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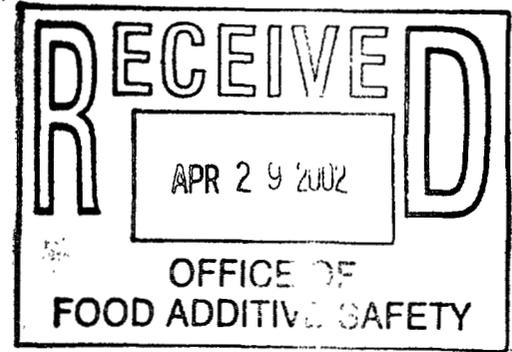
Original Submission

000002



April 24, 2002

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



Dear Dr. Kahl,

We are hereby submitting, in triplicate, a generally recognized as safe (GRAS) notification, in accordance with proposed 21 C.F.R. § 170.36, for Novozymes' glucose oxidase enzyme preparation produced by *Aspergillus oryzae* expressing the gene encoding a glucose oxidase from *Aspergillus niger*. The glucose oxidase enzyme preparation is intended for use mainly in 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,

A rectangular box with a red border, used to redact the signature of the sender.

Lori Gregg  
Regulatory Specialist

Enclosures (3 binders)

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April 22, 2002



RE: GRAS Notification - Exemption Claim

Dear Sir or Madam:

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

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

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

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

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

Glucose oxidase enzyme preparation from *Aspergillus oryzae* expressing the gene encoding a glucose oxidase from *Aspergillus niger*.

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

The glucose oxidase is intended for use mainly in the baking industry. Other possible uses would be as a processing aid in the manufacture of food such as cheese, beer, carbonated beverages and fruit juice. The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements for normal production following Good Manufacturing Practices.

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

This GRAS determination is based on scientific procedures.

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

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

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[Redacted signature box]

24 April, 2002  
Date

John Carroll  
Director, Regulatory Affairs





**A glucose oxidase preparation produced by  
*Aspergillus oryzae* expressing the gene encoding  
a glucose oxidase from *Aspergillus niger***

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

April 2002

**000005**

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## 1. GENERAL INTRODUCTION

The subject of this notification is a glucose oxidase preparation (Novozymes A/S trade name Gluzyme<sup>®</sup> Mono BG) produced by submerged fermentation of an *Aspergillus oryzae* microorganism carrying the gene coding for glucose oxidase from *Aspergillus niger*.

This glucose oxidase preparation performs in the same way as glucose oxidase from *Aspergillus niger* which has not been genetically modified. Glucose oxidase is used in the food industry mainly in the baking industry as a processing aid to strengthen gluten in dough systems. It causes the oxidation of free sulfhydryl units in gluten protein, whereby disulfide linkages are formed. This results in stronger, more elastic doughs with greater resistance to mechanical shock, as well as better oven spring and larger loaf volume.

Other possible uses would be as a processing aid in the manufacture of food such as cheese, beer, carbonated beverages and fruit juice.

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

The safety of the production organism must be the prime consideration in assessing the probable degree of safety of an enzyme preparation intended for use in food<sup>1,2</sup>. The production organism for this glucose oxidase, *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 material<sup>3-8</sup>. The genetic modifications used to construct the production microorganism are well defined and are described in Section 2. The safety studies performed and described in Section 7 show no evidence to indicate that any of the cloned DNA sequences and incorporated DNA encode or express a harmful or toxic substance.

## 2. PRODUCTION MICROORGANISM

### 2.1 Production Strain

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The *A. oryzae* strain, designated Mt1-72, was constructed by transformation of the recipient strain, designated BECh 2 (see Section 2.2), with a purified DNA fragment. This genetically modified production organism complies with the OECD (Organization for Economic Co-operation and Development) criteria for GILSP (Good Industrial Large Scale Practice) microorganisms<sup>9</sup>. It also meets the criteria for a safe

production microorganism as described by Pariza and Foster and several expert groups<sup>3-8</sup>.

The DNA fragment used in the strain construction contains strictly defined fungal chromosomal DNA fragments and synthetic DNA linker sequences. The specific DNA sequences include: a gene encoding an *A. niger* glucose oxidase enzyme; an *Aspergillus nidulans* selectable marker gene, *amdS* (acetamidase)<sup>10</sup>; well-characterized noncoding regulatory sequences including the *A. niger* terminator<sup>11</sup>, the *A. niger* neutral amylase II (NA2) promoter<sup>12</sup>, the 5' nontranslated leader of the *A. nidulans* triose phosphate isomerase gene<sup>13</sup>; and a 48 bp fragment from a known sequence from *Escherichia coli* plasmid pUC19<sup>14</sup> originating from the cloning constructions.

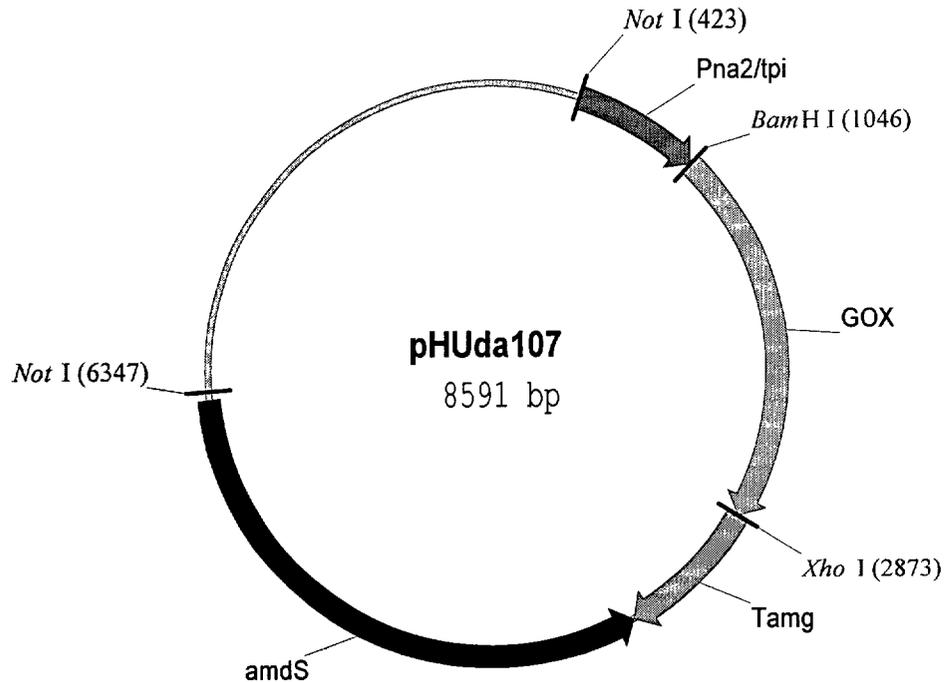
## 2.2 Recipient Organism

The recipient microorganism, designated BECh 2, used in the construction of the glucose oxidase production strain is an amylase negative, alkaline protease (alp) negative, neutral metalloprotease I (Npl) negative, cyclopiazonic acid deficient, kojic acid deficient derivative of the fully characterized, well-known industrial production strain of *A. oryzae* (Ahlburg) Cohn. The strain was obtained from the Institute for Fermentation, Osaka, Japan (IFO) and is designated strain IFO 4177 (synonym A1560). This classification of A1560 as *A. oryzae* has been confirmed by the Centraalbureau voor Schimmelcultures, Baarn, Holland<sup>15</sup>.

