation Procedure of Mutagenicity Study using Bacteria, Ministry of labour, 1988", concerning the general specifications of the test. However, the exposure of the test bacteria in liquid culture ("treat and plate"), as applied in this study with strains of *Salmonella*, is not specifically described in any guidelines.

Four histindine-requiring strains of *Salmonells typhimurium* (TA98, TA100, TA1535 and TA1537) and one tryptophan-requiring strain of *Escherichia coli* (WP2 uvrA) were applied in this study.

000021

Crude enzyme preparations contain free amino acids such as histidine and tryptophan. Lecitase Novo, batch No. PPW 6703, significantly supported 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 artifacts due to growth stimulation, a "treat and plate" assay was therefore applied. Bacterial cultures were exposed to Lecitase Novo, solvent and appropriate positive controls in phosphate buffered test substance and broth were removed by centrifugation of the bacterial suspensions.

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

The study was carried out with and without a metabolic activation system – a liver preparation from male rats pre-treated with Aroclor 1254, and the co-factors required for mixed function oxidase activity (S9 mix). The correct genotypes of all bacterial test strains used were checked.

Two independent and identical experiments were performed. All bacterial strains were exposed to serial dilutions of Lecitase Novo, solvent (sterile deionized water), and positive controls. The final concentrations of the test article achieved were 5.0, 2.5, 1.25, 0.625, 0.313, and 0.156 mg per ml (*S. typhimurium)* or per plate (*E. coli*).

The number of revertants per plate was determined by triplicate plating at each dose on selective agar. The number of viable bacteria in each culture was determined by plate count.

No dose-related and reproducible increases in revertants to prototrophy were obtained with any of the bacterial strains exposed to Lecitase Novo, batch PPW 6703, either in the presence or absence of S-9 mix.

The sensitivity of the individual bacterial strains and the metabolizing potential of the S-9 mix were confirmed in both studies by significant increases in number of revertant colonies induced by diagnostic mutagens under similar conditions.

It was concluded that Lecitase Novo, batch PPW 6703, did not induce gene mutations in bacteria in either the absence or presence of S-9 mix, when tested under the conditions employed in these studies.

***In vitro* cytogenetic test – cultured human lymphocytes**

Covance Laboratories Limited, Study No. 665/326. Novo Nordisk Study No. 996050. Lecitase Novo: Induction of Chromosome Aberrations in Cultured Human Peripheral Blood Lymphocytes.

The effects on chromosomal structure of exposure to Lecitase Novo, batch PPW 6703, were investigated in cultured human lymphocytes in accordance with the current guidelines of OECD (Guideline 473, July 1997) and the ICH Tripartite Harmonized Guideline on Genotoxicity: Specific Aspects of Regulatory Tests (1995).

Heparanized whole blood cultures from three male donors were established, and division of the lymphocytes was stimulated by adding phytohemaglutinin (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 Lecitase Novo 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 three 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 Lecitase Novo on mitotic index. Chromosome aberrations were analyzed at three consecutive dose levels. Cells were arrested in metaphase by colchicines 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 Lecitase Novo treatments and negative controls. Slides were scored blind and aberrations classified according to the scheme described by ISCN (ISCN 1985).

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 Lecitase Novo chosen for analysis, 5000μg/ml, produced approximately 12% and 42% 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 and 1638 μg/ml produced approximately 0% and 53% mitotic inhibition in the absence and presence of S-9, respectively.

Cells treated with Lecitase Novo, either in the absence and presence of S-9, had similar numbers of aberrations to those observed in concurrent solvent controls. There were no 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.

It was concluded that Lecitase Novo, batch PPW 6703, under the conditions of the test, did not induce chromosome aberrations in cultured human blood lymphocytes in either the absence or presence of S-9.

7.5 Estimates of Human Consumption and Safety Margin

As stated in section 6.3 the enzyme activity is largely heat inactivated and/or removed during the baking process, the de-gumming process of oil, the lecithin hydrolysis process or the process used to obtain egg yolk modification.

However, in order to illustrate a "worst case" situation the following calculations are made assuming that all enzyme activity is retained in the baking product, the oil, the hydrolyzed lecithin and the modified egg yolk, respectively.

Lipopan F has an activity of 10000 LENU/g and an approximate content of 4% TOS (Total Organic Substances from the fermentation, mainly protein and carbohydrate components). Lecitase Novo has an activity of 4000 LENU/g and an approximate content of 1.5% TOS.

7.5.1 Estimates of human consumption

<u>Baking</u>

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

000024

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 bread, cakes and bisquits per person per day is 158 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 bread, flutes, pita-bread, cakes, and rye bread per person per day is 123 g.

