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ORIGINAL SUBMISSION

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April 25, 2000

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 Novo Nordisk's lipase enzyme preparation produced by *Aspergillus oryzae* expressing the gene encoding a lipase from *Thermomyces lanuginosus*. The lipase enzyme preparation is intended for use in the fats and oils industry and the baking 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)



Novo Nordisk

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April 25, 2000

RE: GRAS Notification - Exemption Claim

Dear Sir or Madam:

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



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

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

Novo Nordisk BioChem 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 *Thermomyces lanuginosus*.

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

The lipase is intended for use in the fats and oils industry as well as the baking industry. 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* and published safety studies on the enzyme are 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.

John Carroll
Director, Regulatory Affairs

April 25, 2000
Date

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Novo Nordisk

**A lipase preparation produced by
Aspergillus oryzae expressing the gene encoding
a lipase from *Thermomyces lanuginosus***

**Lori Gregg, Enzyme Regulatory Affairs, NNBNA, USA
Rie Tsuchiya, Enzyme Regulatory Affairs, NNA/S, Denmark**

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Novo Nordisk

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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 *Thermomyces lanuginosus* (also described as *Humicola lanuginosa*, see Appendix 1).

There are two main formulations of this lipase preparation. One formula is to be used as a processing aid primarily in the fats and oils industry. The other formula is to be used as a processing aid mainly in the baking industry. Other potential applications are: flavor enhancement in milk products, cheese and pet food ingredients. For simplicity, the name Lipase will be used in this document to describe the lipase preparation produced by submerged fermentation of an *Aspergillus oryzae* microorganism carrying the gene coding for lipase from *Thermomyces lanuginosus*.

A lipase hydrolyzes esters in an aqueous solution, however, it is also known to catalyze esterification of fatty acids and alcohols or rearrange fatty acids in glycerides under certain conditions where water content is low. The specificity of this Lipase depends on the reactants and the reaction conditions. In some reactions, the Lipase shows 1,3 specificity, whereas in other reactions the product functions as a non-positional lipase. Rearrangement of fatty acids in glycerides can improve the physical and nutritional characteristics of the glyceride products¹.

The Lipase for the fats and oils application is formulated as a granulate to be used in packed bed columns or stirred batch reactors for esterification and rearrangement of fatty acids. This granulate formulation is insoluble and physically stable in oil. The Lipase for the baking application is formulated as a granulate to be used in the baking industry. This granulate formulation contains wheat flour and is specific for use in baking flour.

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 *T. lanuginosus*. 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

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intended for use in food². 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 genetic material³⁻⁸. 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 plasmid DNA encode or express a harmful or toxic substance.

Information concerning this enzyme used in the baking application (Novozym 677) was submitted to FDA and discussed at a meeting with FDA on June 27, 1996. This enzyme has been marketed as GRAS in the baking industry since that time.

It should be noted that within some of the attachments, the name "Novozym 677" is used to describe the Lipase.

2. PRODUCTION MICROORGANISM

2.1 Production Strain

The microbial production strain, designated H-1-52/c (synonym AI-11), was constructed by plasmid transformation and classical mutagenesis of a derivative of the industrial strain, *Aspergillus oryzae* strain IFO 4177 (synonym A1560). This genetically modified production organism complies with the OECD (Organization for Economic Co-operation and Development) criteria for GILSP (Good Industrial Large Scale Practice) microorganisms⁹. It also meets the criteria for a safe production microorganism as described by Pariza and Foster and several expert groups³⁻⁸.

Two plasmids were used in the strain construction, one an expression plasmid and the other a selectable marker plasmid. These plasmids contain strictly defined fungal chromosomal DNA fragments and DNA from well-characterized *Escherichia coli* vectors. The specific DNA sequences include: a gene encoding a *Thermomyces lanuginosus* lipase enzyme; an *Aspergillus nidulans* selectable marker gene, *amdS* (acetamidase)¹⁰, well-characterized noncoding regulatory sequences from *Aspergillus niger*¹¹ and *Aspergillus oryzae*¹², and known sequences from *Escherichia coli* plasmids pUC19¹³ and pBR322¹⁴.

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This genetically modified *A. oryzae* strain has been in use by Novo Nordisk for over ten years in the production of a commercial lipase enzyme for technical applications. This strain was the subject of a Premanufacture Notice (PMN) submitted to the Environmental Protection Agency in November, 1988¹⁵.



2.2 Recipient Organism

The recipient microorganism used in the construction of the production strain is a 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, Holland¹⁶.

2.3 Lipase Expression Plasmid

The 5.5 kb lipase expression plasmid, pBoel960, used in the construction of the production strain contains the following genetic material (see Appendix 2, Figure 1):

- 1.14 kb DNA from the *A. oryzae* TAKA amylase gene promoter
- 0.92 kb DNA from the *T. lanuginosus* lipase gene
- 0.75 kb DNA from the *A. niger* glucoamylase gene terminator sequence
- 2.69 kb DNA from the *E. coli* plasmid pUC19

2.4 Selectable Marker Plasmid

The 8.96 kb selectable marker plasmid, p3SR2, used in the construction of the production strain contains the following genetic material (see Appendix 2, Figure 2):

- 5.25 kb DNA from *A. nidulans* encoding the *amdS* gene
- 3.71 kb DNA from the *E. coli* plasmid pBR322

2.5 Stability of the Transformed Plasmid 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 plasmid DNA is stably integrated into the *A. oryzae* chromosome and, as such, is poorly mobilizable for genetic transfer to other organisms and is mitotically stable⁹.

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2.6 Absence of rDNA Sequences in the Enzyme Preparation

No transforming capability was detected in a sample of the enzyme preparation analyzed for transforming DNA.

2.7 Antibiotic Resistance Gene

Both pBoel960 and p3SR2 contain the β -lactamase gene, *bla*, from *E. coli* plasmid pBR322 encoding resistance to ampicillin. These genes are prokaryotic in origin and lack the appropriate sites and signals (promoter, ribosome binding site, etc.) to be functionally expressed when integrated in an eukaryotic chromosome. In addition, the prokaryotic *bla* gene lacks appropriate eukaryotic signal sequences and processing signals necessary for secretion and export. Therefore, any β -lactamase potentially produced by expression of the *bla* gene would be intracellularly localized and not be present in the final product, the lipase enzyme preparation.

This is a basic principle of molecular cell biology and genetics and is generally recognized within the scientific community. Prokaryotic and eukaryotic sites and signals for effective and functional gene expression and gene product synthesis are different¹⁷⁻²⁰.

Tests of *A. oryzae* production strains containing the *bla* gene integrated as part of an expression vector have not shown any evidence of β -lactamase expression.

2.8 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 IFBC³ is satisfactorily addressed.

3. MANUFACTURING PROCESS

This section describes the manufacturing process for the Lipase which follows standard industry practices²¹⁻²³. 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.

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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 industry²¹⁻²³. The raw materials conform to Food Chemicals Codex specifications except those raw materials which do not appear in the FCC. For those not appearing in the FCC, internal specifications have been made in line with FCC requirements. On arrival at Novo Nordisk 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 fermenter and the main fermenter 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.

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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.

3.3 The Recovery Process

The recovery process is a multistep 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 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

The liquid enzyme concentrate is sprayed onto a mixture of silicon dioxide, cellulose and dextrin and the granulate is formed. The granulates are dried in a fluid bed. The product is discharged continuously after sieving.

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Lipase formula for baking applications.

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

The final standardization is made by a dilution of the agglomerate with wheat flour according to the product specification.

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, broad fatty acid specificity
Molecular weight	35 kDa
Isoelectric point (pI)	4.4
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.

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5.1 Quantitative Composition

Lipase for fats and oils applications has the following typical composition:

Silicon dioxide	approx. 59%
Cellulose	approx. 15%
Dextrin	approx. 15%
Enzyme solids (TOS)	approx. 6%
Water	approx. 5%
Sodium chloride	approx. 1%
Potassium sorbate	approx. 0.1%

Lipase for baking applications has the following typical composition:

Wheat flour solids	approx. 80%
Water	approx. 8%
Dextrin	approx. 7%
Sodium chloride	approx. 2%
Enzyme solids (TOS)	approx. 2%
Sorbitol syrup	approx. 1%

5.2 Specifications

The Lipase conforms to the general and additional requirements for enzyme preparations as described in Food Chemicals Codex, 4th edition, 1996²⁴. In addition, 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 Specifications²⁵.

The following Novo Nordisk specifications have been established for the Lipase:

Lipase activity*	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 <i>E. coli</i> /25 g	negative by test
Salmonella/25 g	negative by test
Antibiotic activity	negative by test

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Production organism	negative by test
Mycotoxins	negative by test



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 Novo Nordisk specification and is not mentioned in FCC or JECFA.

*The activity of this enzyme can be measured differently depending on the application. For the fats and oils application, the activity is measured in Interesterification Units Novo (IUN/g) using the Novo Nordisk Interesterification Activity Method. The substrate is a mixture of tristearin and soybean oil. The fatty acids in tristearin are stearic acids, while the main fatty acids of soybean oil are linoleic acid, oleic acid and palmitic acid. As a result of the enzyme action, the fatty acids in the tristearin and soybean oil are rearranged and the tristearin is converted to other types of triglycerides. An Interesterification Unit Novo is defined as 0.01 w/w% converted tristearin per minute. For the baking application the activity is measured in Lipase Units (LU/g) using the Novo Nordisk Lipase/Esterase pH-stat Method on a Tributyrin Substrate. A Lipase Unit is the amount of enzyme which liberates 1 μ mol titratable butyric acid per minute under the given reaction conditions.

6. APPLICATION

6.1 Mode of Action

The enzyme is a lipase (EC 3.1.1.3). A lipase hydrolyzes esters in an aqueous solution. A lipase is also known to catalyze esterification of fatty acids and alcohols or rearrange fatty acids in glycerides under certain conditions where the water content is low.

In the fats and oils industry Lipase catalyzes the rearrangement of fatty acids in glycerides i.e. interestrification of glycerides and acidolysis between glycerides and fatty acids. This can be done to improve the physical and nutritional properties of glyceride products. Typical substrate reaction mixtures comprise triglycerides with or without free fatty acids. The positional specificity of Lipase depends on the reactants and the reaction conditions. In some cases, Lipase shows 1,3 positional

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specificity, whereas in other cases the product functions as a non-positional lipase.

The Lipase is a physically stable enzyme in an oil phase. Packed bed columns or stirred batch reactors may be used for esterification and rearrangement of fatty acids in glycerides. More information on the use of the lipase in fats and oils can be found in the product sheet in Appendix 3.

In the baking application, the Lipase shows 1,3-specificity acting on the primary (1- and 3-position) ester bonds in triglycerides to form diglycerides, monoglycerides and free fatty acids.