## 2.3 Glucose Oxidase Expression Fragment

The plasmid pHUda107 was digested with NotI as indicated in the map below, and the 5925 bp fragment containing the glucose oxidase gene was isolated.

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This purified fragment was used to transform the *A. oryzae* host strain, BECh 2, and contains the following genetic material:

<u>Size (bp)</u>	<u>Element</u>	<u>Origin</u>
616	Pna2/TPI	<i>A. niger</i> BO1
9	Linker	Synthetic
1818	GOX	<i>A. niger</i> 1-3-2
11	Linker	Synthetic
698	Tamg	<i>A. niger</i> BO1
2725	amdS	<i>A. nidulans</i>
48	pUC19	<i>E. coli</i>

Pna2/tpi is the neutral amylase II promoter from *A. niger*. The 5' nontranslated part of this promoter has been replaced with the 5' nontranslated part of the *A. nidulans* triose phosphate isomerase (TPI) promoter.

GOX is the *A. niger* glucose oxidase gene.

Tamg is the amyloglycosidase terminator of *A. niger*.

amdS is the acetamidase gene (including promoter and terminator) from *A. nidulans*.

pUC19 is a fragment of the pUC19 vector including the origin of replication.

## 2.4 Stability of the Introduced Genetic Sequences

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The presence of the introduced DNA sequences was determined by Southern hybridization to assess the stability and potential for transfer of genetic material as a component of the safety evaluation of the production microorganism<sup>3-8</sup>. The transforming DNA is stably integrated into the *A. oryzae* chromosome and, as such, is poorly mobilizable for genetic transfer to other organisms and is mitotically stable<sup>9</sup>.

## 2.5 Antibiotic Resistance Gene

The transforming DNA does not contain antibiotic resistance genes.

## 2.6 Absence of Production Organism in Product

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

# 3. MANUFACTURING PROCESS

This section describes the manufacturing process for the glucose oxidase which follows standard industry practices<sup>16-18</sup>. The quality management system used in the manufacturing process for the glucose oxidase complies with the requirements of ISO 9001. It is also manufactured in accordance with current good manufacturing practices.

## 3.1 Raw Materials

The raw materials used in the fermentation and recovery process for the glucose oxidase enzyme concentrate are standard ingredients used in the enzyme industry<sup>16-18</sup>. The raw materials conform to Food Chemicals Codex specifications except those raw materials which do not appear in the FCC. For those not appearing in the FCC, internal specifications have been made in line with FCC requirements. On arrival at Novozymes A/S, the raw materials are sampled by the Quality Control Department and subjected to the appropriate analyses to ensure their conformance to specifications.

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

## 3.2 Fermentation Process

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The glucose oxidase is manufactured by submerged fed-batch pure culture fermentation of the genetically modified strain of *A. oryzae* described in Section 2. All equipment is carefully designed, constructed, operated, cleaned, and maintained so as to prevent contamination by foreign microorganisms. During all steps of fermentation, physical and chemical control measures are taken and microbiological

analyses are done to ensure absence of foreign microorganisms and confirm strain identity.

### 3.2.1 Production Organism

Each batch of the fermentation process is initiated with a lyophilized stock culture of the production organism, *A. oryzae*, described in section 2. Each new batch of the stock culture is thoroughly controlled for identity, absence of foreign microorganisms, and enzyme-generating ability before use.

### 3.2.2 Criteria for the Rejection of Fermentation Batches

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

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

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

Any contaminated fermentation is rejected.

## 3.3 Recovery Process

The recovery process is a multi-step operation which starts immediately after the fermentation process and consists of both the purification and the formulation processes.

### 3.3.1 Purification Process

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

1. Pretreatment - pH adjustment
2. Primary Separation - vacuum drum filtration
3. Concentration - ultrafiltration and/or evaporation

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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 and/or ultrafiltration if enzyme concentration is too low to reach target yield

### 3.3.2 Formulation and Standardization Processes

The liquid concentrate is spray dried by means of atomization into a fluidized spray dryer. The powder from the primary drying zone is directed into an integrated fluid bed for agglomeration and further drying. The product is discharged continuously after sieving. The product is standardized to the declared enzyme activity by addition of wheat flour.

### 3.4 Quality Control of Finished Product

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

## 4. ENZYME IDENTITY

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

Classification	Glucose oxidase (generic name)
IUB nomenclature:	Glucose oxidase
IUB No.:	1.1.3.4
CAS No.:	9001-37-0
Specificity:	oxidizes glucose to gluconic acid
Amino acid sequence:	the total nucleotide and amino acid sequences have been determined

## 5. COMPOSITION AND SPECIFICATIONS

The glucose oxidase enzyme preparation is presently available in a formula for use in baking applications.

### 5.1 Quantitative Composition

Gluzyme Mono BG has the following typical composition:

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Enzyme solids (TOS)	approx.	23 %
Wheat solids	approx.	39 %
Sodium chloride	approx.	32 %
Water	approx.	6 %

## 5.2 Specifications

The glucose oxidase conforms to the general and additional requirements for enzyme preparations as described in Food Chemicals Codex, 4th edition, 1996<sup>19</sup>. In addition, the glucose oxidase also conforms to the General Specifications for Enzyme Preparations Used in Food Processing as proposed by the Joint FAO/WHO Expert Committee on Food Additives in Compendium of Food Additive Specifications<sup>20</sup>.

The following Novozymes' specifications have been established for the glucose oxidase:

Enzyme 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 $1 \times 10^4$
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
Production organism	negative by test
Mycotoxins	negative by test

Heavy metals, lead, arsenic, antibiotic activity, and mycotoxins are analyzed at regular intervals.

Gluzyme Mono BG has a typical activity of 10000 GODU/g. 1 glucose oxidase unit (GODU) is the amount of enzyme which produces 1  $\mu$ mol hydrogen peroxide per minute under the standard conditions.

## 6. APPLICATION

### 6.1 Mode of Action

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The active enzyme in Gluzyme Mono BG is glucose oxidase, which catalyzes the oxidation of glucose to gluconic acid, oxygen, and water. In baking applications, it

causes the oxidation of free sulfhydryl units in gluten protein, whereby disulfide linkages are formed, resulting in stronger, more elastic dough.

Gluzyme Mono is used in the baking industry as a processing aid to strengthen gluten in dough systems giving improved resistance to mechanical shock, better oven spring and larger loaf volume.

A Product Sheet for this product is not yet available, however further information regarding the general use of glucose oxidase in baking applications is given in the enclosed Product Sheet (Appendix 1).

## 6.2 Use Levels

The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements for normal production following cGMP.

In the baking industry, the recommended dosage of Gluzyme Mono BG is 0.25-5 g per 100 kg flour corresponding to 25-500 GODU/kg flour.

## 6.3 Enzyme Residues in the Final Food

The enzyme is added to the flour or to the liquid and is active during the dough preparation and the leavening of the unbaked bread. During the baking process the high temperatures of the oven cause an inactivation of the enzyme activity.