USA: The Economic Research Service, USDA report on Food Consumption, Prices and Expenditures, 1970-1997 reports that per capita consumption in 1997 of flour and cereal products from white and whole wheat flour was 137.2 lbs.. This corresponds to 170 g per day however this includes cereal and snack products where the Lipase would not be used.

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

a) The calculation is made assuming that all TOS remains in the baking product. Lipopan F contains 4 % TOS.

b) It is assumed that all baking products are produced using Lipopan F as a processing aid, used at the highest recommended dosage.

The maximum recommended dosage of Lipopan F is 500 LENU per kg flour, corresponding to 5 g of Lipopan F per 100 kg flour as described in Section 6.2. Lipopan F contains 4 % TOS. Using a standard recipe, 100 kg flour results in 140 kg bread, giving a theoretical content of 1.4 mg TOS/kg bread.

Based on the highest average daily intake of baking products (158 g), the daily intake per person of Lipopan F corresponds to 1.4 x 0.158 = 0.23 mg TOS per day.

For an average person weighing 60 kg this corresponds to 3.7×10^{-6} g TOS/ kg body weight per day.

000025

De-gumming

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 the worst case, an exaggerated human intake is estimated using the following assumptions.

c) The calculation is made assuming that all TOS remains in the oil, although it would not be the case in reality. Lecitase Novo contains 1.5 % TOS.

d) It is assumed that all oils and fats are produced using Lecitase Novo as a processing aid, used at the highest recommended dosage.

The maximum recommended dosage of Lecitase Novo is 400 LENU/kg triglycerides, corresponding to 1 kg for 10 tons de-gummed oil as described in Section 6.2. Lecitase Novo contains 1.5 % TOS. Therefore, the triglycerides would contain 1.5 mg TOS/kg triglyceride.

Based on the highest average daily intake of oils and fats (144 g), the daily intake per person of Lecitase Novo corresponds to 1.5 x 0.144 = 0.22 mg TOS per day.

For an average person weighing 60 kg this corresponds to 3.6×10^{-6} g TOS/ kg body weight per day.

Lecithin hydrolysis

Lecithin is a food additive used widely for food[37]. According to Lehman[38], the average human intake of food per day is 1500 g.

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

a) The calculation is made assuming that all TOS remains in the hydrolyzed lecithin product. Lecitase Novo contains 1.5 % TOS.

b) It is assumed that half of the human intake of food contains food additives. It corresponds to 750 g as the average human intake of the food containing food additives per day.

c) It is further assumed that all this 750 g of modified food per day contains 0.5 % lecithin, which is processed with Lecitase Novo at the highest recommended dosage level, corresponding to 3.75 g lecithin per day.

The maximum recommended dosage of Lecitase Novo is 10000 LENU/kg crude lecithin, corresponding to 1 kg for 400 kg of hydrolyzed lecithin as described in Section 6.2. Lecitase Novo contains 1.5 % TOS. Therefore, the hydrolyzed lecithin would contain 37.5 mg TOS/kg lecithin.

Based on the daily intake of food containing food additives (corresponding to 3.75 g lecithin per day), the daily intake per person of Lecitase Novo corresponds to 37.5 x 0.00375 = 0.14 mg TOS per day.

For an average person weighing 60 kg this corresponds to 2.3×10^{-6} g TOS/ kg body weight per day.

Egg yolk modification

The modified egg yolk has a superior emulsifying effect. It is used for e.g. mayonnaise.

In accordance with FDA 21 CFR §101.12 Reference amount customarily consumed per eating occasions, consumption of mayonnaise per person per eating occasions is 15 g.

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

a) The calculation is made assuming that all TOS remains in the modified egg yolk. Lecitase Novo contains 1.5 % TOS.

b) It is assumed that mayonnaise is consumed at three eating occasions a day. It corresponds to 45 g mayonnaise daily per person.

c) Mayonnaise contains typically 5% yolk. It is further assumed that all mayonnaise is produced using modified egg yolk, which is processed with Lecitase Novo at the highest recommended dosage level, corresponding to 2.25 g egg yolk per day.

The maximum recommended dosage of Lecitase Novo is 8000 LENU/kg egg yolk, corresponding to 1 kg for 500 kg of modified egg yolk as described in Section 6.2. Lecitase Novo contains 1.5 % TOS. Therefore, the modified egg yolk would contain 30 mg TOS/kg egg yolk.

Based on the daily intake of mayonnaise (corresponding to 2.25 g egg yolk per day), the daily intake per person of Lecitase Novo corresponds to 30 x 0.00225 = 0.07 mg TOS per day.

For an average person weighing 60 kg this corresponds to 1.1×10^{-6} g TOS/ kg body weight per day.