The main triglyceride content in conventional bread dough, containing no added fat, is found in the flour component(s) of the dough and constitutes typically about 1-2% of the weight of the flour. These lipids interact with specific gluten complex proteins to form lipid-gluten aggregates during the dough preparation.

The addition of lipase modifies this interaction and thereby improves properties of dough and baked products. Although the nature of the interaction between lipid and gluten is unknown, it is anticipated that lipase reduces a possible lipid-gluten over-aggregation in the dough by exerting a limited attack on the lipid component of the aggregates without, however, making a total degradation of the aggregates. More information on the use of the Lipase in baking applications can be found in Appendix 4, 5 and 6.

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 Lipase is up to 1 kg to produce 1 ton of triglycerides.

In the baking application, the recommended use level for the Lipase is 1-5 grams/100 kg flour or 500-2000 lipase units (LU)/kg.

6.3 Enzyme Residues in the Final Food

The Lipase is insoluble and physically stable in oil. Furthermore, after coming in contact with the enzyme preparation, the oil will be

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subjected to oil refining methods which include processing steps such as filtration, distillation, deacidification, bleaching and deodorization which would remove any potential residues.

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 food². 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 consume³. 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 JECFA²⁶.

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 enzymes²⁷ (Appendix 7). *A. oryzae* is accepted as a constituent of foods²⁶. *A. oryzae* has been used to produce soy sauce in the United States since before 1958^{3,27}. Therefore, *A. oryzae* meets the criterion of "common use in foods in the US before 1958" and can be considered "generally recognized as safe", GRAS³. 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 1973²⁸. Enzyme preparations from *A. oryzae* have been marketed in the US as GRAS by Novo Nordisk and other companies since that time. Therefore, enzyme preparations from *A. oryzae* are also considered GRAS^{2,3,26}.

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,

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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 plasmids 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.

Because the genetic modifications are well characterized and specific, and the incorporated plasmid 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^{3,29}. To confirm the safety of the enzyme, safety studies³⁰ 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^{2,31,32}. 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 group³¹.

7.2.1 Lipases

Microbial lipases have been reported to be used in food production since 1952^{33,34}. 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 Novo Nordisk as GRAS since that time.

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^{5-8,29,35-37}. Essentially all these groups conclude that if a food ingredient is substantially equivalent to an existing food ingredient

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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^{35,38,39}. 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^{29,36}.

The lipase enzyme from the genetically modified *A. oryzae* is a lipase from *Thermomyces lanuginosus* (previously known as *Humicola lanuginosa*, see Appendix 1). The *T. lanuginosus* lipase coding sequence incorporated into the *A. oryzae* production strain has not been altered. *T. lanuginosus* is an ubiquitous, thermophilic fungus that is not described as pathogenic or as a known toxin producer⁴⁰. The transfer of a gene from a nonpathogenic, nontoxigenic source, *T. lanuginosus*, to a similarly safe host, *A. oryzae*, is regarded as a safe system for enzyme production^{2,3}.

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 another⁴¹. Lipases are classified into four main families according to their structural homology. *Rhizomucor miehei* lipase (Rml) and *Thermomyces lanuginosus* lipase (Tll) both belong to the *R. miehei* family⁴². The amino acid sequences of Rml and Tll lipases have been compared^{43,44}. They are composed of the same number of amino acid residues, 269, and there are substantial structural similarities^{41,43,45}. The three-dimensional structure of the Tll enzyme is essentially identical to that of the Rml^{43,44}. The main elements of the secondary structure are conserved⁴³. The pentapeptide consensus sequence and the active site catalytic residues are the same in both lipases and there are conserved residues within the lid and hinge regions⁴³. *T. lanuginosus* lipase expressed in *A. oryzae* is substantially equivalent to a lipase from *R. miehei* and is safe for use in food.

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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 enzymes²¹⁻²³.

Silicon dioxide is used in the formulation that is intended for use in the oil and fats industry. The enzyme is granulated with silicon dioxide to increase the strength of the granulate and improve porosity of the granulate which allows the oil to penetrate the granulate more easily. There is no covalent bonding between the enzyme and the silicon dioxide.

The enzyme preparation will be used in packed bed columns or stirred batch reactors. The oil passes over the enzyme granulates and the enzyme catalyzes the interesterification reaction. Because the enzyme, as well as, all of the ingredients in the enzyme preparation are insoluble in oil, there should not be any residues left in the oil. Furthermore, after coming in contact with the enzyme preparation, the oil will be subjected to oil refining methods which include processing steps such as filtration, distillation, deacidification, bleaching and deodorization which would remove any potential residues.

Silicon in the form of silicon dioxide occurs abundantly in nature and makes up approximately 25 % of the earth's crust⁴⁶. Silicon dioxide is present in practically all plants, animals and natural waters⁴⁶. Silicon dioxide is present in normal human tissues at between 10 and 200 mg per 100 g tissue⁴⁶.

Silicon dioxide is used in a variety of food applications as anticaking agents, refining agents, filtering aids, washing agents etc⁴⁶. Silicon dioxide is GRAS for use as a substance migrating to food from paper and paperboard products (21 CFR 182.90). Diatomaceous silica is listed as the carrier for amyloglucosidase derived from *Rhizopus niveus* which is approved as a secondary direct food additive (21 CFR 173.110). Diatomaceous earth, a type of silicon dioxide, is part of a lipase enzyme preparation from *Rhizopus niveus* that is affirmed as GRAS as a direct food ingredient (63 FR 24416; 21 CFR 184.1420). Also, there is an FCC monograph on silicon dioxide. Based on the estimates of human consumption and safety margin of this enzyme preparation (see Section 7.5) as well as the current regulatory status of

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silicon dioxide in food, the use of silicon dioxide in this Lipase enzyme preparation is safe for use in food.

7.4 Safety studies

This section describes the studies and analysis performed to evaluate the safety of the use of the Lipase. A summary of the safety studies and analysis has been published by Greenough *et al* (Appendix 8).



7.4.1 Description of Test Material

The test batches of the lipase preparation used for the safety studies were produced in the same manner as in production scale. Key characteristics of the test batches are given in Table 1, Appendix 8.

7.4.2 Studies

The following studies were performed:

- 1) Acute oral toxicity in rats.
- 2) Acute inhalation toxicity in rats.
- 3) Subacute oral toxicity in rats.
- 4) Skin irritation in rabbits.
- 5) Eye irritation in rabbits.
- 6) Skin sensitization: delayed contact hypersensitivity in guinea pigs.
- 7) Gene mutation (Ames test with *S. typhimurium*/*E. coli*).
- 8) Chromosome aberrations (*in vitro* cytogenetics with human lymphocytes).
- 9) Aquatic organism toxicity: *Daphnia* and carp.
- 10) Algal growth inhibition test.
- 11) Biodegradability.
- 12) Pathogenicity of *A. oryzae*: spores of host organism and genetically modified recombinant strain.

7.5 Estimates of Human Consumption and Safety Margin

This section discusses the potential human exposure to the Lipase from food. The first part is based on the exposure from fats and oils and the second part is based on exposure from bakery products. As described in section 6.4, due to the stability of the lipase in the oil application and the type of further processing that the final oil product is subjected to after interesterification with the enzyme product, there should be very little if any enzyme residue in the final oil product. However in each case (both fats and oils applications and baking

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applications), it is assumed that all enzyme activity is retained in the food so that the worst-case scenario is presented.

Fats and Oils Application

The maximum dosage of the lipase in the fats and oils application is 1 kg to produce 1 ton of triglycerides. The Lipase formula used for this application contains 6% TOS. Therefore, the triglycerides would contain 60 mg TOS/kg triglyceride.



According to the Economic Research Service, USDA report on Food Consumption, Prices and Expenditures, 1970-1997:

In the US, the average consumption of vegetable oil per person per day is 69 g.

Based on the average daily intake of vegetable oil (69 g), the daily intake per person of the Lipase would be:

$$0.069 \text{ kg oil} \times 0.06 \text{ g TOS/kg oil} = 0.0041 \text{ g TOS/person/day}$$

For an average person weighing 70 kg, this corresponds to :

$$\underline{0.000058 \text{ g TOS/kg bw/day.}}$$

Safety margin:

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

The test batch used for the 13 week rat study has a 27% TOS content.

The dose level in the 13 week oral toxicity study in rats, which could be given without any toxic effects was 5 g enzyme concentrate/kgbw/day or 1.35 g TOS/kgbw/day which is the NOAEL.

The safety margin for the fats and oils application is calculated as follows:

$$1.35 \text{ g TOS/kg bw/day divided by } 0.000058 \text{ g TOS/kg bw/day} =$$

$$\mathbf{2.3 \times 10^4}$$

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Baking Application

A theoretical calculation of human exposure to the lipase through consumption of bread can be made on the following assumptions:

The maximum dosage is 2500 LU/kg flour, corresponding to 5 g of Novozym 677 BG (50 KLU/g) per 100 kg flour.



The maximum TOS content of Novozym 677 BG is estimated to be 5%. Bread contains approximately 75% flour.

Therefore, the maximum TOS in bread is: 1.88×10^{-6} g TOS/g bread.

(The enzyme is inactivated during the baking process, however, for the estimation of human consumption we assume that all activity is retained in the bread.)

An average bread consumption of 160 g/person/day and a body weight of 60 kg results in: 2.67 g bread/kg body weight/day.

The maximum estimated daily intake (EDI) of TOS through bread is therefore: EDI = 0.000005 g TOS/kg body weight/day.

(Although, not all bread types will contain lipase, we assume that all bread will contain lipase to consider the worse case scenario.)

Safety margin:

The test batch used for the 13 week rat study had a 27% TOS content.

The dose level in the 13 week oral toxicity study in rats which could be given without any toxic effects was 5 g enzyme concentrate/ kg bw/day or 1.35 g TOS/ kg bw/day which is the NOAEL.

The safety margin for the baking application is calculated as follows:

$$1.35/0.000005 = 2.70 \times 10^5$$

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.

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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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8. LIST OF APPENDICES

1. Taxonomy of *Thermomyces lanuginosus*, internal Novo Nordisk memorandum, May 13, 1996.
2. pBoel 960 plasmid map (Figure 1), p3SR2 plasmid map (Figure 2)
3. Novo Nordisk Product Sheet for Lipozyme TL IM (B 1276a-GB)
4. Novo Nordisk Product Sheet for Novozym 677 BG (B 719d-GB)
5. Application Sheet on Breadmaking with Novozym 677 BG (B 720b-GB, September 1995).
6. Effect of Lipase on Breadmaking in Correlation with Their Effects on Dough Rheology and Wheat Lipids, International Symposium and Exhibition on New Approaches in the Production of Foodstuffs and Intermediate Products from Cereal Grains and Oil Seeds, ISE '94, ICC/AACC/CCOA/AOCS, November 16-19, 1994, Beijing, China.
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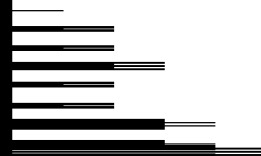
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To: PE
From: KMO
Copy: MiSa, IGC, HDa

Taxonomy of *Thermomyces lanuginosus*

In relation to a filing to FDA, PE has requested documentation in support of the change of species name from *Humicola lanuginosa* (Griffon and Maublanc) Bunce to *Thermomyces lanuginosus* Tsiklinsky.