# 7. SAFETY EVALUATION

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## 7.1 Safety of the Production Strain

The safety of the production organism must be the prime consideration in assessing the probable degree of safety of an enzyme preparation intended for use in food<sup>2</sup>. If the organism is nontoxigenic and nonpathogenic, then it is assumed that food or food ingredients produced from the organism, using current Good Manufacturing Practices, is safe to consume<sup>3</sup>. Pariza and Foster (1983) define a nontoxigenic organism as "one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure" and a nonpathogenic organism as "one that is very unlikely to produce disease under ordinary circumstances". *A. oryzae* meets these criteria for nontoxigenicity and nonpathogenicity. In addition, *A. oryzae* is not considered pathogenic by JECFA<sup>21</sup>.

Barbesgaard et al. reviewed the safety of *A. oryzae* and describe it as having a very long history of safe industrial use, being widely distributed in nature, and being commonly used for production of food grade enzymes<sup>22</sup> (Appendix 2). *A. oryzae* is

accepted as a constituent of foods<sup>21</sup>. *A. oryzae* has been used to produce soy sauce in the United States since before 1958<sup>3,22</sup>. Therefore, *A. oryzae* meets the criterion of “common use in foods in the US before 1958” and can be considered “generally recognized as safe”, GRAS<sup>3</sup>. A GRAS petition, 3G0016, proposing affirmation that enzyme preparations from *A. oryzae* are GRAS for use in food was submitted to FDA and accepted for filing in 1973<sup>23</sup>. Enzyme preparations from *A. oryzae* have been marketed in the US as GRAS by Novozymes and other companies since that time. Carbohydrase and protease enzymes from *A. oryzae* are now the subject of GRAS notice No. 90. Therefore, enzyme preparations from *A. oryzae* are also considered GRAS<sup>2,3,21</sup>.

An evaluation of the genetically modified production microorganism for the glucose oxidase, embodying the concepts initially outlined by Pariza and Foster, 1983 and further developed by IFBC in 1990, the EU SCF in 1991, the OECD in 1992, ILSI Europe Novel Food Task Force in 1996, FAO/WHO in 1996, JECFA in 1998 and Pariza and Johnson, 2001 demonstrates the safety of this genetically modified production microorganism strain. The components of this evaluation: the identity of the host strain, a description of the incorporated DNA fragment, the sources and functions of the introduced genetic material, an outline of the genetic construction of the production strain, and some characteristics of the production strain and the enzyme derived from it are given in Section 2.

Because the genetic modifications are well characterized and specific, and the incorporated DNA does not encode and express any known harmful or toxic substances, the glucose oxidase enzyme preparation derived from the genetically modified *A. oryzae* is considered safe<sup>3,24</sup>. To confirm the safety of the enzyme, safety studies were performed on the enzyme preparation and are described in Section 7.4.

## 7.2 Safety of the Glucose Oxidase Enzyme

Enzyme proteins themselves do not generally raise safety concerns<sup>2,25,26</sup>. As indicated in section 4, this is a glucose oxidase, IUB EC 1.1.3.4, which causes the oxidation of free sulfhydryl units in gluten protein, whereby disulfide linkages are formed.

### 7.2.1 Glucose Oxidases

Fungal glucose oxidases have been reported to be used in food production since 1957<sup>27,28</sup>. Glucose oxidase from *A. niger* is part of the GRAS petition 3G0016 that was submitted to FDA and accepted for filing in 1973. Glucose oxidase from *A. niger* is also the subject of GRN No. 89.

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## 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<sup>5-8,24,29-31</sup>. Essentially all these groups conclude that if a food ingredient is substantially equivalent to an existing food ingredient known to be safe, then no further safety considerations other than those for the existing ingredient are necessary.

In addition, FDA has applied this concept in the determination that several enzyme preparations are safe for use in food<sup>29,32,33</sup>. 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<sup>24,30</sup>.

The glucose oxidase that is the subject of this petition is the same enzyme as glucose oxidase from *A. niger* that has been in use since 1957. The *A. oryzae* production strain contains the gene encoding the glucose oxidase from *A. niger*.

The advantage of using the glucose oxidase produced by the genetically modified *Aspergillus oryzae* compared to glucose oxidase from *Aspergillus niger* that has not been genetically modified is a more efficient production process which leads to increased enzyme yields, higher quality and greater purity of the final enzyme product. In addition, due to the increase in yield, less raw materials are required and there is less waste formation, thereby improving natural resources management, reducing environmental pollution and reducing costs.

The substantial equivalence of Gluzyme Mono BG to Gluzyme BG (Novozymes' glucose oxidase enzyme preparation produced by *A. niger*) is confirmed by enzymatic activity determination and application tests showing similar catalytic properties and functional effects.

## 7.3 Safety of the Manufacturing Process

The glucose oxidase 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 glucose oxidase preparation is produced in accordance with current good manufacturing practices, using ingredients that are acceptable for general use in foods, and under conditions that ensure a controlled fermentation. These methods are based on generally available and accepted methods used for production of microbial enzymes<sup>16-18</sup>.

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## 7.4 Safety studies

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

### 7.4.1 Description of Test Material

The safety studies described below were conducted on a liquid glucose oxidase enzyme concentrate that was prepared according to the description given in Section 3 except that stabilization and standardization were omitted.

### 7.4.2 Studies

The following studies were performed:

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

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

## 7.5 Estimates of Human Consumption and Safety Margin

As stated in section 6.3 the enzyme activity is largely heat inactivated during the baking process, however, in order to illustrate a “worst case” situation, the following calculation is made assuming that all enzyme activity is retained in the baking product.

Gluzyme Mono BG has a typical activity of 10,000 GODU/g and an approximate content of 23% TOS (Total Organic Substances from the fermentation, mainly protein and carbohydrate components).

### 7.5.1 Estimates of human consumption

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

#### United Kingdom:

*The Ministry of Agriculture, Fisheries and Food: 1987 Annual Report of the National Food Survey Committee, Household Food Consumption and Expenditure: Consumption of bread, cakes and bisquits per person per day is 158 g.*

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Denmark:

*"Levnedsmiddelstyrelsen": Development of Food Consumption in Denmark, 1955-1990, Description of the Danish Diet based on food statistics and nutrition calculated data: Consumption of bread, flutes, pita-bread, cakes, and rye bread per person per day is 123 g.*

USA:

Industrial Outlook 1992 (Food Beverages): Consumption of bread and related products per person per day is 109 g.

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

- a) The calculation is made assuming that all TOS remains in the baking product. Gluzyme Mono BG contains 23 % TOS.
- b) It is assumed that all baking products are produced using Gluzyme Mono as a processing aid, used at the highest recommended dosage.

The maximum recommended dosage of Gluzyme Mono BG is 500 GODU per kg flour, corresponding to 5 g of Gluzyme Mono BG per 100 kg flour as described in Section 6.2. Gluzyme Mono BG contains 23 % TOS. Using a standard recipe, 100 kg flour results in 140 kg bread, giving a theoretical content of 8.2 mg TOS/kg bread.