Safety margin

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

The test batch used for the 13 week rat study has 8.3% TOS. The NOAEL dose level in the 13 weeks oral toxicity study in rats was 10 ml/kg/day corresponding to 0.83 g TOS/kg/day.

The estimated human consumption is:

Baking:	3.7×10^{-6} g TOS/kg/day
De-gumming:	3.6×10^{-6} g TOS/kg/day
Lecithin hydrolysis:	2.3×10^{-6} g TOS/kg/day
Egg yolk modification:	1.1×10^{-6} g TOS/kg/day

The safety margin can thus be calculated to be:

Baking:	$0.83/3.7 \times 10^{-6} = 2.2 \times 10^{5}$
De-gumming:	$0.83/3.6 \times 10^{-6} = 2.3 \times 10^{5}$
Lecithin hydrolysis:	$0.83/2.3 \times 10^{-6} = 3.5 \times 10^{5}$
Egg yolk modification:	$0.83/1.1 \times 10^{-6} = 7.4 \times 10^{5}$

7.6 Results and Conclusion

The results of the tests described in section 7.4.2 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. oryzae,* 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 and baking industries as well as in other food or non-food applications.

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Appendix 1

Lecitase® Novo

Application

Lecitase Novo is intended for the full or partial hydrolysis of phospholipids for performing vegetable oil degumming, obtaining lyso-phospholipid emulsifiers with altered emulsifying properties and improving the emulsifying properties of egg yolk.

Description

Lecitase Novo is a carboxylic ester hydrolase (E.C.3.1.1.3) produced by submerged fermentation of a genetically modified *Aspergillus oryzae* microorganism. Lecitase Novo has inherent activity towards both phospholipid and triglyceride structures, although the activity towards triglyceride will not be expressed in any of the applications mentioned above when the respective recommendations described in the Activity section below are followed. Lecitase Novo acts on phospholipids (phosphatides, lecithins) as a phospholipase type A_1 to yield the corresponding lyso-1-phospholipid (2-acylglycerophospholipid) plus a free fatty acid (FFA). Lecitase Novo is free of any significant side-activities.

Specifications

Lecitase Novo is a liquid preparation standardized in Lecitase Novo Units per g (LENU/g).

Lecitase Novo .....4,000 LENU/g

The unit is defined relative to a Lecitase Novo standard. One unit is equivalent to the amount of enzyme producing 1 μmole of free fatty acid per minute under standard conditions (substrate: 1-(S-decanoyl)-2-decanoyl-1-thio-sn-glycero-3-phosphocholine (CAS# 129940-52-9), temp. 37°C, pH 7.0). The analytical method, EAL-SM-0503.02, is available on request. Lecitase Novo is a pale brownish liquid. The colour may vary from batch to batch and colour intensity is not an indication of product strength. The density of Lecitase Novo is approx. 1.2 g/cm^3.

Purity
The product complies with the recommended purity specifications for food-grade enzymes given by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Food Chemicals Codex (FCC).

Activity

The optimal working conditions for Lecitase Novo will vary for the various applications. The following sections give the optimal conditions as determined by parameter optimization studies for the following applications/conditions: (I) vegetable oil degumming, (II) lecithin hydrolysis, (III) egg yolk modification, (IV) analytical conditions in aqueous solution.

Novo Nordisk

I. **Vegetable oil degumming** (see Application Sheet B 1325):
Typical reaction matrix: vegetable oil (rapeseed, sunflower seed, soybean), 5% water vs. oil, citric acid 0.1% w/w vs. oil, NaOH 0.03% w/w vs. oil (1.5 equiv. vs. citric acid), temp. 40-45°C, Lecitase Novo dosage 200 LENU/kg oil ~ 50 ppm. Optimal working conditions for Lecitase Novo are a pH around 4.5 (1.5 equiv. NaOH) and a reaction temperature around 40-45°C (see Figs. 1 and 2). Oil degumming performance vs. enzyme dosage is shown in Fig. 3.
The activity optima as a function of pH and/or temperature and/or dosage may vary according to the quality of oil and the exact conditions.

