GR

ORIGINAL SUBMISSION

novozymes®
Rethink Tomorrow

RECEIVED
DEC 30 2008
BY:

December 22, 2008

Overnight Express Mail
Robert Martin, Ph.D.
Deputy Division Director
Division of Biotech and GRAS Notice Review
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 20740-3835

Dear Dr. Martin:

A new GRAS notification entitled "A branching glycosyltransferase produced by *Bacillus subtilis* expressing the *Rhodothermus obamensis* branching glycosyltransferase gene" was sent by overnight mail to you on Friday, December 19, 2008 for delivery on Monday, December 22, 2008 (3 copies). I regret to inform you that the Exemption Claim was inadvertently left out of the Notification. Three copies are enclosed for incorporation into the Notification. In addition, a pdf version of the Exemption Claim was emailed to you today. We trust that this immediate disclosure to correct the oversight and the methods of providing the Exemption Claim are satisfactory and will not delay the FDA from granting a December 2008 receipt date.

Thank you for your attention to this matter. Should you have any questions, please contact me directly at (919) 494-3152 or by email at DCBe@novozymes.com.

Sincerely,

Denise Bernstein
Staff Specialist

Enclosures (3 Exemption Claim letters for incorporation into GRAS Notification binders)

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. Unless separate agreements exist, the contents of this document are subject to change without further notice

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

Tel:919-494-3000 **Fax: 919-494-3420** **www.novozymes.com**

novozymes®
Rethink Tomorrow

December 19, 2008

RECEIVED
DEC 30 2008
BY:----------

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 branching glycosyltransferase preparations produced by submerged fermentation of *Bacillus subtilis* expressing the *Rhodothermus obamensis* branching glycosyltransferase gene 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.*

Branching glycosyltransferase preparation produced by submerged fermentation of *Bacillus subtilis* expressing the *Rhodothermus obamensis* branching glycosyltransferase gene

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

The branching glycosyltransferase is intended for use in the in the food industry as a processing aid to obtain dextrins with improved physical properties such as higher solubility, lower viscosity, and reduced retrogradation. The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements for normal production following Good Manufacturing Practices.

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

This GRAS determination is based on scientific procedures.

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

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

19 December, 2008

John Carroll — Date
Director, Regulatory Affairs

novozymes®
Rethink Tomorrow

December 19, 2008

RECEIVED
DEC 23 2008
BY:

Overnight Express Mail
Robert Martin, Ph.D.
Deputy Division Director
Division of Biotech and GRAS Notice Review
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 20740-3835

Dear Dr. Martin,

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' branching glycosyltransferase enzyme preparation produced by *Bacillus subtilis* expressing the *Rhodothermus obamensis* branching glycosyltransferase gene. The enzyme preparation is intended to be used in the starch industry as a processing aid to obtain dextrins with improved physical properties such as higher solubility, lower viscosity and reduced retrogradation.

Thank you for your attention to this matter. Should you have any questions or require additional information, please contact me by direct telephone at 919 494-3152, direct fax at 919 494-3420 or email at DCBe@novozymes.com.

Sincerely,

Denise Bernstein
Staff Specialist

Enclosures (3 binders)

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. Unless separate agreements exist, the contents of this document are subject to change without further notice.

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

Tel:919-494-3000 ***Fax: 919-494-3420*** ***www.novozymes.com***

2008-37573

novozymes®
Rethink Tomorrow

GRAS Notification

A branching glycosyltransferase produced by *Bacillus subtilis* expressing the *Rhodothermus obamensis* branching glycosyltransferase gene

Denise Bernstein, Regulatory Affairs, Novozymes North America, Inc. USA
Dorthe Helnov, Regulatory Affairs, Novozymes A/S, Denmark

December 2008

TABLE OF CONTENTS

		PAGE
1.	**GENERAL INTRODUCTION**	3
2.	**ENZYME IDENTITY**	3
3.	**PRODUCTION MICROORGANISM**	4
3.1	Recipient Organism	4
3.2	Construction of the Recombinant Microorganism	5
3.3	Stability of the Introduced Genetic Sequences	6
3.4	Antibiotic Resistance Gene	6
3.5	Absence of the Production Organism in Product	6
4.	**MANUFACTURING PROCESS**	6
4.1	Raw Materials	6
4.2	Fermentation Process	7
4.3	Recovery Process	7
4.4	Quality Control of Finished Product	8
5.	**COMPOSITION AND SPECIFICATIONS**	8
5.1	Quantitative Composition	8
5.2	Specifications	8
6.	**APPLICATION**	9
6.1	Technological function	9
6.2	Types of Foodstuffs	9
6.3	Maximum Dosage of the Enzyme Preparation	9
6.4	Enzyme Residues in the Final Food	9
7.	**SAFETY EVALUATION**	9
7.1	Safety of the Production Strain	9
7.2	Safety of the Enzyme Product	11
7.3	Safety of the Manufacturing Process	12
7.4	Safety Studies	12
7.5	Estimates of Human Consumption and Safety Margin	13
7.6	Results and Conclusion	15
8.	**LIST OF APPENDICES**	15
9.	**LIST OF REFERENCES**	15

1. GENERAL INTRODUCTION

Branchzyme® is the Novozymes A/S trade name for a branching glycosyltransferase preparation produced by submerged fermentation of a *Bacillus subtilis* strain carrying a gene encoding a branching glycosyltranserase from *Rhodothermus obamensis*.