This memo is an attempt to summarize the results of a literature search for such data.

The generic name *Thermomyces* was introduced by Tsiklinsky in 1899 for one species, *T. lanuginosus*. The species was described and photographs taken to illustrate the morphology. No type specimen was deposited.

The species as described by Tsiklinsky is very characteristic morphologically and physiologically and there is little doubt that Tsiklinsky's *Thermomyces lanuginosus* is the same as isolated and described by later workers (such as for instance Bunce (1961) and Cooney & Emerson (1964)) as *Humicola lanuginosa*.

According to The International Code of Botanical Nomenclature the correct name is the earliest legitimate one. Thus *Thermomyces lanuginosus* should be the correct name, provided it is legitimate. However due to Tsiklinsky's rather incomplete description of the species this legitimacy has been questioned by many mycologists. Thus the main reason for the taxonomical confusion relates to different views upon whether Tsiklinsky's description meets the requirements for a valid publication.

The decision to favour the name *Thermomyces* at Novo Nordisk is based upon the following circumstances:

- All major commercial culture collections (ATCC: American Type Culture Collection, IMI: International Mycological Institute, CBS: Centraalbureau voor Schimmelcultures) now use the name *T. lanuginosus*.
- A neotype named *T. lanuginosus* has been selected at IMI (IMI 84400)
- Most recent publications discussing this problem are in favour of using the name *T. lanuginosus*:

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Fassatiova O. (1967) Česká Mykologie 21, 78-89

Manoch L. et al. (1986) Trans. Brit. mycol. Soc. Japan

However the name *Humicola lanuginosa* is still seen in literature. The extended use of this name is probably due to the popularity of the book of Cooney & Emerson (1964) "The Thermophilic Fungi", which recommends the use of the name *Humicola lanuginosa*.

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Appendix 2

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Figure 1. pBoel 960 plasmid map

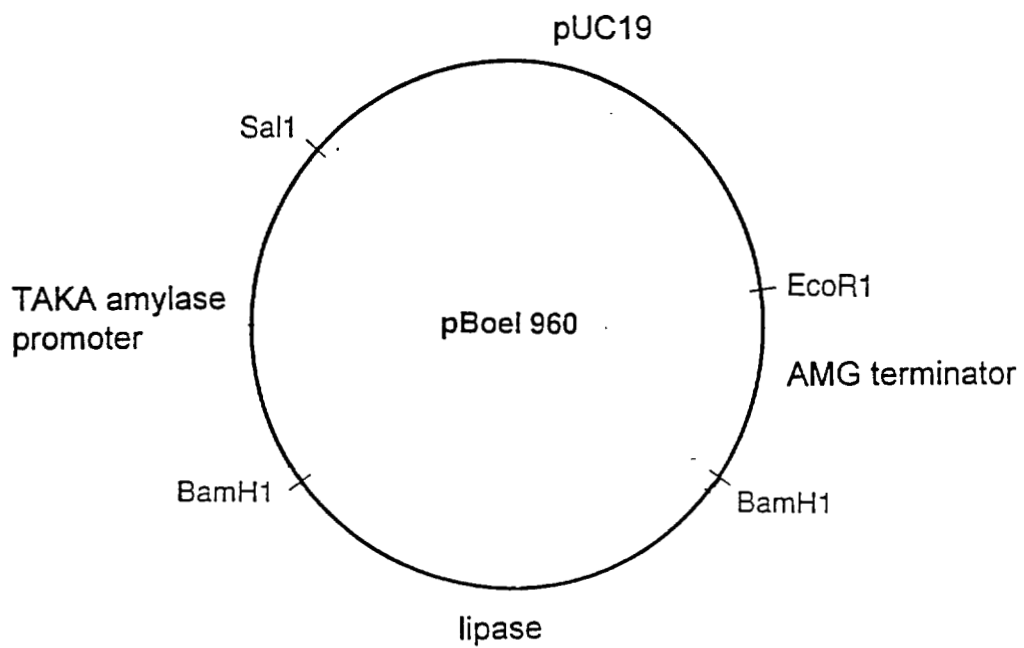
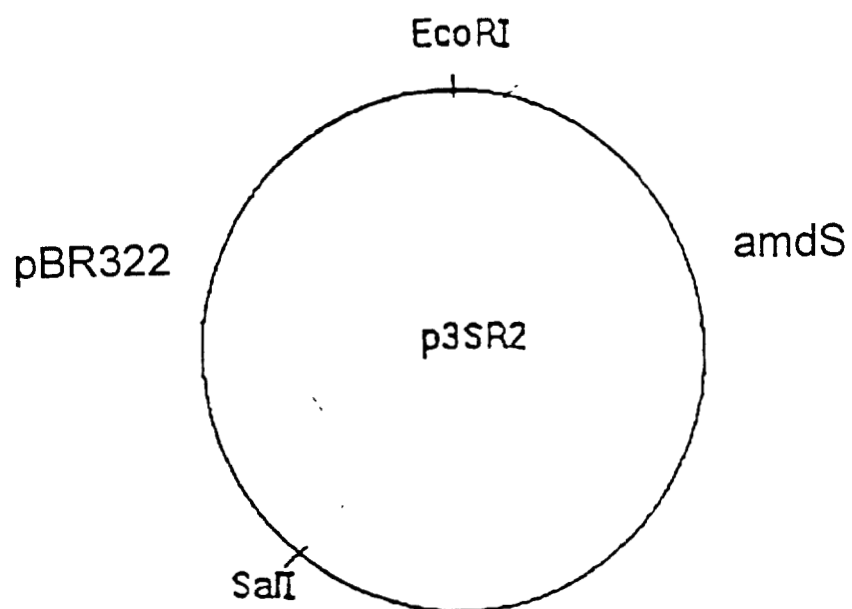


Figure 2. p3SR2 plasmid map



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Appendix 3

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Lipozyme TL IM

- Application** Lipozyme TL IM is intended for interesterification of bulk fats, production of frying fats, shortenings and margarine components.
- Description** Lipozyme TL IM is a food grade granulated silica preparation of a microbial 1.3 specific lipase (EC 3.1.1.3) from *Thermomyces lanuginosus* produced by submerged fermentation of a genetically modified *Aspergillus oryzae* micro-organism.
- Specification**
- | | |
|---------------------|---------------|
| Catalytic activity: | 75 IUN/g |
| Bulk density: | 0.54 g/ml |
| Particle diameter: | 0.30 - 1.0 mm |
| Water content: | 5 w/w% |
- 1 Interesterification Unit Novo is defined as 0.01 w/w% converted tristearin/minute (initial rate) at the following batch interesterification conditions: Substrate (full hydrogenated soybean oil/ soybean oil; 27/73 w/w%). Temperature: 70°C. No co-solvents. Enzyme dosage: 10 w/w %.
- Description of the analytical method is available upon request.
- Activity** Lipozyme TL IM is most active in the temperature range 55-70°C. For initial interesterification trials, Lipozyme TL IM dosages between 6-10 w/w %, contact times in the range 2-6 hours and temperatures around 60-70°C are recommended.
- Stability** Lipozyme TL IM is a mechanical stable granulate and can be used both in batch and in fixed bed operations. However, Lipozyme TL IM is intended for the use in non-aqueous media only since the granules will disintegrate in water.

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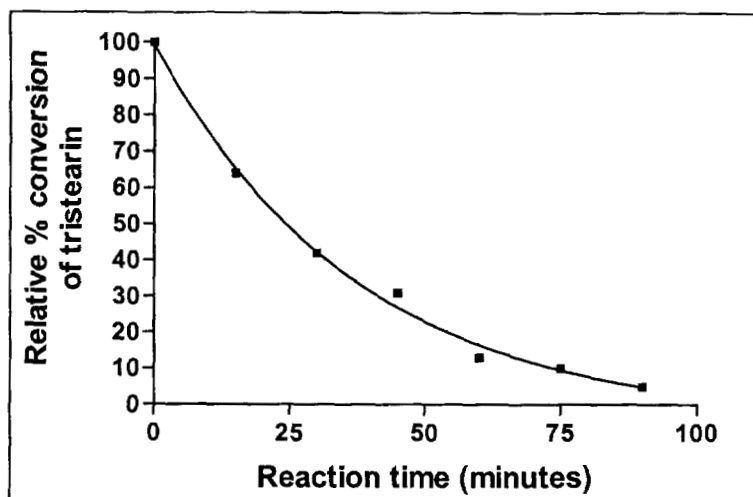


Fig. 1.

Batch interesterification of full hydrogenated soybean oil and refined, bleached, deodorized soybean oil (27/73 w/w%, respectively).

The reaction is followed by monitoring the conversion of tristearin.

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).

Safety

Enzymes are proteins and inhalation of dust or aerosols may induce sensitisation and may cause allergic reactions in sensitised 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. Leftover material may dry out and create dust. Material Safety Data Sheets and separate material describing how to handle the product safely are available upon request. A separate Novo Nordisk leaflet (B 143), "How to handle liquid Novo Nordisk enzymes - safely", is available on request.

Enzyme Business

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2880 Bagsvaerd
Denmark

Tel. +45 4444 8888
Fax +45 4444 1021
Telex 37560

Laws, regulations and rights may prevent customers from using certain products. It is the sole responsibility of the customer to ensure that the use of Novo Nordisk products is in compliance with relevant laws and regulations and, furthermore, does not infringe patents or other rights belonging to third parties. Subject to change without further notice.



Storage

Enzymes gradually lose activity over time depending on storage temperature. Cool conditions are recommended.

When stored below 25°C Lipozyme TL IM will maintain its declared activity for at least 3 months.

Extended storage and/or adverse conditions, including higher temperature, may lead to higher dosage requirement.

**Standard
Packing**

Lipozyme TL IM is available in 25 kg (55 lb.) fibre drums.

Note that units in parentheses are US units NOT UK units!

Enzyme Business

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Appendix 4

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Novozym[®] 677 BG

Description

Novozym 677 BG is a purified 1,3-specific lipase from *Humicola lanuginosa* produced by a submerged fermentation of a genetically modified *Aspergillus oryzae*.

Application

Novozym 677 BG is used in baking as a dough/bread improver. Its use yields doughs with improved stability and loaves with increased volume, whiter crumb and improved crumb structure.

Novozym 677 BG works well together with hemicellulases and fungal alpha-amylases. A combination of Novozym 677 BG and a hemicellulase results in loaves with significantly larger volume and better crumb structure with less dough stickiness than loaves baked with the same hemicellulase alone.