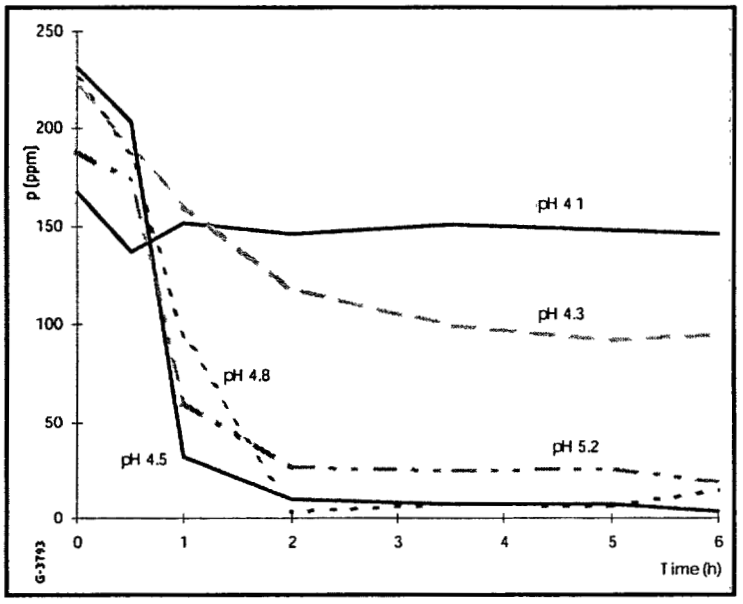

Fig. 1. Degumming with Lecitase Novo. pH variation.
Water-degummed rapeseed oil, 40°C, 800 LENU/kg oil.

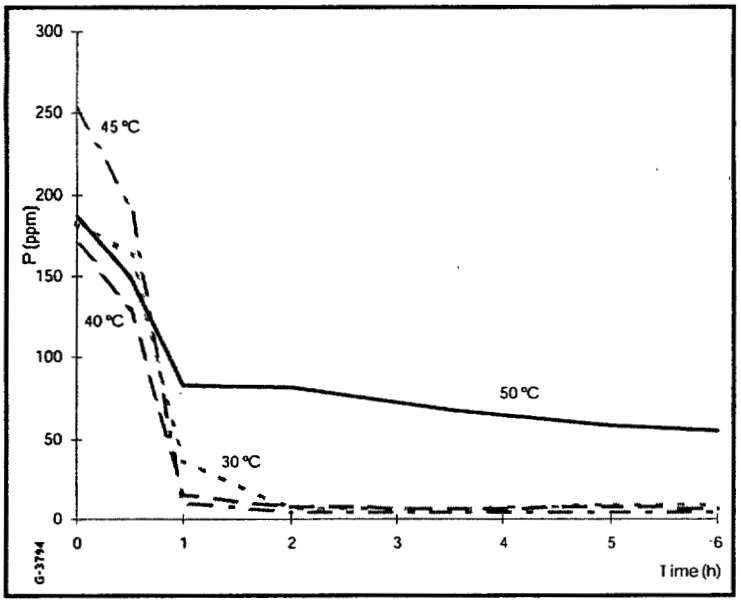

Fig. 2. Degumming with Lecitase Novo. Temperature variation.
Water degummed rapeseed oil, pH 4.5, 800 LENU/kg oil.

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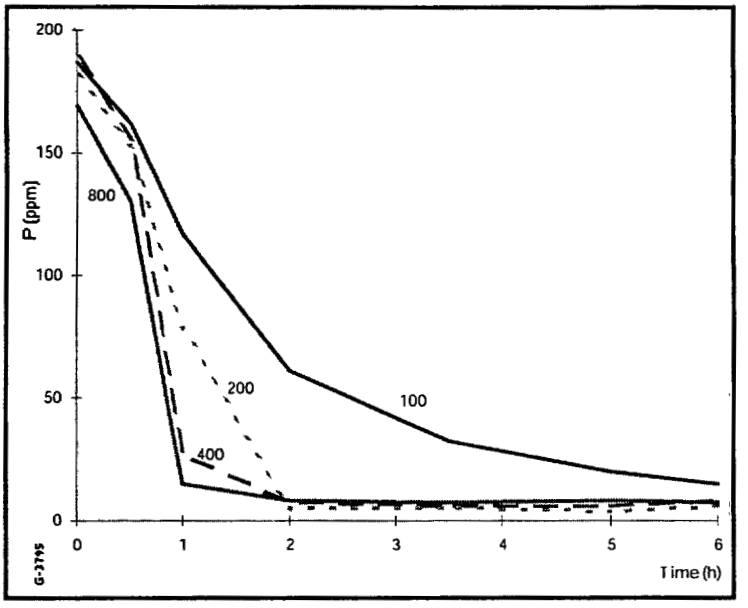

Fig. 3. Degumming with Lecitase Novo. Dose response.
Water-degummed rapeseed oil, 40°C, 800 LENU/kg oil.

II. **Lecithin hydrolysis**:
Typical reaction matrix: crude lecithin mix (60% lecithins, 40% triglycerides), 5% water vs. crude lecithin, temp. 40-45°C, Lecitase Novo dosage 10,000 LENU/kg crude lecithin ~ 0.25%. Optimal working conditions for Lecitase Novo are a pH around 6.5-7 and a reaction temperature around 40-45°C.