Based on the highest average daily intake of baking products (158 g), the daily intake per person of Gluzyme Mono corresponds to  $10.3 \times 0.158 = 1.3$  mg TOS per day.

For an average person weighing 60 kg this corresponds to  $2.2 \times 10^{-5}$  g TOS/ kg body weight per day.

**Safety margin**

The safety margin is calculated as dose level with no adverse effect (NOAEL) divided by the estimated human consumption. The NOAEL dose level in the 13 weeks oral toxicity study in rats was 10 ml/kg/day corresponding to 1.14 g TOS/kg/day.

However, because of the observed slight decrease in food consumption and associated decrease in body weight/body weight gain in the highest dosed females, a conservative NOEL of 340.6 mg TOS per kg body weight per day is used for the safety margin calculation.

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The estimated human consumption is  $2.2 \times 10^{-5}$  g TOS/kg/day.

The safety margin can thus be calculated to be:  $0.3406 / 2.2 \times 10^{-5} = 1.6 \times 10^4$

## 7.6 Results and Conclusion

The results of the tests described in section 7.4.2 show that the glucose oxidase enzyme preparation does not exhibit any mutagenic activity, clastogenic activity, or toxic effect under the conditions of each specific test. On the basis of the evaluation contained in Sections 7.1-7.5, a review of the published literature, the history of use of *A. oryzae*, and the limited and well defined nature of the genetic modifications, the glucose oxidase enzyme preparation can be safely manufactured and used as a processing aid in the baking industry as well as in other food or non-food applications.

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## 8. LIST OF APPENDICES

1. Novozymes Product Sheet for Gluzyme BG
2. Barbesgaard, Peder, Heldt-Hansen, Hans Peter and Diderichsen, Børge. On the Safety of *Aspergillus oryzae*: a Review, , Appl. Microbiol. Biotechnol., 36:569-572, 1992.
3. Summary of Toxicity Data (File 2002-00377-02)

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2. Pariza, M.W. and Foster, E.M.. Determining the Safety of Enzymes Used in Food Processing. *Journal of Food Protection*, 46:5:453-468, 1983.
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13. McKnight, G.L., O'Hara, P.J., and Parker, M.L.. Nucleotide Sequence of the Triosephosphate Isomerase Gene from *Aspergillus nidulans*: Implications for a Differential Loss of Introns. *Cell* 46: 143-147, 1986.
14. Yanisch-Perron, C., Vieira, J. and Messing, J.. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors, *Gene*, 33:103-119,1985.
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Appendix 1

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# Product Sheet

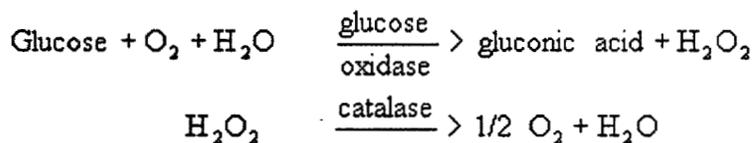
novozymes 

## Gluzyme® BG

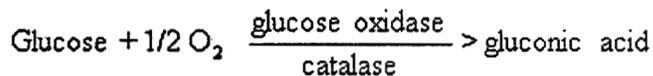
### Description

Gluzyme is a glucose oxidase preparation with catalase side activities, produced by submerged fermentation of a selected strain of *Aspergillus niger* which has not been genetically modified.

The product catalyzes the oxidation of glucose to gluconic acid, oxygen and water:



The overall reaction is:



### Product Properties

#### Product type

Gluzyme is standardized using a special wheat flour with a narrow particle size distribution. Gluzyme is a mix of yellowish/grey, free-flowing, non-dusting, agglomerated granulate and flour. It has an average particle size of 150 microns within the range of 50-212 microns.

#### Activity

Gluzyme is available as:

Gluzyme 2.500 BG .....2500 GODU/g  
 Gluzyme 10.000 BG .....10,000 GODU/g

GODU = Glucose Oxidase Units.

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The product contains a non-standardized amount of catalase activity. The products are standardized with wheat flour by Documented Addition in a strictly ISO-controlled process. See the Analytical Method for further information.

## Solubility

The active components of Gluzyme are readily soluble in water at all concentrations that occur in normal usage. However, water solutions will be turbid due to the wheat flour used for standardization of the enzyme.

## Food-grade Status

Gluzyme BG 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).

## Other Characteristics

Gluzyme is stable in the pH range of 3.5 to 7.0 and up to at least 50°C (122°F); i.e. it can probably be used at up to at least 60°C (140°F).

## Packaging

See the standard [Packaging List](#) for more packaging information.

## Application

Gluzyme can be used to strengthen gluten in dough systems. It causes the oxidation of free sulfhydryl units in gluten protein, whereby disulphide linkages are formed, resulting in stronger, more elastic doughs with greater resistance to mechanical shock, better oven spring and larger loaf volume. Gluzyme is active in the dough, but will be inactivated during baking.

## Reaction Parameters

### Dosage

The recommended dosage of Gluzyme 10.000 BG is within the range of 0.25-5 g per 100 kg flour (i.e. 2-50 ppm, 0.1-2.2 g/cwt), corresponding to 25-500 GODU per kg flour. The optimum dosage of Gluzyme can vary, depending on flour quality, formulation and process, and should be determined through baking trials. In bread-baking procedures using overnight fermentation, overdosing or dosing at higher than the recommended levels may result in off-flavour. For application in non-yeast-raised dough systems a higher dosage, e.g. 100-500 ppm is recommended.

## Safety

Enzymes are proteins. Inhalation of dust or aerosols may induce sensitization and may cause allergic reactions in sensitized individuals. Some enzymes may irritate the skin, eyes and mucous membranes upon prolonged contact. This product has been developed to resist mechanical effects. However, excessive mechanical wear and tear or crushing may create dust.

All spills, however minor, should be removed immediately. Use respiratory protection. Major spills should be carefully shovelled into plastic-lined containers. Minor spills and the remains of major spills should be removed by vacuum cleaning or flushing with water (avoid splashing). Vacuum cleaners and central vacuum systems should be equipped with HEPA filters. Wear suitable protective clothing, gloves and eye/face protection as prescribed on the warning label. Wash contaminated clothes.

## Handling precautions

Gluzyme BG can easily be mixed with flour or starch. Preparing a 1:10 pre-mix can facilitate its use.

A [Material Safety Data Sheet](#) is supplied with all products. See the [Safety Manual](#) for further

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information regarding how to handle the product safely.

## Storage

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

When stored at recommended conditions, the product is best used within 6 months.

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Appendix 2

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Appendix 3

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Toxicology

Date: 15 Jan 2002  
File: 2002-00377-02  
Ref.: SGE/PBjP

## SUMMARY OF TOXICITY DATA

**Gluzyme GMM from *Aspergillus niger*  
expressed in *Aspergillus oryzae***

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## ABSTRACT

Gluzyme™ (Batch No. PPX 7029) is a liquid enzyme concentrate, in which the main activity is glucose oxidase (GOX) activity with a minor catalase side activity. It is produced by submerged fermentation of a strain of *Aspergillus oryzae*, containing the glucose oxidase gene originating from *Aspergillus niger*.