Novozym 677 BG works best in a fat-free formula. When used at recommended dosages, the enzyme has no detrimental effect on emulsifiers.

An application sheet (B 720) is available on request.

Activity

Novozym 677 BG is available in the following strength:

Novozym 677 BG 50 KLU/g

The product is standardised in **Kilo Lipase Units**. The analytical method (AF 95) is available on request.

Novozym 677 BG contains no significant side-activities.

Dosage

The recommended dosage for Novozym 677 BG is 0.5-2.5 grams/cwt. flour (1-5 grams/100 kilos; 500-2500 LU/kg). The optimum dosage for a specific formula should be determined through baking trials.

A dosage of up to 2.5 grams/cwt. flour (5 g/100 kg flour) does not result in any off-flavours; dosages significantly higher than this may give a detectable off-flavour to the bread.

Product Specification

Novozym 677 BG complies with FAO/JECFA and FCC recommended specifications for food grade enzymes, supplemented with maximum limits of 5×10^4 /g for total viable count and 10^2 /g for mould.

Product Characteristics

Novozym 677 BG is active over a broad pH interval (5-8). The enzyme stability at normal temperatures is excellent. It is active during the mixing, fermentation and proofing stages of bread production and is inactivated during baking.

Product Type

Novozym 677 BG is standardized using a special wheat flour with a narrow particle size distribution. The product is light brown, free-flowing, non-dusting, agglomerated with an average particle size of around 150 microns. No particles are larger than 200 microns and less than 1% are smaller than 50 microns.

Packaging

Novozyme 677 BG is available in 60-litre fibre drums with a net weight of 25 kg.

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Breadmaking with Novozym 677 BG

Introduction

Novozym 677 BG is a lipase. Its use in breadmaking has been found to offer the following advantages:

- 1) increased dough stability and larger volume of baked goods
- 2) improved crumb softness
- 3) improved crumb structure and a whiter crumb
- 4) synergic effect with pentosanases
e.g. less sticky dough and bigger volume

The above advantages are most pronounced in no or very low fat recipes like French baguettes or German rolls.

For the production of special low calorie bread types, the fat content can be reduced and the added lipase will secure the same bread quality.

The main triglyceride content in conventional bread dough, containing no added fat, is found in the flour component(s) of the dough and constitutes typically about 1 - 2 % of the weight of the flour. These lipids interact with specific gluten complex proteins to form lipid-gluten aggregates during the dough preparation.

The addition of lipase modifies this interaction and thereby improves properties of dough and baked products. Although the nature of the interaction between lipid and gluten is unknown, it is anticipated that lipase reduces a possible lipid-gluten over-aggregation in the dough by exerting a limited attack on the lipid component of the aggregates without, however, making a total degradation of the aggregates.

Enzymes

Novozym 677 BG, an experimental 1,3-specific lipase with a specific amount of LU/g (Lipase Units per gram). The analytical method, AF 95/5, is available on request.

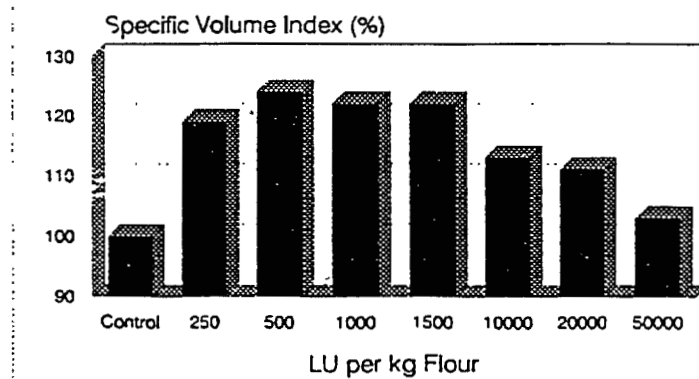
A preliminary product sheet, B 719, gives more details on Novozym 677 BG.

Application

Novozym 677 BG can be used in different baking processes (sponge and dough, straight dough) with different flour qualities and recipes. For illustration purposes, we have used a straight dough process with a standard Danish flour.

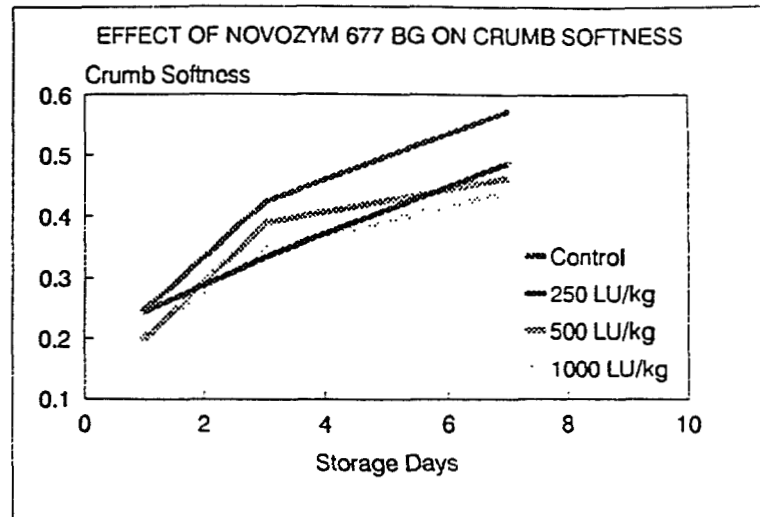
The optimal dosage is in the range of 500 - 2000 LU/kg, here around 1000 LU/kg. Higher dosages result in an over-modification of the dough, i.e. a too strong dough.

EFFECT OF NOVOZYM 677 BG ON THE
SPECIFIC VOLUME INDEX OF HARD ROLLS



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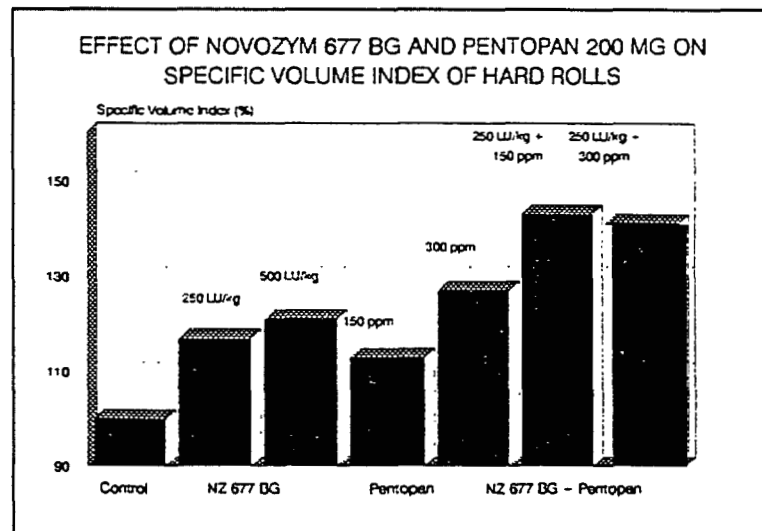
The volume increase automatically gives improved softness as illustrated, but not necessarily an anti-staling effect.



Novamyl^R is an anti-staling enzyme, giving no volume increase, but, by modifying the starch, it retards staling. Thus, optimal softness and anti-staling can be achieved by combining Novozym 677 BG and Novamyl.

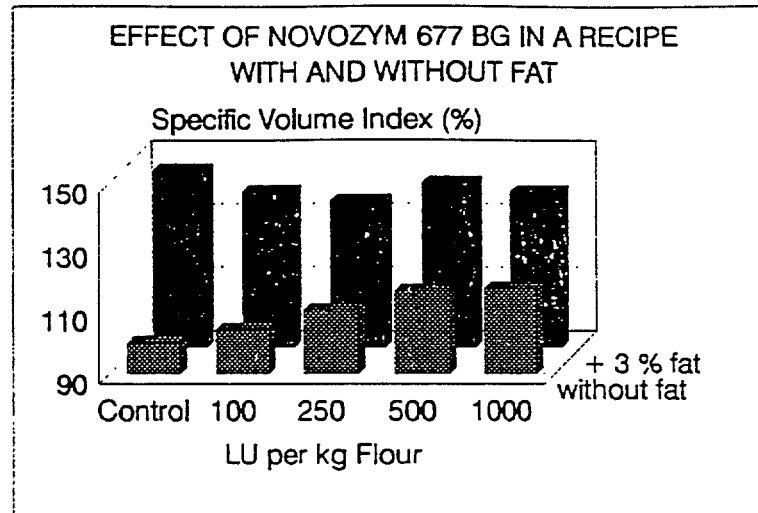
Novozym 677 BG gives a whiter crumb with uniform crumb structure.

Pentosanases like Pentopan 200 MG give improved volume. Used in combination with Novozym 677 BG, a pronounced synergic effect on volume and softness is observed.



In addition, the lipase reduces the dough stickiness typically seen at higher pentosanase dosages. Thus, on a scale of 1 (almost liquid) to 5 (dry), the average score increased from 2.5 (too sticky) to 4 (normal) by adding 500 LU/kg of Novozym 677 BG together with Pentopan 200 MG.

The effect of lipases in fat-containing (soy oil) recipes is much less pronounced. The addition of the fat in itself brings about the traditional volume increase.



However, for special low calorie bread varieties, Novozym 677 BG can be used to compensate for a reduction of the fat content. Thus, bread baked with 250 LU per kg flour without fat is equally fresh as bread with 6 % added fat.

Inactivation

Novozyym 677 BG is completely inactivated during normal baking conditions.

Appendix 6

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Effect of Lipase on Breadmaking in Correlation with Their Effects on Dough Rheology and Wheat Lipids

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Enzymes, such as α -amylases and pentosanases, have been extensively used to improve the quality of baked bread. However, the use of lipase for breadmaking is almost unknown. It was reported¹¹ that combination of lipase with vital gluten and lecithin can results in a smaller volume, but with an improved the mouth feeling and crumb texture.

Lipid is a minor component of wheat flour. The knowledge of the chemistry of wheat lipid in breadmaking is still fragmentary, although several investigations have been carried out²¹. Bekes et al³¹ reported that the loaf volume decreases with increase of non-polar/polar lipids ratio. The flour lipids, especially the glycolipids, form an integral part of gluten. It was suggested that glycolipids, through hydrogen bonds and hydrophobic interactions, form a linkage between gliadin and glutenin moleculars in gluten⁴⁵¹. Bekes et al⁶¹ reported that there is a strong positive correlation between the lipid mediated aggregates and loaf volume. However, over-aggregates of the gluten may be responsible for the deterioration of breadmaking quality⁷¹.

The present study was undertaken to illustrate the effect of lipase on breadmaking and the synergetic effect of lipase in combination with other enzymes. In order to understand the mechanism of lipase in breadmaking, the gluten dough was studied using dynamic rheological methods and the effect of lipase on the free lipids in wheat flour dough was also studied.