III. **Egg yolk modification**:
Typical reaction conditions: egg yolk is heated to 45°C, Lecitase Novo is added at a dosage of 1 kg per 1000 kg yolk, reaction is carried out for 3 hours, pasteurized, and further processed, e.g. dried.
When treated egg yolks are used in the production of mayonnaise and other emulsions there will be some changes due to the improved emulsification. The most obvious change is an increase in viscosity and an improved heat stability of the emulsion.

IV. **Analytical conditions**:
Typical reaction matrix: 2% purified soy lecithin, 2% Triton X-100, 20 mM BR. The enzyme shows an alkaline pH optimum using lecithin as a substrate with a pH optimum at pH 9 or above and a temperature optimum around 40°C at pH 5 using lecithin as a substrate.

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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. A Material Safety Data Sheet describing how to handle the product safely is supplied with the product. Further information on how to handle the product safely is available on request.

Storage

Enzymes gradually lose activity over time depending on storage temperature. Cool conditions are recommended. When stored at 0-4°C, Lecitase Novo will maintain the stipulated activity for at least three months. Do not store at temperatures above 4°C. Extended storage and/or adverse conditions, including higher temperature, may lead to higher dose requirements.

Standard packaging

Lecitase Novo is available in jerry cans containing 25 kg (approx. 20.8 l).

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Appendix 2

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SUBMISSION END

AM

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June 18, 2001

Rudaina Alrefai, 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

RE: GRAS Notice (GRN) No. 000075

Dear Dr. Alrefai,

In response to your request, we are hereby submitting an amendment to Novozymes' GRN No. 000075. On page 12, Section 6.3, the second sentence should read, "The Lipase is hydrophilic." We incorrectly used the term "hydrophobic" in that sentence.

Also at the request of FDA, we have included additional information on the donor strain for the lipase gene. Attached please find a short section on the safety of the donor strain, *Fusarium oxysporum*. Finally, please find enclosed copies of references 2-9 from the notification.

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

Sincerely,

Lori Gregg
Regulatory Specialist

Novozymes North America, Inc.
77 Perry Chapel Church Road
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000048

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

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Safety of the donor strain

The donor for the lipase enzyme is *Fusarium oxysporum*. It belongs to the section Elegans of the genus *Fusarium* within the class of imperfect fungi Hyphomycetes. In general, these fungi are not regarded as primary human pathogens. They are adapted to a living as saprophytes and many of them as plant parasites. Fungal spores or hyphal fragments, which are deposited on the skin or enter the body via one of its orifices, are generally incapable of further growth and development.

An increasing number of reports have been published connecting *Fusarium* species with opportunistic mycosis in humans. They have been held responsible for causing ulcers, necrosis, and other lesions of the skin as well as disseminated mycosis and infections in some organs and tissues in patients with severe underlying diseases. A number of general reviews concerning these opportunistic *Fusarium* infections of man and other animals have been written[1,2,3]. *F. oxysporum* has mainly been associated with eye infections in humans and specific strains of this species have been reported to produce various secondary metabolites, including fusaric acid, monoliformine and zearalenone.

However, the pathogenic and toxigenic potential of the particular strain of *F. oxysporum* used as donor for the lipase gene should not be of particular importance to the safety of our lipase enzyme preparation[4]. Pariza and Johnson[5] confirm that the safety of the production strain is the primary concern in evaluating enzyme safety. The primary issue in evaluating the safety of a production strain is its toxigenic potential. It is important to note that the only *F. oxysporum* DNA introduced into the *Aspergillus oryzae* production organism is the protein coding sequence from the *F. oxysporum* lipase gene contained in the lipase expression fragment. No *F. oxysporum* genetic sequences related to secondary metabolite production or pathogenic properties were introduced into the *A. oryzae* production microorganism. The steps used during strain construction ensure that the incorporated *F. oxysporum* DNA is limited solely to the lipase coding sequence. The initial identification and isolation of the *F. oxysporum* lipase gene from a cDNA plasmid library derived from purified *F. oxysporum* mRNA, assures that the library contains only *F. oxysporum* coding sequences. The specific lipase DNA fragment found in pMStr20 (see section 2.3 of the notification) is generated

[1] Austwick, PKC (1984). Fusarium infections in man and animals. In, The Applied Mycology of Fusarium. Symposium of the British Mycological Society held at Queen Mary College, London, September 1982, edited by Moss, MO & Smith, JE, Cambridge University Press, UK.

[2] Summerbell RC et al. (1988). Fusarium proliferatum as an agent of disseminated infection in an immunosuppressed patient. Journal of Clinical Microbiology 26: 82-87.

[3] Joffe (1989). Human Infections associated with Fusarium species. In: Fusarium species: Their Biology and Toxicology. John Wiley & Sons. p. 293-298.