Toxicological tests were undertaken to evaluate the safety of this enzyme preparation with respect to general toxicity, cytotoxicity and mutagenicity. The main conclusions can be summarised as follows :

Oral administration to rats of up to 10 ml/kg body weight/day in males and 3 ml/kg body weight/day in females (equivalent to 1135 and 340 mg Total Organic Solids/kg/day, respectively) for 13 weeks did not revealed any significant toxic effects attributable to the test substance Gluzyme.

Gluzyme has shown no *in vitro* mutagenic activity either at the gene level in bacteria or chromosome level in human lymphocytes.

Gluzyme has shown no cytotoxic activity in the Neutral Red Uptake (NRU) assay applying the mouse fibroblast cell line L929 as test system.

It is overall concluded that Gluzyme can be considered safe for its intended use.

## 1. INTRODUCTION

In order to register Gluzyme for use in food processing, this glucose oxidase preparation was investigated with respect to general toxicity, cytotoxicity and mutagenicity. The results of these studies are summarized in this paper.

The studies were carried out at Inveresk (Eye, Scotland), Covance (Harrogate, North Yorkshire, England), and Novozymes A/S (DK-2880 Bagsværd, Denmark) during the period March 2001 to December 2001.

All studies, except NRU, were carried out in accordance with current guidelines of the Organisation for Economic Cooperation and Development (OECD) and they were conducted in compliance with the principles of Good Laboratory Practice (OECD, as revised in 1997).

## 2. TEST SUBSTANCE

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### 2.1 Characterisation

Gluzyme, batch PPX 7029, which was applied in the present toxicological investigations, was a mixture of four separate fermentation sub-batches. They were fermented and recovered according to the same procedures as are used for the production of commercial Gluzyme preparations. The product complies with the Food and Agriculture Organization/World Health organization (FAO/WHO), Joint Expert Committee on Food Additives (JECFA) and Food Chemical Codex (FCC) recommended purity specifications for food grade enzymes.

The principal enzyme is a glucose oxidase ( $\beta$ -D-Glucose : oxygen -1-oxidoreductase, E.C. 1.1.3.4). Glucose oxidase oxidizes  $\beta$ -D-Glucose in the presence of oxygen to form gluconolactone and hydrogen peroxide. The enzyme activity is measured in an assay based on the oxidation of glucose under constant standard conditions. One glucose oxidase unit (GODUF) is the amount of

enzyme which produces 1 µmol hydrogen peroxide per minute under standard conditions using the FIA method.

The preparation appears as a brown liquid with a specific density of 1.061 g/ml. It is completely miscible with water. The molecular weight is 89 kD and the specific activity is 260 GODU(F)/mg enzyme protein.

This batch has been analyzed for chemical and microbial content; and all the analytical results were within the set limits.

The main enzyme activity was measured to be 4790 GODU(F)/g with an amount of 10.7 (w/w) % Total Organic Solids (TOS = 100% - % water - % ash). The pH was measured to 6.8.

Analyses of the antibiotic activity and microbial contamination showed that this batch complies with the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 1992) and Food Chemical Codex (FCC IV, 1996).

## 2.2 Production micro-organism

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

*A. oryzae* has a long history of safe use and it has been used since the beginning of the last century in the production of enzymes, and in the past decades as recombinant organism for production of a variety of bio-industrial products.

*A. oryzae* is generally regarded as non-pathogenic and non-toxigenic. Certain strains may produce one or more of the secondary metabolites cyclopiazonic acid, kojic acid and β-nitropropionic acid. The toxicity of these metabolites is low to moderate and the production is tied both to strain specificity and culture conditions. The amounts of these metabolites produced during industrial fermentation processes are small, and there are no reports that their production has resulted in adverse effects on human health.

Gluzyme, batch PPX 7029, has been analysed for the presence of aflatoxin B<sub>1</sub>, ochratoxin A, sterigmatocystin, T2 toxin and zearalenone as well as cyclopiazonic acid, kojic acid and β-nitropropionic acid. None of these mycotoxins were detected in significant amounts. The present production strain does not contain any resistance markers and the test article does not contain the production strain.

## 3. STUDY SUMMARIES

### 3.1 General toxicity

#### 3.1.1 13-weeks Subchronic Oral Toxicity in Rat

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*Inveresk Research Group, Inveresk Report Number No. 20161, Novozymes reference number 20016004 : Gluzyme, PPX 7029. 13-week Toxicity Study including Neurotoxicity Screening in Rats with administration by Gavage..*

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

Three groups of 10 male and 10 female (Low, Intermediate and High dose groups) Sprague-Dawley rats were dosed orally by gavage once daily at levels 1, 3 and 10 ml.kg<sup>-1</sup>.day<sup>-1</sup> (equivalent to 5082, 15247 and 50822 GODU-F.kg<sup>-1</sup>.day<sup>-1</sup> or 113.5, 340.6 and 1135.3 mg TOS.kg<sup>-1</sup>.day<sup>-1</sup>). A similar constituted group received the vehicle (sterile water for irrigation) and served as the control. The dose formulations for group 2 and 3 were prepared by diluting the undiluted test substance with vehicle to the appropriate concentration (w/v). The dose volume was 10 ml.kg<sup>-1</sup>.day<sup>-1</sup>. The rats were dosed for at least 91 days and until the day before necropsy.

No dose-related deaths occurred. There were no treatment related clinical observations and the male and female treated up to dosages of 10.0 ml/kg/day showed no evidence of neurotoxicity during the Functional Observation Battery procedures.

Bodyweight gain, food consumption and the efficiency of food utilisation were in males unaffected by the treatment. In the females however a very slight decrease in food consumption and an associated, non-statistically significant, but consistent decrease in body weight/body weight gain was observed in High dose females.

There was no treatment-related ocular. There was no treatment related alteration in the haematological or biochemical composition of the blood. Organ weights were unaffected by treatment. There were no findings at necropsy or after histopathological evaluation that were considered to be due to administration of Gluzyme.

The No Effect Level (NOEL) was considered to be 10 ml.kg<sup>-1</sup>.day<sup>-1</sup> for males and 3 ml.kg<sup>-1</sup>.day<sup>-1</sup> for females due to the slight decrease in food consumption and associated a very slight decrease in food consumption and associated, non-statistically significant, but consistent decrease in body weight/body weight gain in High dose females. 10 ml.kg<sup>-1</sup>.day<sup>-1</sup> for males and 3 ml.kg<sup>-1</sup>.day<sup>-1</sup> for females corresponds to 1135.3 and 340.6 mg TOS kg<sup>-1</sup>.day<sup>-1</sup> equivalent to 50822 and 15247 and GODU-F kg<sup>-1</sup>.day<sup>-1</sup>, respectively.

## 3.2 Mutagenicity

### 3.2.1 Assessment of gene mutations in bacteria

*Novozymes AIS Report No.: 20018029. (file nr. 2001-09756-01). Gluzyme (batch No. PPX 7029): Test for mutagenic activity with strains of Salmonella typhimurium and Escherichia coli. June 2001.*

Gluzyme, batch PPX 7029, was examined in order to determine the ability to induce gene mutations in strains of *Salmonella typhimurium* and *Escherichia coli*.