Material & Methods

Flour The Manitoba flour used for test baking is an European type baking flour.

Enzymes SP677, a Fungal lipase with 510,000 LU/g; Pentopan 200MG, a fungal pentosanase with 1250 FXU/g. Both are produced by Novo Nordisk A/S.

Baking procedure The formulation is 100% flour, 5% pressed yeast, 1.5% salt and 1.5% sugar. A typical Danish pan bread procedure was used. The samples were cooled before they were packed in polyethylene bags, sealed and stored at 22 °C.

Specific volume index The mean value of 30 rolls is measured using the traditional rape seed method. The specific volume (ml/g) of the control is defined as index 100. The specific volume index is calculated relatively to the control.

Crumb firmness was measured using the Texture Analyzer TA-XT2. A 20 mm Ø probe with a compression rate of 2mm/sec is used to compress 5 mm of a 20 mm bread slice. The average of 5 repeating compressions per slice were recorded. 4 slices per bread and two breads per sample were measured and the average results were recorded.

Wheat flour and Gluten dough A wheat flour dough of 10 g flour and 5,9 g water with or without enzyme was made. The dough was packed in polyethylene bag, sealed and it then was incubated at 32°C for 90 min. For the lipids analysis, the flour dough was lyophilized and ground to fine powder. For rheological study, the gluten was isolated using a Glutomatic 2200.

Dynamic rheological measurements The gluten was placed in PP30 of Bohlin VOR Rheometer and rested for 10 min. before measuring started. 1) Oscillation strain sweep: 1 Hz with amplitude from 0.1% to 100%; The results were mean values of triple tests. 2) Shear stress relaxation: 10% amplitude and strain rise time of 0.3 sec. The results was mean value of 10 tests.

Free lipids analysis The lyophilized wheat flour dough was extracted with hexane for 1 hr. using a Soxtec-extractor (system HT6).

Results & Discussion

Breadmaking effects

The effect of lipase on bread volume is shown in Figure 1. An addition of 500 LU/kg flour results in a volume increase of 24%. When the dosage of lipase is higher than 10,000 LU, the dough become dry and stiff with a reduced volume increase. Table 1 shows the effect of lipase on dough consistence, crumb structure and colour. The dosage of pentosanase is often limited due to dough stickiness caused by larger dosage of pentosanase. When combining the lipase with pentosanase, not only is the dough nice and elastic, it can also achieve a greater volume increase than when either enzyme is used alone.

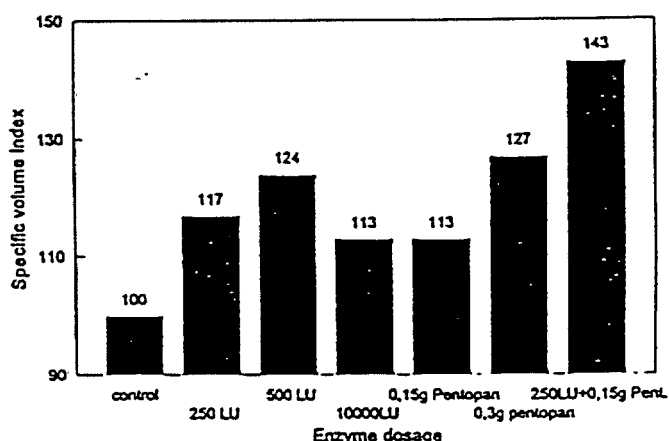


Figure 1, Effect of lipase on bread volume.

Enzyme/kg flour	Dough consistence	Crumb structure	Crumb colour
Control	Normal	Poor	Normal
Lipase, 250 LU	Normal	Uniform	Slight white
Lipase, 500 LU	Normal	Uniform	White
Lipase, 10,000 LU	Dry - Stiff	Fine	White
Pentopan, 0,15 g	Normal - soft	Uniform	Normal
Pentopan, 0,3 g	Soft - sticky	Uniform-silky	Normal
250LU + Pen.0,15 g	Normal	Uniform-silky	White

Table 1, Effect of lipase on dough and crumb properties.

Crumb softness

The effect of lipase on crumb softness during storage without added fat is shown in Figure 2. Without added fat, the bread with lipase has significantly softer crumb than the control. A hypothesis of lipase's effect was reported⁹¹ as due to its effect to produce emulsifiers (i.e. monoglycerides) in situ. However, our results show that an increasing amount of lipase does not have significantly more effect on crumb softness. The staling rate of the bread with lipase, (i.e. the slope of the curve) is similar to the control. It appears that the softer crumb obtained with lipase is due more to the effect of large volume and a more uniform crumb structure⁹¹. If the previous hypothesis was true, addition of lipase in bread with added fat would have even more effect on crumb softness due to additional monoglycerides produced in situ. However, our results shown in figure 3 indicate that the addition of lipase does not increase the crumb softness of the bread with 3% added fat. As fat itself has a good anti-staling effect. Besides, an addition of monoglycerides would not have such an significant effect on the bread volume increase as shown in Fig. 1.

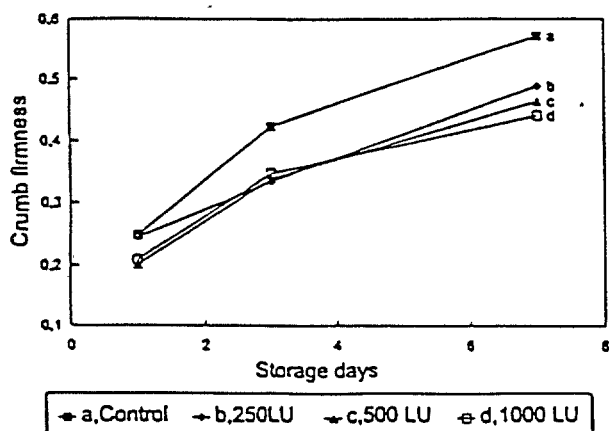


Figure 2, Crumb firmness without added fat.

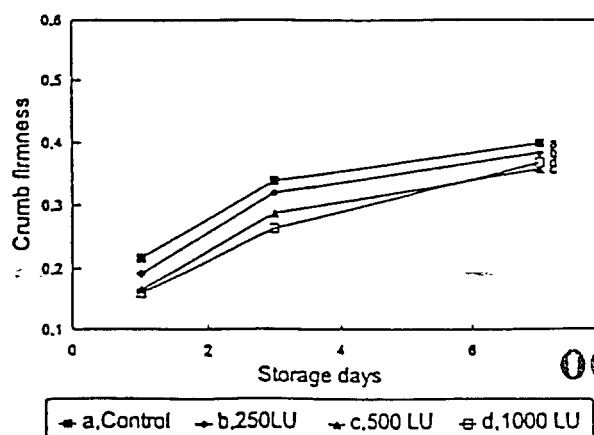


Figure 3, Crumb firmness with 3% fat.

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Our hypothesis for the mechanism of lipase is that the lipase changes the interaction between gluten and some lipids fragments during dough mixing resulting in an improved gluten net-work, which ensures a more stable dough and a larger bread volume with more uniform crumb structure.

Rheology properties of gluten

Figure 4 illustrates the effect of lipase on the storage modulus G' and phase angle δ of the gluten from lipase treated wheat flour dough measured with oscillation method. The gluten complex treated with lipase is significantly stronger with an increased G' and also more elastic with a lower δ than the control. An overdosage of lipase results in a too strong gluten complex, as seen with a very high G' , resulting in a too stiff dough and smaller volume increase. Previous studies^{10,11} reported that increase of crosslinkage in dough, e.g. through an oxidant, resulted in changes of stress relaxation, i.e. increased rigidity modulus and relaxation time. Our results from the stress relaxation measurements indicate that the gluten treated with lipase has more crosslinks than the control. As it is shown in Figure 5, the gluten with lipase has a high G -filt, increased relaxation time and with more "shoulders" on the Spectrum indicating increase of crosslinks in the net-work. These extra crosslinks give a higher gluten strength and more elasticity. It is not possible to predict the nature of these extra crosslinkss. More studies must be done in order to elucidate these.

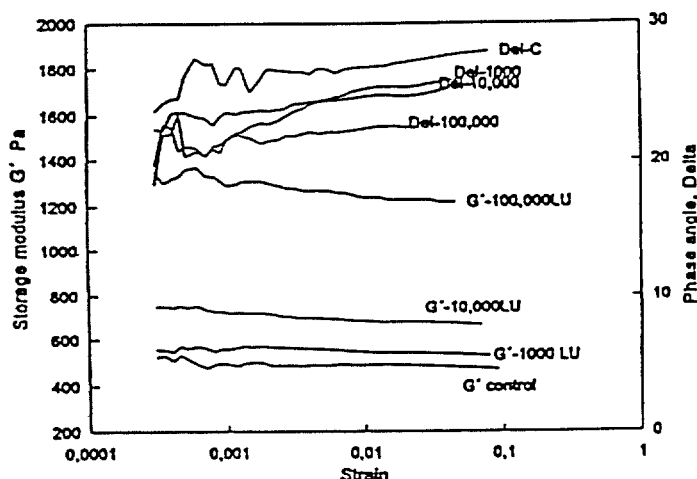


Figure 4, Oscillation strain sweep, effect of lipase on G' and delta.

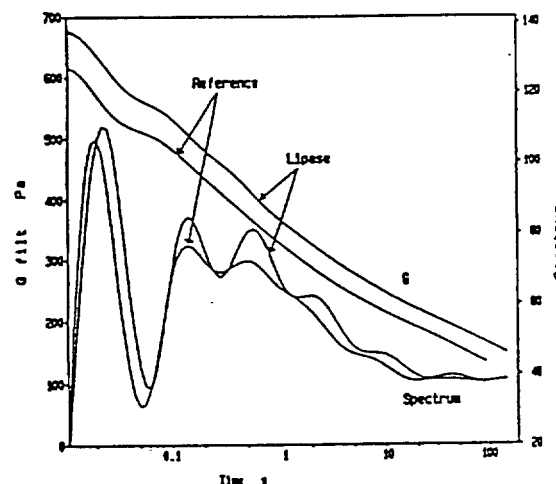


Figure 5, Stress relaxation, effect of lipase on G -filt and relaxation time.

Free lipids

Table 2 shows the free lipid in the wheat flour and in dough. The amount of free lipids in dough is significantly lower than that in the flour, indicating binding of lipids to the gluten or starch during dough mixing. The content of the free lipids in the lipase treated dough is approx. 10% higher than the control dough. Hoseneý et al¹² indicated that the free lipids in dough is more important to breadmaking than bond lipids. The more free lipids, mainly the free fatty acids, released by the lipase treatment may have a higher mobility resulting in an improved dough rheological property and better quality of breadmaking as mentioned above. However, it is not yet possible to indicate the relation between these results and the results of our rheological measurements. More studies will be conducted to elucidate the effect of lipase on the polar and non-polar lipids in dough, as well as the composition of the lipid fragments in the free lipid fraction will be studied.