The enzyme is a glycosyltranferase (EC 2.4.1.18) that transfers a segment of a 1,4-alpha-D-glucan chain to a primary hydroxy group in a similar glucan chain with 1,6-alpha linkages to increase the number of branched points.

Branchzyme is to be used in the starch industry as a processing aid, where it is utilized to obtain dextrins with improved physical properties such as higher solubility, lower viscosity and reduced retrogradation.

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

It should be noticed that in some reports, the Branching glycosyltransferase preparation is described by its trade name, Branchzyme, or by PPY 27209, which refers to an internal production batch code. Furthermore, in some cases branching glycosyltransferase is described as branching enzyme.

2. ENZYME IDENTITY

2.1 Enzyme Identity

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

Generic name:	Branching Glycosyltransferase
Systematic name:	1,4-alpha-D-glucan:1,4-alpha-D-glucan 6-alpha-D-(1,4-alpha-D-glucano)-transferase
IUB nomenclature:	1,4-alpha-glucan branching glycosyltransferase
IUB No.:	EC 2.4.1.18
CAS No.:	CAS 9001-97-2
Specificity:	The enzyme transfers a segment of a 1,4-alpha-D-glucan chain to a primary hydroxy group in a similar glucan chain with 1,6-alpha linkages, which thereby increases the number of branched points.
Sequences:	Total nucleotide sequences have been determined.

3. PRODUCTION MICROORGANISM

This notification is directed to a genetically modified strain of *Bacillus subtilis* that contains a synthetic gene encoding a branching glycosyltransferase. The branching glycosyltransferase enzyme preparation is produced by submerged fermentation of *Bacillus subtilis* strain JA 1343 expressing a branching glycosyltransferase from *Rhodothermus obamensis*. The genetically modified strain was constructed using standard genetic modification techniques.

3.1 Recipient Organism

The host strain *Bacillus subtilis* JA 1343 is a genetically modified derivative of *Bacillus subtilis* 168 b(6) constructed by plasmid transformation whereby the following resident genes have been inactivated: *sigF*, *nprE*, *aprE, and amyE*, resulting in a sporulation negative, protease deficient, and amylase negative strain that is marker free. JA1343 expresses a synthetic gene based on the amino acid sequence resulting from the translation of the wild-type branching glycosyltransferase coding region of the *Rhodothermus obamensis* gene. The branching bglycosyltransferase gene was designed by creating a new DNA sequence based on the amino acid sequence of the branching glycosyltransferase gene cloned from *Rhodothermus obamensis* to accommodate codon usage in Bacillus subtilis (4). The new gene encoding the branching glycosyltransferase, called *BEK*, encodes for the same enzyme as the wild type branching glycosyltransferase gene from *Rhodothermus obamensis*. JA 1343 contains one copy of the synthetic *BEK* gene integrated in its chromosome. The *BEK* gene was inserted at the *amyE* locus of *Bacillus subtilis* under control of the $P_{amyQ(sc)}/P_{cry3A}$ double tandem promoter and the ribosome binding site of *Bacillus clausii*, and followed by the transcription terminator (*aprH*) of *Bacillus clausii*. The resulting strain JA1343 is sporulation negative, protease deficient, amylase negative, and antibiotic marker free. The parent strain *Bacillus subtilis* 168 is an auxothrophic mutant and the genome has been fully sequenced (1).

This is the same recipient organism as the one used for Novozymes' production strains expressing an alpha-Acetolactate decarboxylase (2; 21 CFR 173.115); a Maltogenic amylase (GRASP 7G0328, see Appendix A, Table 1, p. 147; 3); and hyaluronic acid (4).

3.1.1 Introduced Genetic Material

The *BEK* gene was designed by creating a new DNA sequence based on the amino acid sequence from the wild-type branching glycosyltransferase gene cloned from *Rhodothermus obamensis*. The source of the wild type branching glycosyltransferase coding region of *Rhodothermus obamensis* is JCM 9785. In order to facilitate the expression of the branching glycosyltransferase gene in *Bacillus subtilis*, the *BEK* synthetic gene comprises codons mostly used in *Bacillus subtilis*, which was designed based on the amino acid sequence resulting from the translation of the wild type branching glycosyltransferase gene from *Rhodothermus obamensis* (5). The *BEK* gene encodes the same amino acid sequence as the wild type branching glycosyltransferase gene from *Rhodothermus obamensis*.