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

Crude enzyme preparations contain free amino acids such as histidine and tryptophan. Gluzyme™, batch No. PPX 7029, significantly supported growth of the histidine-requiring *S. typhimurium* strains by direct incorporation of the test substance on selective agar media. Therefore, in order to avoid the risk of artefacts due to growth stimulation, a "treat and plate" assay was applied with all *Salmonella* strains. Bacterial cultures were exposed to Gluzyme™, solvent and appropriate positive controls in phosphate buffered nutrient broth for three hours at 37°C. After this period, all nutrients originating from the test substance and broth were removed by centrifugation of the bacterial suspensions.

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

Further Gluzyme causes pronounced cytotoxicity and dose related increases of induced mutations, when a plate incorporation assay as well as a liquid culture assay ("treat and plate") is applied. The principal enzyme activity of Gluzyme is a glucose oxidase. In the presence of glucose hydrogen peroxide is produced by the catalytic action of glucose oxidase. Hydrogen peroxide is a well known cytotoxic and mutagenic compound in vitro. Therefore, in this study the glucose oxidase was inactivated by heat treatment for 30 minutes at 60°C at pH 2 and subsequent adjustment to neutral pH.

The study was conducted in accordance with OECD Guideline for Testing of Chemicals No. 471 (1997) concerning the general specifications of the test. However, the exposure of the test bacteria in liquid culture ("treat and plate"), as applied in this study with strains of Salmonella, is not specifically described in any guidelines. The treat and plate assay is recommended by JMHW (1984) when highly toxic compounds, e.g. antibiotics, are being assayed for bacterial mutagenicity, and by UKEMS (1990) when feeding effects are suspected and if the test substance is of biological origin (e.g. foodstuff or biological fluid).

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

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

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

No dose-related and reproducible increases in revertants to prototrophy were obtained with any of the bacterial strains exposed to Gluzyme™, batch PPX 7029, either in the presence or absence of S-9 mix.

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

It was concluded, that Gluzyme™, batch PPX 7029, did not induce gene mutations in bacteria in either the absence or presence of S-9 mix, when tested under the conditions employed in these studies.

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

Covance Laboratories Limited Report No.197415-D6172. Novozymes reference No.20016017  
*Gluzyme: Induction of Chromosome Aberrations in Cultured Human Peripheral Blood Lymphocyte. October 2000.*

The effects on chromosomal structure of exposure to Gluzyme™, batch PPX 7029, were investigated in cultured human lymphocytes in accordance with the current guidelines of OECD (Guideline 473, July 1997) and the ICH Tripartite Harmonised Guideline on Genotoxicity: Specific Aspects of Regulatory Tests (1995).

Heparinised whole blood cultures from three female donors were established, and division of the lymphocytes was stimulated by adding phytohaemagglutinin (PHA) to the cultures.

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Two independent experiments were performed both in the absence and presence of metabolic activation by a rat liver post-mitochondrial fraction (S-9) from animals induced with Aroclor.

Sets of duplicate cultures were treated with the solvent (sterile purified water), test chemical or positive controls (-S-9: 4-Nitroquinoline 1-oxide, +S-9: Cyclophosphamide). Treatments with Gluzyme™ covered a broad range of doses, separated by narrow intervals, where the highest dose level used was 5376 µg/ml, which was in excess of the recommended maximum concentration for chromosome aberration studies according to current regulatory guidelines.

The lymphocyte cultures were exposed to the test substance for three hours and cells were harvested 17 hours later. In the second experiment treatment in the absence of S-9 was continuous for 20 hours. 9. The test article dose levels for chromosome analysis were selected by evaluating the effect of Gluzyme™ on mitotic index.

Chromosome aberrations were analysed at three consecutive dose levels. Cells were arrested in metaphase by colchicine and after centrifugation and hypotonic treatment, metaphase spreads were prepared and stained with Giemsa. A total of 200 cells were scored per dose level (100 from each replicate culture) from Gluzyme™ treatments and negative controls. Slides were scored blind and aberrations classified according to the scheme described by ISCN (ISCN 1985).

The proportion of cells with structural aberrations in all cultures of the solvent controls (purified water) was within the limits of the historical ranges. The positive controls induced statistically significant increases in the proportion of cells with structural aberrations, thus demonstrating the sensitivity of the test procedure and the metabolic activity of the S-9 mix employed.

In the first experiment the highest concentration of Gluzyme™ chosen for analysis, 5376 µg/mL, produced approximately 32% and 0% mitotic inhibition (reduction in mitotic index) in the absence and presence of S-9, respectively. In the second experiment the highest concentration chosen for analysis, 2109 µg/mL and 5000 µg/mL, produced approximately 49% and 0% mitotic inhibition in the absence and presence of S-9, respectively.

Treatment of cultures with Gluzyme in the absence and presence of S-9 (both experiments) resulted in numbers of cells with structural aberrations which were similar to those observed in concurrent vehicle controls in the majority of cases. Exceptions to this were observed at the intermediate dose analysed (4301 mg/ml) for the pulse 3 hour +S-9 treatment in Experiment 1 and for the highest dose analysed (5376 mg/ml) for the pulse 3 hour -S-9 treatment in Experiment 1. In both cases one replicate culture had numbers of aberrant cells (excluding gaps) that exceeded the historical negative control (normal) range. However, these increases were small, were absent from the replicate cultures, not dose related and not reproduced between experiments. As such, these increases were considered spurious and of no biological importance.

Normal frequencies of cells with numerical aberrations were seen under all treatment conditions.

It was concluded that Gluzyme™, batch PPX 7029, under the conditions of test, did not induce chromosome aberrations in cultured human blood lymphocytes in either the absence or presence of S-9.

### 3.3 Cytotoxicity

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#### 3.3.1 Neutral Red Uptake

*Novozymes AIS Study summary No.: 20018027. (file nr. 2001-16652-01). Gluzyme (batch No. PPX 7029): In vitro Cytotoxicity Test: Neutral Red Uptake in L929 Monolayer Culture. October 2001.*

The purpose of this study was to screen for the cytotoxic potential of the enzyme preparation.

The neutral red uptake assay is a quantitative, colorimetric method to measure the cell viability. Neutral red is actively taken up by the cells and retained in the lysosomes/endosomes. The amount of neutral red taken up by the cells after exposure to the test substance is an indication of the number of viable cells and thus provides a measure of general toxicity. The basis of the present test system is that a cytotoxic substance regardless of site or mechanism of action will interfere with the viability and growth of the continuously dividing fibroblasts and, thus, result in a reduction of the cell number. The degree of inhibition of growth, related to the concentration of the test substance, provides an indication of toxicity.

The test system L929 is an established mouse fibroblast cell line. It was selected for the ease with which these cells are maintained and grown as monolayer culture and it is commonly used as first order test system for general cytotoxicity.

L929 was grown in EMEM with 10% foetal calf serum (FCS). 96-well microplates were added 150  $\mu$ l (5 x 10<sup>5</sup> cells per ml) cell culture per well. Plates were incubated for 24 hours at 37°C, establishing a near confluent monolayer. The test substance was dosed in five concentration (30,000, 10,000, 3,000, 1,000 and 300  $\mu$ g neat test substance per ml growth medium (EMEM 10% FCS)) to accurately determine the cytotoxicity in this model. SDS in different concentrations (120, 100, 80  $\mu$ g per ml growth medium) was used as concurrent positive controls. The exposure time was fixed to 24 hours.