	Wheat flour	Wheat flour dough	Dough with lipase
Free lipid %	1,22	0,52	0,57

Table 2, Free lipid in flour and dough with/without lipase.

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Conclusion

Addition of lipase results in a significantly improved breadmaking quality in terms of larger volume, improved crumb structure and whiter crumb colour. The combination of lipase with pentosanase or xylanase can improve the dough consistence and result in a bread volume increase which is not possible to achieve when either enzymes is used alone. The effect of lipase on crumb softness during storage is probably due to its effect on bread volume increase and crumb structure, rather than the anti-staling effect of monoglycerides produced in situ. The effect of lipase on breadmaking may be because of its ability to improve the rheological properties of the gluten net-work, i.e. it improves the gluten strength and elasticity due to formation of extra crosslinks. The free lipid content in the lipase treated dough is approx. 10% higher than the control. These extra free lipids released by lipase may influence the formation of gluten complex during dough mixing. More studies will be done to study the effect of lipase on polar and non-polar lipids and the composition of the lipid in the free lipid fraction.

References

- 1) JP patent, JP-A-62-285749 (published 1987-12-11)
- 2) Carr, N.O. et al. 1992. Lipid interactions in Breadmaking. *Critical Reviews in Food Science and Nutrition* 31(3):237.
- 3) Bekes, F. et al, 1986. Relationship between lipid content and composition and loaf volume of twenty-six common spring wheats. *Cereal Chem.* 63(4):327.
- 4) Hoseney et al, 1970. Functional (breadmaking) and biochemical properties of wheat flour components. VI. Gliadin-lipid-glutenin interaction in wheat gluten. *Cereal Chem.* 47:135.
- 5) Buchyk, W, 1984. Carbohydrate and lipid complexes with gliadin and glutenin. Contribution No. 675. Supported by a grant from the Natural Sci. and Eng. research council of Canada.
- 6) Bekes, F. et al, 1992. Lipid mediated aggregates in flour and in gluten. *J. of Cereal Sci.* 16:129.
- 7) Weegels & Hamer, 1992. Improving the bread making quality of gluten. *Cereal Food World* 37:379.
- 8) Hille, J. Nov. 1993. The use of enzymes in breadmaking to replace emulsifiers. *Jornadas de Aplicaciones Industriales de las Enzimas, Barcelona*
- 9) Fearn, T and Russell, P.I. 1982. A kinetic study of bread staling by DSC. The effect of loaf specific volume. *J.Sci. Food Agric.* 33:537
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- 11) Mita, T. 1983. Shear stress relaxation of chemically modified gluten. *Cereal Chem.* 60:93
- 12) Hoseney et al, 1969. Functional (breadmaking) and biochemical properties of wheat flour components. V. Role of total extractable lipids. *Cereal Chem.* 46:606

$$T_{\mu\nu} = \frac{1}{2} \left(\frac{\partial \phi}{\partial x^\mu} \frac{\partial \phi}{\partial x^\nu} - \frac{1}{2} g_{\mu\nu} \left(\frac{\partial \phi}{\partial x^\alpha} \frac{\partial \phi}{\partial x^\alpha} \right) \right)$$

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Pages 000051 - 000054 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

Appendix 8

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Pages 000056 - 000061 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

SUBMISSION END

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Reference List for Industry Submission, GRN 000043

<i>Pages</i>	<i>Author</i>	<i>Title</i>	<i>Publish Date</i>	<i>Publisher</i>	<i>BIB_Info</i>
000051 - 000054	Barbesgaard, Peder; Heldt-Hansen, Hans Peter; Diderichsen, Berge	On the safety of Aspergillus oryzae: a review	1992	Applied Microbiology Biotechnology	Volume 36, pgs 569-572
000056 - 000061	Greenough, R.J.; Perry, C.J.; Stavnsbjerg, M.	Safety Evaluation of a Lipase Expressed in Aspergillus oryzae	1996	Fd. Chem. Toxic.	Volume 34, Number 2, pgs 161-166

NA- Not applicable



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Via FedEx

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Food and Drug Administration
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Re: Lipase (subject of GRAS Notice No. GRN 43) for use in brewing

Dear Dr. Cheeseman:

As discussed recently by phone, my client submitted GRAS Notice No. GRN 43 for a lipase preparation produced by *Aspergillus oryzae* and expressing the gene encoding a lipase from *Thermomyces lanuginosus*. GRAS Notice No. GRN 43 covers the use of the lipase preparation in baking and in fats and oils applications. My client has now extended the use to include use as a processing aid in brewing.

As you are aware, use in brewing requires an additional approval by the Alcohol and Tobacco Tax and Trade Bureau (TTB), and TTB has historically looked to FDA to acknowledge the safety of food substances. Based on the information in GRAS Notice No. GRN 43, including a published safety article (Attachment 1), as well as an evaluation of the brewing application and calculation of human exposure and a safety margin, my client has determined that this lipase preparation is generally recognized as safe for use in the brewing application.

Since the lipase preparation is subject to GRAS Notice No. GRN 43 and the extension of the use to brewing applications does not change the safety profile of the enzyme based on human exposure to the enzyme preparation, we believe that filing an additional GRAS notification would be duplicative. We are requesting a letter from your office to confirm our understanding that an additional GRAS notification need not be filed for the extension of the intended use to the brewing industry.

We are including the following information on the safety of the lipase preparation for use in the brewing industry.

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Enzyme Identity

The enzyme is a triacylglycerol lipase (IUB No. 3.1.1.3) that has specificity toward the 1, 3 position ester bonds in triglycerides. It is a food grade enzyme and complies with the Food Chemicals Codex and FAO/WHO JECFA recommended purity specifications for food grade enzymes. As mentioned earlier, the lipase preparation is the subject of GRAS Notice No. GRN 43.

The lipase preparation is used in a mixture that blends six enzymes. The commercial name of the enzyme mixture is Onda Pro and is intended for use in the brewing industry. All of the enzymes in Onda Pro, except the lipase, are already included in the list of enzymes approved for use in brewing in the Adjunct Reference Manual.

Enzyme Application

The lipase makes it possible to make a standard fermentable wort from 100% barley under normal brewing conditions. The application and benefits of the lipase are described in the attached Product Application Sheet (Attachment 2). The lipase is an important part of the enzyme mixture as the lipase is used to increase the free fatty acid profile in wort from 100% barley to the same level as in wort from 100% malt. Lipase in 100% barley brewing improves filtration speed and reduces haze in the wort, thereby helping to deliver the desired clear wort.

Safety of the Enzyme

The safety of the enzyme is presented in GRAS Notice No. GRN 43. Included in GRAS Notice No. GRN 43 is a published safety article on the lipase preparation (Greenough, R. J., Perry, C. J. and Stavnsbjerg, M. Safety Evaluation of a Lipase Expressed in *Aspergillus oryzae*. Food Chem. Toxic. 34 (2):161-166, 1996.). We have included a copy of the article with this letter (Attachment 1).

Estimate of Human Consumption and Safety Margin

The lipase enzyme is added via the enzyme blend during the mashing process and will be completely inactivated during wort boiling. In addition, the enzyme protein should be mostly removed during filtration and further processing of the beer. Based on our knowledge of the brewing process, we would expect that the final beer product would only have negligible levels of enzyme residue.

However, in order to illustrate the maximum potential intake, the following calculations were made assuming that all enzyme activity is retained in the final beer; that all of the total organic

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solids (TOS) remain in the final beer; and that all beers are produced using the lipase preparation as a processing aid at the recommended dosage.

The table below shows estimated consumption of the lipase based on the recommended dosage of the lipase for a 100% barley brewing process and the U.S. per capita consumption of beer in 2001 of 81.76 liters/year:

100 % Barley process Using 2.5 kg Ondea Pro (contains 45,500,000 Lipase Units (LU))								
1 ton of barley gives	5550	L Beer	containing	45,500,000	LU	corresponding to	8198	LU/L Beer
Consumption per day	0.224	L Beer	containing	8198	LU/L Beer	corresponding to	1836	LU/day
For a person weighing	60	Kg				corresponding to	30.60	LU/Kg bw/day

The recommended dosage of Ondea Pro is 1.2-2.5¹ kg per metric ton of barley to produce 5550 liters of beer.

In the US in 2001, the per capita consumption of beer was 81.76 liters according to the 2003 ERS/USDA Data: Food consumption (per capita) data system.

Based on the information about the test substance in the safety article:

1 gram lipase contains 208 KLU and 270 mg TOS

giving:

1 KLU = 270/208 = 1.298 mg TOS

1 mg TOS = 208/270 = 0.770 KLU = 770 LU

From the table above we have 30.60 LU/Kg bw/day

¹ The attached Product Application Sheet (Attachment 2) indicates that the recommended dosage of Ondea Pro is 1.2-2.5 kg per metric ton of barley. It also recommends using a dosage of 2 kg Ondea Pro per metric ton of barley for specification trials with gradual lowering of the dosage for optimal performance. Although our client has not seen any brewers using Ondea Pro in the upper end of the dosage range, such use is still theoretically possible. Thus, we use the 2.5 kg Ondea Pro per metric ton of barley figure in our safety calculations to estimate maximum potential intake.

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$$30.60 \text{ LU} = (1.298 \text{ mg TOS}) / (1000) * (30.60) = .0397188 \text{ mg TOS} = \text{EDI}$$

$$\text{Safety margin} = \text{NOEL} / \text{EDI} = 1.351 * 1000 / 0.0397188 = \mathbf{34,014}$$

Note, in GRAS Notice No. GRN 43, it is estimated that the safety margin for the use in fats and oils is 23,000 and the safety margin for the use in baking is 270,000. The use in brewing falls between the two, and in all cases, the safety margin is very high.

Conclusion

Based on the information contained in this letter as well as the information in GRAS Notice No. GRN 43, our client has determined that the use of this lipase preparation in brewing is Generally Recognized as Safe. Since the lipase preparation is subject to GRAS Notice No. GRN 43 and the use in brewing does not change the safety profile of the enzyme, we believe that filing an additional GRAS notification would be duplicative. We would appreciate your confirmation of our understanding.

Sincerely,

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Gary L. Yingling

Attachments

cc: Novozymes

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Pages 000084-000089 have been removed in accordance with copyright laws. The removed reference is:

Greenough RJ1, Perry CJ, Stavnsbjerg M., "Safety evaluation of a lipase expressed in *Aspergillus oryzae*" Food Chem Toxicol. 1996 Feb;34(2):161-6.

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Brewing

Application Sheet

Barley Beer Solutions

Barley has always been the main raw material for making beer. To convert the barley grain into fermentable wort, enzymes are needed, for example, to break down cell walls and to release the amino acids and simple sugars that encourage yeast growth.