[4] IFBC (International Food Biotechnology Council). (1990). 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.

[5] Pariza, M.W. and Johnson, E.A. (2001). Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing: Update for a New Century. Reg. Tox and Pharm 33: 173-186.

by *in vitro* polymerase chain reaction (PCR) amplification of the coding sequence of the *F. oxysporum* lipase gene from the lipase cDNA plasmid. The synthetic oligonucleotide primers used are homologous and complementary to sequences immediately 5' and 3' to the *F. oxysporum* lipase coding sequence. The only DNA that can be amplified using these primers is the *F. oxysporum* lipase gene coding sequence found between the two primers. Finally, the lipase gene sequence in the fragment was verified by DNA sequencing.

Therefore, following the concepts outlined in Pariza and Johnson (2001), we can conclude that all new DNA introduced into the *A. oryzae* production strain is safe and we have not changed the strain's toxigenic potential. As stated in the GRAS notice in section 7.1 on page 14, 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. oryzae* is considered safe (IFBC, 1990; Kessler et al., 1992). The safety of the enzyme preparation is confirmed by performing safety studies as described in Section 7.4 of the notification.

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codex alimentarius commission

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS

WORLD HEALTH ORGANIZATION

JOINT OFFICE: Via delle Terme di Caracalla 00100 ROME: Tel. 57971 Telex: 610181 FAO I. Cables Foodagri Facsimile: 6799563

Agenda Item 15

ALINORM 89/39

JOINT FAO/WHO FOOD STANDARDS PROGRAMME

CODEX ALIMENTARIUS COMMISSION
Eighteenth Session
Geneva 3-12 July 1989

IMPLICATIONS OF BIOTECHNOLOGY ON INTERNATIONAL FOOD STANDARDS AND CODES OF PRACTICE

(Document prepared for the Secretariat by Drs. David Berkowitz and Jim Maryanski, USA)

1. The new technologies for production of food, food ingredients, and food additives by biotechnology including genetic engineering are rapidly approaching the stage in development where their impact on food safety evaluation, Codex standards and codes of practice should be addressed. Many Member States of the Codex Alimentarius Commission (the Codex) have already dealt with issues such as safety evaluation of the intended uses for products of biotechnology at the country level. This paper addresses general issues that may arise in the Codex system's consideration of the products of biotechnology including:

(i) the role of new technologies in food production;

(ii) the effect of biotechnology on existing Codex definitions;

(iii) the safety evaluation of foods, food ingredients, and food additives developed through biotechnology, and

(iv) the designation of substances derived by biotechnology on the label of food products.

I. GENERAL CONSIDERATIONS OF THE ROLE OF BIOTECHNOLOGY

2. Biotechnological production of foods, food ingredients, and food additives has a long, safe history. Biotechnology, in the broad sense, is the technology used for any food production accomplished by living organisms or their components. Traditional food products such as vinegar, bread, cheese, and alcoholic beverages, among others are prepared by biotechnological processes. There have been successive improvements in quality of these traditional foods by changes in the microorganisms used in production. Additionally, new varieties of foods produced by conventional genetic techniques of breeding and selection are an integral part of the global food supply.

3. Food products produced through "new" biotechnology, such as recombinant DNA techniques and cell fusion, are emerging from research and development. These methods represent new tools that permit the scientist to transfer

genetic traits among diverse organisms. In most cases, genetic traits manipulated by recombinant DNA methods result from single (or a few) genes that encode well-known cellular functions, and these can be transferred to desired host organisms more rapidly and precisely than would be possible by conventional genetic methods.

4. This enhanced technology to modify food products will yield an expanding array of new food, food ingredients, and food additives and new processes to produce food ingredients and additives that are currently in use. Most of the applications of new biotechnology to food substances will be well-defined, incremental changes to improve economic, nutritional, or organoleptic properties. The nature of these new modifications will not differ fundamentally from changes accomplished by conventional means and can be assessed by standard molecular, chemical and toxicological methods.

5. Considerable public attention has been focused on recombinant DNA methods, but other methods of finding, selecting and manipulating genetic traits are being applied to develop new food and food ingredients, including cell and protoplast culture, somaclonal variation, electroporation, microinjection and microprojectile techniques. These methods are often used in concert with conventional methods of plant and animal breeding and microbial strain selection and improvement. The safety evaluation should focus on the characteristics of the finished product. Knowledge of the methods used is important to identify relevant scientific questions that should be answered, just as knowledge of a new chemical process is necessary to determine whether chemical impurities may be present in the finished product and whose safety must be examined.