The concentration of Gluzyme that was required to reduce the viability of the treated test system to 50% of that of the untreated control test system was determined as the endpoint (NRU<sub>50</sub>). The NRU<sub>50</sub> value for Gluzyme, batch PPX 7029, was estimated to be >30 mg/ml. The positive control, SDS, met the acceptance criteria of a valid test.

The results shown in this report indicate, that the sample of Gluzyme, batch PPX 7029, is non-cytotoxic *in vitro* in the Neutral Red Uptake assay applying the mouse fibroblast cell line L929 as test system.

The study was performed according to the Good Laboratory Practice Regulations described in OECD Principles of GLP, Dok. C (81) 30 (final), Paris, France (1981), but the summary report cited is a preliminary report before audit from the QA department has been finalized.

#### 4. CONCLUSION

Based on the toxicological data summarised above and the fact that the production strain has a safe industrial history in use, it is concluded that Gluzyme can be considered safe for food processing.

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Submission End

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## *Reference List for Industry Submission, GRN 000106*

<i>Pages</i>	<i>Author</i>	<i>Title</i>	<i>Publish Date</i>	<i>Publisher</i>	<i>BIB Info</i>
000029-000032	Barbesgaard, Peder, Heldt-Hansen, Hans Peter, Diderichsen, Borge	On the safety of Aspergillus oryzae a review	1992	Applied Microbiology and Biotechnology	Vol. 36, pp. 569-572

*NA- Not applicable*

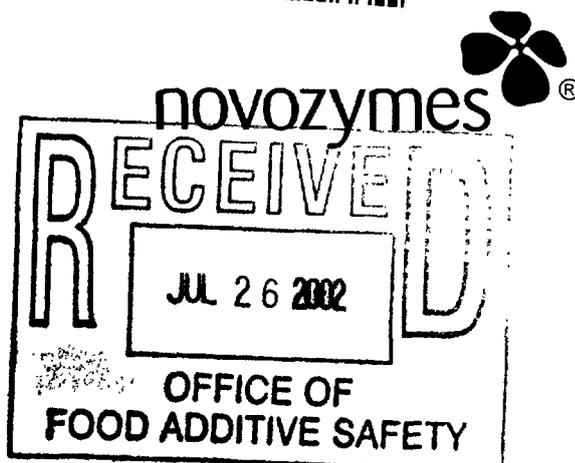
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June 21, 2002

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

In preparation for our teleconference on Monday, June 24, 2002, Novozymes would like to provide a revised Section 6.1, Mode of Application. Since our last teleconference with the agency on June 3, 2002, Novozymes has reviewed our GRAS notice No. 000106 and recognizes that Section 6.1 could have been written more clearly to describe the mechanism of glucose oxidase, the subject of this notice.

We also realized that the product sheet included as Appendix 1 in the notification could also be a source of confusion as to the reaction that is taking place. As indicated in the notice, the product sheet for this product, Gluzyme Mono BG (glucose oxidase from *A.oryzae*, carrying the gene encoding glucose oxidase from *A.niger*), was not available at the time of submission. The product sheet that is included as Appendix 1 in the notice is for a glucose oxidase product (from *A.niger*). This product sheet shows the glucose oxidase reaction and side reaction of the catalase. Historically both reactions, the primary of the glucose oxidase and the side of the catalase, have been reported in the product data sheet. However, the catalase reaction is not beneficial in the baking application because it is the production of hydrogen peroxide that is important for the purpose of improving resistance to mechanical shock, better oven spring and larger loaf volume. Attached please find a preliminary product sheet for Gluzyme Mono BG which properly describes this product and its use in baking.

We fully appreciate that the Monday conference call has been arranged to provide us an opportunity to more fully understand why FDA has concerns with the GRAS notification. While we are anxious to understand any and all of the agency's concerns, we remain hopeful that it will be possible to quickly address any confusion within the context of a submission to this notice that will clarify the matters covered.

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

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Novozymes GRAS Notice No. GRN 000106

#### Revised 6.1 Mode of Action

The functional enzyme activity in Gluzyme Mono BG is glucose oxidase. Glucose oxidase catalyzes the oxidation of glucose to gluconic acid and hydrogen peroxide. In baking applications, generation of hydrogen peroxide is known to improve the characteristics of dough. Hydrogen peroxide formed by the glucose oxidase reaction causes the oxidation of free sulfhydryl units in gluten protein which causes formation of disulfide bonds resulting in a stronger more elastic dough.

Gluzyme Mono BG is used in the baking industry as a processing aid to strengthen gluten in dough systems giving improved resistance to mechanical shock, better oven spring and larger loaf volume. Gluzyme Mono BG can be used as an alternative to chemical oxidizers such as potassium bromate and azodicarbonamide.

A preliminary product sheet on Gluzyme Mono BG and its use in the baking industry is enclosed.

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**PRELIMINARY PRODUCT SHEET**

# Gluzyme Mono<sup>®</sup> BG

## Description

Gluzyme Mono BG is a glucose oxidase preparation from *Aspergillus niger*, produced by submerged fermentation of a genetically modified *Aspergillus oryzae* microorganism. Glucose oxidase catalyzes the oxidation of glucose to gluconic acid and H<sub>2</sub>O<sub>2</sub>.

## Product Properties

### Product type

Gluzyme Mono BG is standardized using a special wheat flour with a narrow particle size distribution. Gluzyme Mono BG is a mix of yellowish/grey, free-flowing, non-dusting, agglomerated granulates and flour. It has an average particle size of 150 microns within the range of 50-212 microns.

### Activity

Gluzyme Mono BG is available with a declared activity of:

Gluzyme Mono BG .....10,000 GODU/g

GODU = Glucose Oxidase Units.

The product is standardized with wheat flour by Documented Addition in a strictly ISO-controlled process. See the Analytical Method for further information. The product contains a non-standardized amount of catalase side activity.

### Solubility

The active components of Gluzyme Mono BG are readily soluble in water at all concentrations that occur in normal usage. However, water solutions will be turbid due to the wheat flour used for standardization of the enzyme.

### Food-grade Status

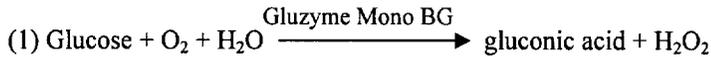
Gluzyme Mono BG complies with the recommended purity specifications for food-grade enzymes given by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Food Chemicals Codex (FCC).

### Packaging

See the standard Packaging List for more packaging information.

## Application

Gluzyme Mono BG can be used to strengthen gluten in dough systems. The oxidation of glucose results in the formation of gluconic acid and hydrogen peroxide (1). The formed hydrogen peroxide is capable of oxidising free sulphhydryl groups in gluten protein, whereby disulfide linkages are formed (2).



The catalase side activity does not affect the performance of glucose oxidase. The addition of Gluzyme Mono BG results in stronger and more elastic dough with greater resistance to mechanical shock, better oven spring and larger loaf volume. Gluzyme Mono BG is active in the dough but will be inactivated during baking.