Novozymes Ondea® Pro makes it possible to make a standard fermentable wort from 100% barley under normal brewing conditions.

Worts made from 100% barley using Novozymes Ondea Pro can be used as base for creating any type of great tasting beer.

Benefits

- Maximize raw material cost savings
 - Higher than usual extract yield
 - Enables the use of non-malting barley
 - Efficient wort and beer filtration
 - Control of FAN and fermentability
 - Improved foam stability
 - Works with standard equipment and standard mashing time
 - Non, or very few, investments needed
 - Decoction with a high percentage of adjunct possible
 - High Gravity Brewing is possible (up to 26°Plato of first wort)
 - Produces high quality final beer
- Reduce carbon foot print
 - In some case, CO₂ emissions are reduced by 8% .
 - Save up to 3 kg CO₂ per hl of beer

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- Increase the platform for new branding
 - A beer based on 100% barley
 - Flexibility to match the current beer profile

Product

Novozymes Ondea Pro secures efficient viscosity reduction, turbidity reduction, protein degradation and attenuation control. The enzyme works in synergy with the barley's own enzymes to produce fermentable worts.

Raw material quality

Synergy between Novozymes Ondea Pro, the barley proteases and barley β -amylase is essential, meaning that the presence of natural barley enzymes is a must to achieve a successful brewing process and barley grains with active enzymes are required.

Novozymes has developed a method for gauging the suitability of barley for a 100% barley beer production: "Barley evaluation test for 100% barley beer" see Appendix 1

With Novozymes Ondea Pro, the extract yield from barley is the same as the extract yield from malt on a dry basis.

Milling

Milling barley is significantly harder than malt. Barley has a higher initial water content (approximately 12-14%), so in order to ensure effective performance, the mill needs a higher power uptake.

For use with a Mash Filter, a standard hammer milling procedure (with adjusted power uptake) or any comparable system, such as a dispax, is sufficient to secure perfect mash filtration. When cleaning the mash filter, spent grain fall from the filter sheet most efficiently when the dry matter load was comparable to malt.

When using a Lauter Tun, the milling and the resulting grist composition is critical for a good lauter performance. Preservation of the husk is of great importance in order to effectively build up the filter bed.

The best experience is achieved with a grist composition in the following range:

Husk	25-30 %
Sieve 2	15-20 %
Sieve 3	30-40 %
Sieve 4	10-15 %
Sieve 5	2-5 %
Bottom	8-12 %

Method: MEBAK, Volume 2, Test No 1.1.1

These figures can mainly be reached with a six roller mill. With a four roller mill the grist will remain coarser (45% husk fraction) and a two roller mill leads to very coarse grist (flakes). All coarse grist compositions can be processed, but it has to be considered that mashing time and/or the enzyme dosage has to be increased when using coarse grist.

From experience, the following table describes possible roller clearances to obtain best grist composition:

Roller	Mill Clearance, mm								
	Dry			Conditioned			Wet		
Two							0.3-0.4		
Four	1-1.2	0.3-0.45					1.0-1.2	0.3-0.4	
Six				1.60	0.80	0.3-0.40			

Performance

Novozymes Ondea Pro ensures the enzymatic breakdown of the barley grain components, resulting in high-quality standard wort. (Figure 1).

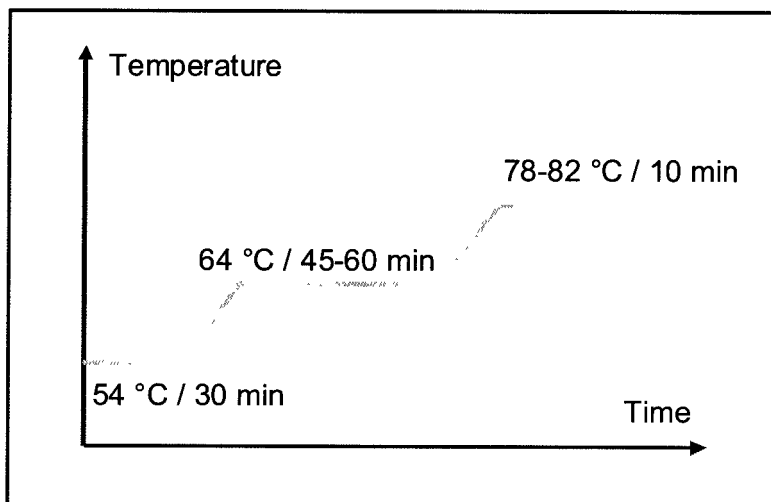


Fig 1 Simple infusion mashing for a 70% RDF target within 2 hours using a dosage of 2 kg Ondea® Pro per ton barley

- Mashing temperature:** The first temperature of 54 °C is essential. The synergy between the heat sensitive proteases of the barley and the Novozymes Ondea Pro protease can fail if the initial temperature is overshoot by incorrect heating units or temperature measurement. Ensure that the highest temperature at mashing in and throughout the first 30 minutes is not greater than 54 °C, preferably staying closer to 52 °C than risking rising to 56 °C or more. Ensure that the remaining mashing time is stable at 64 °C to support the performance of the heat sensitive barley β -amylase.

- Mashing pH: The mashing pH should not be adjusted. The natural mashing pH of barley is normally > 5.7. The lowest acceptable pH is 5.6. If the pH is higher than 6.1, use a moderate acidification with your choice of acid.
- Mashing time: The dosage and saccharification time can be optimized to fit a prevailing production slot.
- Mash agitation: Continuous stirring is required during the entire mashing time to prevent the premature settling of the grist and secure the effective breakdown of the enzyme.
- Iodine test: At the end of saccharification, the iodine test may be positive. Even at the end of the mashing-off, the wort may not be completely iodine negative. There is no need to extend mashing-off as the starch degrading enzymes are still active during wort separation. The final wort will always be iodine negative.
- Wort pH: Contrary to the mash, the wort has to be acidified to a pH of 5.2 during boiling to support the pH drop during fermentation to a beer pH of 4.5.
- Hopping: Experience with 100% barley beer shows that 10-20 less hopping leads to the same bitterness perception found by taste panels.

Wort quality

The combination of the mashing profile shown above and 2 kg of Novozymes Ondea Pro per ton of barley is designed to produce a wort of standard quality to be used in the production of a pilsner type beer with the lowest possible viscosity due to the effective break down of β -glucan and arabinoxylans.

- FAN requirement: Novozymes Ondea Pro's proteolytic component works in synergy with the endogenous enzymes in the barley:

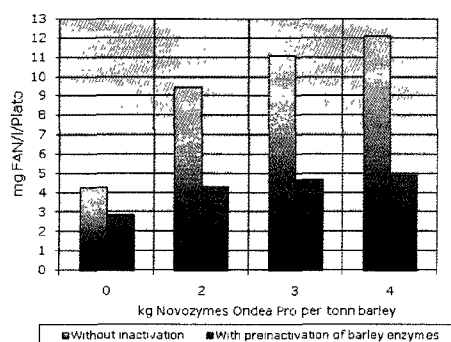


Fig 2 Synergism between Novozymes Ondea Pro and barley proteases

- In addition the FAN from barley has a much higher value for the yeast fermentation so barley wort needs only 9-10 mg FAN/ °P/l to secure good fermentation.
- Sugar profile and fermentability: The recommended mashing profile (Fig 1) and 2 kg Novozymes Ondea Pro per ton barley will result in a wort with an RDF of about 70% and a sugar profile with a higher maltose value and a lower glucose value compared with malt.

Sugar profile

Glucose	4.0%
Fructose	1.8%
Maltose	62.6%
Maltotriose	14.9%
Dextrins (DP4+)	16.8%

Fig 3 Typical sugar profile of a 100 % barley wort processed with Ondea Pro

Beer quality

100 % barley beer brewed with Novozymes OndeaPro has successfully been made in industrial scale. Typical composition as a European pilsner type is:

Plato	Alc vol %	EBC haze	EBC color	pH	Gravity	CO2	Niben foam	RDF
10.47	4,9	0,5	5,9	4,48	1,00425	0,5 %	258	69,8

Taste evaluation shows that the beer is of excellent quality (Fig 4)

	100 % barley		100 % malt			100 % barley		100 % malt	
	fresh	forced aged	fresh	forced aged		fresh	forced aged	fresh	forced aged
Aroma	4.3	3.8	4.2	3.6	Aroma	1.0	1.4	1.0	1.8
Taste	3.9	3.7	4.0	3.8	Taste	1.0	1.6	1.0	1.8
Body	4.4	4.3	4.3	4.3	Bitterness	1.0	1.6	1.0	1.7
Carbonation	4.5	4.5	4.5	4.6	Score	1.0	1.5	1.0	1.7
Bitterness	4.6	4.0	4.0	3.4	Acceptance %	100	77	100	70
DLG Score	4.3	4.0	4.1	3.9					

Fig 4 DLG (= Deutsche Landwirtschaft Gesellschaft) Weihenstephan taste evaluation. 5 = Very good, 1 = unacceptable
 Aging conditions: 24 hours shaking (100rpm) at 20 deg C and 4 days storage at 40 degC. 1= fresh, 4=extremely aged

- Filtration and stabilization: The process needs to be optimized due to the change in raw materials. Good results have been witnessed with 40 gram of silica and 15 gram of PVPP per hl. This can be optimized further.

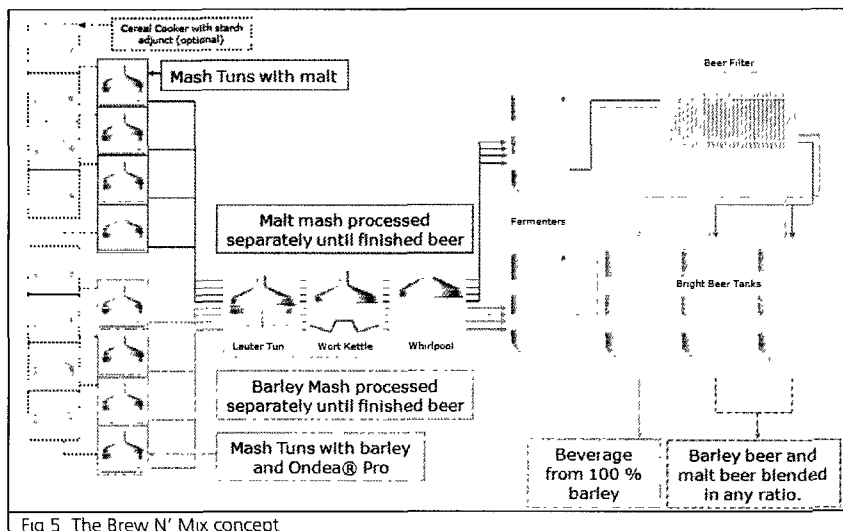
Usage

Novozymes Ondea Pro produces high quality, great tasting beer from 100% barley. The beer has no off-flavors and can be made with standard brewing equipment using standard mashing profile and time.