6. Research and commercial experience, especially in pharmaceutical products, in recombinant DNA technology have demonstrated the power and safety of this technique to modify genetic structure and function. There is no evidence of unique hazards associated with the new technologies and potential risks that may occur are the same in kind as those associated with conventional methods. Safety evaluation should be based on accumulated experience and scientific knowledge based on the characteristics of the finished food substance. Policies and practices that the Codex system may develop should be flexible to accommodate evolving scientific developments and the diversity of application of biotechnology.

II. DEFINITIONS

7. Applications of food biotechnology will involve most aspects of the Codex system. The Codex through its Commission and subsidiary committees has established definitions for food additives, contaminants, pesticide residues, and veterinary drug and hormone residues in food. These definitions have been used successfully in international food trade for many years. Applications of food biotechnology will not necessitate changes in the existing Codex definitions, rather the definitions will provide an established, accepted framework for new technology.

III. SAFETY EVALUATION AND BIOTECHNOLOGY

FOOD ADDITIVES

8. The definition of food additive as adopted by the Codex Alimentarius Commission (Procedural Manual of CAC, 6th Ed. p. 33) includes general substances that:

(i) are not normally consumed as food;

(ii) are not normally used as a typical ingredient of the food;

(iii) are intentionally added for a technological purpose, and

(iv) are intended to become a component (or affect the characteristics) of food.

9. The goal of many applications of new biotechnology will be to design improved source (production) microorganisms or to employ new organisms to produce food additives that are already on the market. Food additives produced by improved strains include well-defined substances with substantial history of safe use such as citric acid, propionic acid, succinic acid, lactic acid, glutamic acid, tryptophan, lysine and xanthan gum. The safety evaluation of those food additives produced by improved microorganisms should focus on factors such as:

(i) the identity of the host organism;

(ii) any evidence of pathogenicity or toxin production;

(iii) the function of the inserted gene(s);

(iv) the identity of organisms that contribute genetic material to the final construct;

(v) characterization of the inserted genetic material to ensure the absence of sequences that may encode harmful substances;

(vi) insertional and genomic stability;

(vii) chemical specifications;

(viii) dietary use and exposure and other relevant information.

This information can be used to determine whether the finished food additive is sufficiently equivalent (including potential impurities from the source organism) to the conventional product. Food additives produced by new technology that are sufficiently equivalent would not require extensive toxicological evaluation or new ADI's to be established. Food additives that deviate significantly from their conventional counterparts should be evaluated on a case-specific basis.

10. The Codex system has established procedures to review biological or natural products through the Codex Committee on Food Additives and Contaminants (CCFAC) and the Joint Expert Committee on Food Additives (JECFA). The products of new technology can be reviewed through the existing system. It will be important to establish priorities for review of food products developed through biotechnology because:

(i) many applications of biotechnology will result in minor changes to food additives or the production (source) organism;

(ii) the scientific questions involved do not differ fundamentally from those for conventional products, and

(iii) a review of all products of new biotechnology would greatly strain the resources of organizations such as the JECFA.

11. Biotechnology may yield some new food additives that have not been used in food or evaluated previously. New food additives can be evaluated by the established Codex system using fundamentally the same criteria and guidelines. The preliminary review should focus on the factors discussed above concerning the nature of the modified organism, chemical specifications, and dietary exposure that will establish the appropriate level of toxicological concern.

ENZYMES

12. The Codex considers enzyme preparations to be food additives or processing aids based generally on whether the enzyme is removed from the finished food during processing. In either case, the safety assessment procedures are conducted through the established mechanisms of JECFA and CCFAC. As is the case for other food additives, enzyme preparations derived from microorganisms modified by recombinant DNA methods will often involve incremental changes in traditional enzymes and microorganisms that may not warrant extensive review. for example, the gene for common enzymes such as amylase, glucoamylase, protease, lipase, catalase, lactase and glucose isomerase will be obtained from new organisms and cloned into traditional production organisms such as Bacillus subtilis, Aspergillus niger or Aspergillus oryzae. Such enzymes and organisms may be modified to increase gene expression by amplification or altered regulatory signals, to increase resistance to higher temperature or lower pH, to improve substrate affinity, or to improve other properties. Generally, common enzyme preparations and production organisms can be assessed in the light of the factors discussed above for food additives. Those enzymes judged to be sufficiently equivalent to conventional products would not require review. New enzymes or source organisms should be evaluated on a case-specific basis.

VIABLE MICROORGANISMS AS STARTER CULTURES

13. A number of foods are produced traditionally by the fermentative action of microorganisms; these include dairy products, meat and vegetables. Many applications of biotechnology will be to improve the functional properties of the culture, and these modifications can be evaluated by the factors discussed for food additives. In some cases, certain factors may present specific concern for starter cultures. A new gene product expressed by a starter culture may be considered in some cases as a new food additive, in which case it may need to be evaluated as such. The use of antibiotic markers that also have therapeutic uses may need to be carefully evaluated in the light of possible transfer of antibiotic resistance to pathogenic food-borne contaminants during processing or to human intestinal flora. Because of the above considerations, new microorganisms should be evaluated on a case-specific basis before being accepted for use as starter cultures.