## Reaction Parameters

### Dosage

The recommended dosage of Gluzyme Mono BG is within the range of 0.25-5 g per 100 kg flour (i.e. 2-50 ppm, 0.1-2.2 g/cwt), corresponding to 25-500 GODU per kg flour. The optimum dosage of Gluzyme Mono BG can vary, depending on flour quality, formulation and process, and should be determined through baking trials.

## Safety

Enzymes are proteins. Inhalation of dust or aerosols may induce sensitization and may cause allergic reactions in sensitized individuals. Some enzymes may irritate the skin, eyes and mucous membranes upon prolonged contact. This product has been developed to resist light mechanical effects. However, excessive mechanical wear and tear or crushing may create dust.

All spills, even small spills should be removed immediately. Use respiratory protection. Major spills should be carefully shovelled into plastic-lined containers. Small spills and the remains of major spills should be removed by vacuum cleaning or flushing with water (avoid splashing). Vacuum cleaners and central vacuum systems should be equipped with HEPA filters. Wear suitable protective clothing, gloves and eye/face protection as prescribed on the warning label. Wash contaminated clothes.

A [Material Safety Data Sheet](#) is supplied with all products. See the [Safety Manual](#) for further information regarding how to handle the product safely.

## Storage

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

When stored at recommended conditions, the product is best used within 6 months.

***Laws, regulations and third party rights may prevent customers from importing, processing, applying and/or reselling certain products in a given manner. It is the responsibility of the customers that their specific use of products from Novozymes does not infringe relevant laws and regulations and, furthermore, does not infringe patents or other third party rights.***

*The contents of this document are subject to change without further notice.*

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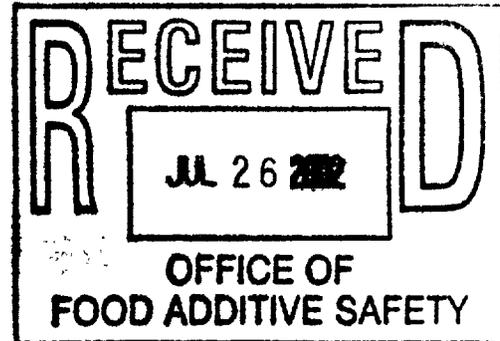
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novozymes 

July 19, 2002

Susan J. Carlson, Ph.D.  
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Re: GRAS Notice No. GRN 000106

Dear Dr. Carlson,

In reference to our telephone conversation yesterday, July 18, 2002, Novozymes would like to further clarify the technical effect of our glucose oxidase enzyme preparation produced by *Aspergillus oryzae* expressing the gene encoding a glucose oxidase from *Aspergillus niger* which is the subject of our GRAS notice No. 000106. As we have stated in the notice, the main use for this enzyme will be in the baking industry. In a letter dated June 21, 2002, we explained the chemical reactions occurring in the baking application and the technical effect in the final food. In baking applications, the glucose oxidase generates hydrogen peroxide which causes the oxidation of free sulfhydryl units in gluten protein which causes formation of disulfide bonds resulting in a stronger, more elastic dough.

In our notice, we also mentioned that this enzyme can be used in other food applications such as cheese, beer, carbonated beverages, and fruit juice. If the enzyme is used in these types of products, the technical effect in the food will be different than that in the baking application. In these systems, glucose oxidase would be used for the removal of oxygen from food. The glucose oxidase would still generate hydrogen peroxide in this system, but when used in combination with a catalase enzyme preparation the ultimate technical effect would be removal of oxygen from the food product.

In all cases, the enzyme activity is the same, glucose oxidase. However, depending on the food system it is used in, the technical effect will be different.

We hope this serves to clarify the technical effect of glucose oxidase in various food systems. 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

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August 28, 2002

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**Subject: GRN 000106 (Gluzyme Mono BG) "Other possible uses..."**

Dear Dr. Carlson:

Thank you for the opportunity to supply additional feedback for GRN 000106.

The thrust of GRN 000106 is the use of glucose oxidase (Gluzyme Mono BG) in the baking application in which glucose oxidase is used to strengthen the dough. This is the major food application for this enzyme. Novozymes is not actively promoting other uses. In an attempt to round off the discussion of uses, we briefly mentioned that, "Other possible uses would be as a processing aid in the manufacture of food such as cheese, beer, carbonated beverages and fruit juice." These other uses carried through from GRP 3G0016; parts of which were converted to GRN 000089. GRN 000089 discusses five enzyme preparations from *Aspergillus niger*, one of which is a glucose oxidase enzyme preparation.

We do not have much information in-house on the uses in cheese, beer, carbonated beverages and fruit juice therefore we have looked to outside literature in order to obtain and frame information on: use levels, fate of the enzyme, and estimates of human consumption.

#### Use Levels

Our overall conclusion is that the use-level ranges for these other possible applications fall well within the dosage range of the 25-500 glucose oxidase units that we provided for the Gluzyme Mono BG baking application. (In the baking case the dosages given were per kilogram of flour.) Indeed the dose ranges given for these other possible applications are at the lower end of what we indicated for the use in flour. Since the enzyme is used in a different way and the desired technical effect for these other applications is typically the reduction or removal of oxygen, it is reasonable that the dose range could be in the lower end of what we indicated for the baking application.

In the beer industry, the suggested dose range for stabilizing beer taste is 15-40 glucose oxidase units per liter.

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In the juice industry, the recommended dosage range is 16-48 glucose oxidase units per liter.

**Fate of the Enzyme:**

Glucose oxidase may be added to beer and fruit juices to avoid color and taste changes. Thus, the addition of glucose oxidase to beer and fruit juices before sealing suggests that the enzyme would remain active at bottling and become deactivated during storage.

**Estimates of Human Consumption for Some of these Food Types:**

The average human intake of beer and fruit juices is estimated using well-established statistics. For example, in the United States, Determinants of Food Consumption and Demand, Food Consumption, Prices, and Expenditures, 1970-97 estimates average annual per capita consumption of beer is 22 gallons and of fruit juice is 9.2 gallons. From these figures we can estimate the average daily per capita consumption of beer is 228 mls. and for fruit juices is 95 mls.

Although glucose oxidase can be used as a processing aid in the manufacturing of cheese, beer, carbonated beverages and fruit juice, the use of this enzyme in these applications is rather limited. Indeed, alternative technology has largely supplanted the use of glucose oxidase for cheese packaging, so we believe that this use has moved from limited to 'very limited.' In the past, glucose oxidase was used as a seal against oxygen in cheese packaging. Glucose oxidase was sprayed on the packaging material to create an oxygen barrier. Vacuum packaging is currently the preferred method of packaging cheese to avoid contact with oxygen.

We hope that this helps to frame the other possible applications referred to in GRN 000106. Please contact me at 919-494-3150 if you have any further questions or require additional information.

Sincerely,

 John Carroll  
Director, Regulatory Affairs

**Some References:**

Reed, G., *Enzymes in Food Processing*, 2<sup>nd</sup> ed., Academic Press, New York, 1975.

Uhlig, H., *Industrial Enzymes and Their Applications*, John Wiley & Sons, Inc., Canada, 1998.

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