This opens up the possibility to use this concept for any beer type.

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Novozymes Ondea Pro is designed to work in synergy with the enzymes within barley. Only when used with 100% barley are the benefits of easy processing and high quality final beer achieved. It is not recommended to use Novozymes Ondea Pro on a blend of barley and malt. Blending should take place after wort boiling and preferably after fermentation (Figure 5). The blending of 100% barley beer and other beers can be made in any ratio.



Novozymes Ondea Pro is very well suited for production of flavoured beers or any type of non-alcoholic grain based beverage, as well as for production of syrups

Novozymes Ondea Pro is recommended to be dosed at the start of mashing (when 30 % of the grits has been mashed in) at a dosage of 1.2 – 2.5 kg per ton of raw barley depending on mashing regime.

As a liquid with a viscosity of 12 cP the product is ideally suited for automatic dosing equipment.

Optimization of saccharification time and dosage is possible to achieve the desired wort profile and attenuation degree.

Labelling

Novozymes Ondea Pro is inactivated during wort boiling so no relevant functionality is observed in the final beer. No labelling is required on the final beer.

Detailed application information

For more information on the use of Novozymes Ondea Pro, please refer to www.ondeabrewing.com or contact Novozymes Customer Solution experts.

Safety, handling and storage

Safety, handling and storage guidelines are provided with all products.

Appendix 1

"Barley evaluation test for 100% barley beer"

Test barley for suitability for the 100% Barley Beer application and the possible laboratory optimization of mashing

The test and optimization work can be done with the same equipment as used to test malt quality: Method EBC 4 5.1 (Extract of malt: **Congress mash**)

Test program:

The barley is milled (GAP 0.2) and weighed (2 x 62.5 g and 1 x 80 g) into 3 mashing cups, with 200 g water (56°C) added to each cup.

The water used can be either brewing water or distilled water + 3.0 ml Ca++ solution (11 g CaCl₂, 2H₂O /500 ml). Place the stirrer and cup in the mashing equipment.

Ondea Pro is added at a dosage of 2 kg per 1000 kg barley: Cup 1 (0.125 g/62.5 g barley) Cup 2 0.187g/62,5 barley) and Cup 3 : 0,240 g/ 80 g barley and the mashing is started.

Mashing diagram: 54°C for 30 minutes, increase (1 °C/minute) to 64°C and maintain for 45 minutes, increase (1 °C/minute) to 80°C and maintain for 10 minutes, cool to 20°C.

All cups receive additional water until it reaches a total of 300.0 g before filtration.

Filtration occurs as described in the EBC Congress mashing test.

(Funnels, 200 mm diameter, fluted filter papers, 320 mm diameter. Schleicher and Schullno. 597 ½, Macherey-Nagel & Co.No. 6141/4, F.C. Binzed No. 12 and 53 or equivalent)

The filtration is recorded, the filtrate wort analyzed and the results recorded.

Results:

	Test conditions		Filtration ml		Wort analysis						
	Ratio Barley/ water	Ondea Pro1 Dosage Kg/Mt	10 minutes	final	Haze NTU	pH	Plato	Maltose %	Glucose %	Dextrin %	FAN ppm
Cup1	1:3.2	2									
Cup2	1 3.2	3									
Cup3	1.2.5	3									

Specification for suitable barley:

Filtration (ratio 1:3.2): 10 minutes > 90 ml, final > 180 ml

Filtration (ratio 1:2.5): 10 minutes > 55 ml, final > 150 ml

Haze (NTU): < 40 NTU

pH: 5.6 - 6.1

Dextrin%: 19-21% = 69-71 RDF%

FAN: >120ppm

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The optimization of mashing to reach the target of the wort/beer or/and adjustment to the barley quality is achieved by changing the mashing diagram

Saccharification (RDF%) is achieved by extending the 64°C rest.

Dextrin% is a direct correlation to RDF% of the beer. If a higher RDF% is required, the 64°C rest is extended to 60 or 75 minutes.

FAN, and to some degree filtration, depends on the 54°C rest.

If more ppm FAN is needed, the 54°C rest is extended to 45 or 60 minutes.

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Novozymes is the world leader in bioinnovation. Together with customers across a broad array of industries we create tomorrow's industrial biosolutions, improving our customers' business and the use of our planet's resources. Read more at www.novozymes.com.

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**West-Barnette, Shayla**

From: LOBG (Lori Gregg) [LOBG@novozymes.com]**Sent:** Thursday, January 21, 2010 10:52 AM**To:** West-Barnette, Shayla**Cc:** Yingling, Gary L.

Dear Shayla,

As discussed in our telephone conversation last week and requested in your email, I have been looking for current data for beer consumption so that I can recalculate the safety margin. I was able to find more recent data from ERS/USDA but it is still per capita data and actually as I read it now it is "Food Availability (per capita) Data", however, they say that this type of data "typically overstates actual consumption or intake".

From www.ers.usda.gov/data/foodconsumption I can see that in 2007 the per capita consumption is 21.8 gallons. In my calculations I had used 21.6 (from 2001) which is only slightly different. I have some information from "Beer Marketers Insight" which says that in 2008 the per capita consumption of beer was 21.7 (which is also close). I do have information from a website called www.beerinfo.com that shows that the per capita consumption varies by state with the highest consumption in Nevada at 44.0 gallons per year.

I have not been able to find data that is not "per capita". I'm wondering if you can give me some guidance on where to find the type of information you suggest that I should use. In the list of questions it says "based on per person calculation". I have not been able to find this type of data.

Please let me know what you suggest or if I should use the 21.8 gallons from USDA's data tables from 2007.

Best regards, Lori

Lori Gregg

Sr.Regulatory Specialist

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FEB 17 2010

January 27, 2010

BY: (b)(6)

novozymes®

Rethink Tomorrow

Shayla West-Barnette
Consumer Safety Officer
Office of Food Additive Safety
FDA, CFSAN
Shayla.westbarnette@fda.hhs.gov

RE: Additional correspondence on GRAS Notice No. 43; lipase for use in brewing

Dear Ms. West-Barnette,

Novozymes enzyme preparation Ondea Pro is a blend of six different enzymes for use in the brewing industry. All of the enzymes in Ondea Pro, except the lipase, are included in the list of enzymes approved for use in brewing in the Adjunct Reference Manual. The lipase is the same lipase that is the subject of GRAS notification No. 43. GRN 43 describes the use of the lipase in baking and fats and oils applications. Novozymes' has determined that this lipase is generally recognized as safe for use in brewing.

The commercial product for use in brewing, Ondea Pro, contains approximately 10% TOS from the six different enzyme concentrates. However, we are focusing only on the lipase portion of the commercial product, Ondea Pro. Ondea Pro contains approximately 0.4% lipase TOS.

We are providing the following information in response to your questions regarding the use of the lipase described in GRAS notice No. 43 in brewing.

1) Please provide the Total Organic Solids (TOS) content and the lipase activity for the preparation intended to be used in beer manufacturing.

The preparation intended to be used in brewing is Ondea Pro. Ondea Pro contains 18.2 KLU (kilo lipase units) per gram and 0.4% lipase TOS. (The total TOS in the commercial product from all enzymes is 10%)

2) Please provide the TOS content of the lipase batch used in the toxicology studies.

The toxbatch used in the safety studies (described in Greenough et al, 1996) contains 27% TOS. The test batch used for the safety studies was produced in a similar manner as described in the manufacturing section of the GRAS notification No. 43 except that standardization and stabilization have been omitted.

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3) Please provide the use level of lipase based on TOS.

The recommended dosage of Ondea Pro in brewing is 1.2-2.5 kg Ondea Pro per metric ton of barley to produce 5550 liters of beer. We use 2.5 kg per ton barley in the calculations to estimate the maximum potential intake.

$$2500 \text{ g Ondea Pro} \times .004 \text{ g lipase TOS} = 10 \text{ g lipase TOS per 1000 kg barley}$$

4) Please revisit the exposure calculations.

We realized that in our original letter dated November 20, 2009, we essentially did the exposure calculations based on activity. Below, we have redone the calculations based on TOS from the commercial product and using current data for beer consumption (users only not "per capita").

For your convenience, information provided in our letter of November 20, 2009 is included again here for reference for the following calculations.

The lipase enzyme is added via the enzyme blend during the mashing process and will be completely inactivated during wort boiling. In addition, the enzyme protein should be mostly removed during filtration and further processing of the beer. Based on our knowledge of the brewing process, we would expect that the final beer product would only have negligible levels of enzyme residue. However, in order to illustrate the maximum potential intake, the following calculations were made assuming that all enzyme activity is retained in the final beer; that all of the total organic solids remain in the final beer; and that all beers are produced using the lipase preparation as a processing aid at the recommended dosage.

As noted above the maximum recommended dosage is 2.5 kg Ondea Pro per ton (1000 kg) barley which yields 5,550 liters of beer.

$$10 \text{ g lipase TOS per 1000 kg barley} = 0.01 \text{ g lipase TOS per kg barley}$$

$$0.01 \text{ g lipase TOS} \times 1000 \text{ kg barley} / 5550 \text{ l beer} = 0.0018 \text{ g TOS per liter beer}$$

$$0.0018 \text{ g TOS per liter beer} \times 1000 = 1.8 \text{ mg TOS per liter beer}$$

From consumption data provided by FDA from the Foods Analysis and Residue Evaluation Program, we used 726.9 g beer consumed per day by actual beer drinkers.

$$1.8 \text{ mg TOS per liter beer} \times .7269 \text{ liters beer per day} = 1.3 \text{ mg TOS per day}$$

For a person weighing 60 kg, that is 1.3/60 or 0.022 mg TOS/kgbw/day

The dose level in the 13 week oral toxicity study in rats which could be given without any toxic effects was 1.35 g TOS/kgbw/day which is the NOAEL.

$$\text{Safety margin} = \text{NOAEL/EDI} = 1350 / 0.022 = \mathbf{61,363}$$

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5) Please use current data for consumption (based on per person calculation)

In the calculations above, we used updated consumption data provided by FDA from the Foods Analysis and Residue Evaluation Program.

6) Please provide a statement about the current use of lipase

The lipase described in GRAS notification No. 43 is currently used in fats and oils, baking, and brewing applications. In the fats and oils industry it is used for interesterification which allows for the production of fats with the desired melting properties but without trans fatty acids. In baking it is used to improve the properties of the dough. In brewing, the lipase improves filtration speed and reduces haze in the wort made from 100% barley.

If you have any questions or need additional information, please do not hesitate to contact me at 919-494-3151 or lobg@novozymes.com.

Sincerely,

(b)(6)

Lori Gregg
Sr. Regulatory Specialist

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