TRANSGENIC AGRICULTURAL FOOD CROPS

14. The first transgenic food crops have been tested in small-scale field tests and are expected to reach the commercial market by 1992. These plants

contain single (or a few) well characterized genes that encode resistance to pests or disease, tolerance to herbicides, improved protein content, or improved shipping or processing qualities (e.g., delayed fruit ripening). The safety assessment should focus on the inserted traits and their stability in the food, rather than on the food per se.

15. Some food crops have been genetically engineered to express a pesticide in the plant. For example, the gene that encodes the delta endotoxin of Bacillus thuringensis can be inserted and expressed into tomatoes to make the plant resistant to insects. In this, or similar cases, Codex may wish to consider whether the incorporated pesticidal function should be included in existing maximum residue levels, whether new permitted levels should be set, or whether any other specification is needed.

TRANSGENIC FOOD ANIMALS

16. The first transgenic animals used for meat and poultry will be developed through the introduction of one or a few well characterized genes into traditional livestock breeding lines. The safety of these new breeds can be evaluated by asking fundamentally the same scientific questions that would be posed for new traits introduced by classical breeding based on the knowledge of the genetic changes introduced into transgenic animals and the health history of the modified animal line. If the product of the introduced gene is an animal drug, its safety should be evaluated in a manner similar to other animal drugs.

IV. DESIGNATION OF SUBSTANCES DERIVED BY BIOTECHNOLOGY ON LABELS OF FOOD PRODUCTS

17. There have been successive improvements in foods (bread, cheese, alcoholic beverages, vinegar) by changes in the production microorganisms, and these changes have not resulted in new names for the foods but the traditional name has been maintained. Likewise, new varieties of foods produced by conventional plant breeding have retained their traditional names. The Codex may consider that those products of biotechnology that are deemed to be safe and that are identical to traditional foods, food ingredients, or additives shall be designated on the labels by the common name of the food, food ingredient, or additive.

18. From the points of view of quality and identity, however, Codex may also have to give some consideration, in specific cases, as to whether genetically altered edible fruits, vegetables or animal products essentially retain the quality factors and composition of the original product, or whether this food represents a new product or a sub-species of the original food and would therefore warrant the use of a new common name.

SUMMARY

19. The Codex through its component organizations has been used successfully in international food trade for many years. Applications of new biotechnology can be evaluated under the existing Codex system as are other foods, food ingredients, food additives, pesticide residues, and animal feeds and drug residues. FAO and WHO should serve to disseminate information concerning these new technologies to member nations and to develop food safety assessments and guidelines as necessary to meet the needs of new technology.

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Reference List for Industry Submission, GRN 000075

Pages	*Author*	*Title*	*Publish Date*	*Publisher*	*BIB_Info*
000041 - 000044	Barbesgaard, Peder; Heldt-Hansen, Hans Peter; Diderichsen, Berge	On the safety of Aspergillus oryzae: a review	1992	Appl. Microbiol Biotechnol	Volume 36, pgs 569-572
000051 - 000067	Pariza, M. W.; Foster, E. M.	Determining the Safety of Enzymes Used in Food Processings	May 1983	Journal of Food Protection	Volume 46, Number 5, pgs 453 - 468
000068 - 000075	NA	Safety Evaluation of Foods and Food Ingredients Derived from Microorganisms	1990	Regulatory Toxicology And Pharmacology	Volume 12, pgs 114 - 128
000076 - 000085	NA	Guidelines for the presentation of data on food enzymes	1992	Reports of the Scientific Committee for Food 27 Series	pgs 13-22
000086 - 000125	NA	Safety Evaluation of Foods Derived By Modern Biotechnology	1993	Reports of the Scientific Committee for Food 27 Series	pgs 13-22
000131 - 000164	FAO WHO	Biotechnology and food safety	1996	FAO Food and Nutrition Paper	Number 61, pgs 1-27
000165 - 000174	Jonas, D.A.; Antignac, E.; Antonie, J.M.; Classen, H.G.; Huggett, A.; Knudsen, I.; Mahler, J.; Ockhuizen, T.; Smith, M.; Teuber, M.; Walker, R.; Vogel, P. DE	The Safety Assessment of Novel Foods	1996	Food and Chemical Toxicology	Volume 34, pgs 931 - 940
000175 - 000199	Organisation for Economic Co-operation and Development	Safety Considerations for Biotechnology	1992	NA	NA

NA- Not applicable