The specific introduced DNA sequences include *amyE* fragments of the wild type *Bacillus subtilis*, double tandem promoter $P_{amyQ(sc)}/P_{cry3a}$, where $P_{amyQ(sc)}$ is a modified version of the promoter of the *Bacillus amyloliquefaciens* alpha-amylase gene *amyQ* (6) and P_{cry3a} is the promoter of the *Bacillus thuringiensis* subsp. *tenebrionis* crystal protein gene cry3A (7); an alkaline protease (aprH) terminator from *Bacillus clausii*; an inactive vector fragment from pBR322, and a ribosome binding site from *Bacillus clausii* (8). Elements of plasmids pBR322 (8), pUB110 (9), and pC194 (10) were used during the construction of the branching glycosyltransferase production strain, but only a small (<100 bp) inactive fraction of the pBR322 was actually introduced into the chromosome of the production strain as a

result of the genetic modification. Elements of the DNA inserted into the *amy*E locus of the final *BEK* production strain are summarized in Table 1 below.

Table 1. A summary of the 4.5-kb segment of DNA inserted into the *amyE* locus of the JA 1343 production strain.

Position (kb)	**Size (kb)**	**Element**	**Origin**
0-1.20	1.20	*amyE* back	*Bacillus subtilis*
1.20-1.29	0.09	Inactive vector fragment	pBR322
1.30-1.47	0.17	amyQ promoter	*Bacillus amyloliquefaciens*
1.47-2.02	0.55	cry 3A promoter w. mRNA stabilizing sequence	*Bacillus thuringiensis*
2.03-2.05	0.02	Ribosome binding site	*Bacillus clausii*
2.05-3.92	1.87	*BEK* (branching glycosyltransferase gene)	*Rhodothermus obamensis (synthetic gene)*
3.93-3.99	0.06	Terminator	*Bacillus clausii*
3.99-4.53	0.54	*amyE* front	*Bacillus subtilis*

Note: the *amyE*back and *amyE*front fragments are actually part of the wild type *Bacillus subtilis* chromosome and are not "foreign". They are included here to show exactly where the introduced DNA lies within the chromosome.

P*amy*Q(sc)/P*cry3a* is a double tandem promoter used in place of the native promoter to drive *BEK* expression in *Bacillus subtilis*. None of the toxin gene coding region of the *Bacillus thuringiensis* subsp tenebrionis crystal protein is present. The cry3A promoter contains a long untranslated leader region with a Shine-Darlgarno-like sequence (*cry3*A stabilizer) at the 3' end, which confers enhanced stability to mRNAs transcribed from the two promoters.

3.2 Construction of Recombinant Microorganism

The construction of the recombinant host strain, *Bacillus subtilis* JA1343 is outlined in the following steps:

1.) The branching glycosyltransferase gene was obtained in an *E.coli* plasmid. This plasmid was digested with restriction enzymes to release the *BEK*-bearing fragment proceeded by a ribosome binding site, and the plasmid was digested with the same enzymes to remove the *aprH* coding region.

2.) The *BEK* DNA fragment was ligated with the plasmid vector fragment from step 1, placing the *BEK* gene under control of the double tandem promoter $P_{amyQ(sc)}/P_{cry3A}$. The ligation was used to transform *Bacillus subtilis* **SHa273** to chloramphenicol resistance. SHa273 is a derivative of *Bacillus subtilis* 168. Integrants were screened for neomycin sensitivity, indicating double-crossover integration. One such integrant was designated **PL4206-1** and contains the chloramphenicol resistance gene and the double tandem promoter/*BEK* expression cassette inserted at the *amyE* locus.

3.) The *BEK* expression cassette was transferred to *Bacillus subtilis* host JA1343 by transformation of the host with genomic DNA from PL4206-1. One such *BEK* integrant was designated **PL4230-1**.

4.) Finally, the chloramphenicol resistance gene (*cat*) was deleted in order to make the *BEK* production strain marker free. **PL4230-1** was transformed with a *cat*-deletion plasmid. Transformants with the plasmid inserted into the chromosome via the homology regions between plasmid and

chromosome (*amyE*back and mRNAstab) were selected by growth on erythromycin at a temperature non-permissive for plasmid replication. The plasmid was then allowed to excise from the chromosome by non-selective growth at a temperature permissive for plasmid replication, and colonies were screened for sensitivity to both erythromycin (indicating loss of the plasmid) and chloramphenicol (indicating deletion of the *cat* gene). One such marker free strain was selected as the production strain (for the chromosomal organization.

The linear construct of the chromosomal organization of the *BEK* gene is depicted below.

3.3 Stability of the Introduced Genetic Sequences

The recombinant production organism is stable during production fermentation, as the inserted DNA is integrated into the chromosome. The stability of the integrated branching glycosyltransferase gene was tested after fermentation. The strain stability during fermentation was analyzed by Southern blotting. No instability of the strain was observed.

Because the integration plasmid is chromosomally integrated, it is poorly transferred to other organisms. As explained above, the absence of free plasmids in the final production strain was confirmed by its sensitivity to erythromycin.

3.4 Antibiotic Resistance Gene

The DNA introduced into the resultant *Bacillus subtilis* production strain does not contain antibiotic resistance genes.

3.5 Absence of the 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 (11) is satisfactorily addressed.

4. MANUFACTURING PROCESS

This section describes the manufacturing process for the branching glycosyltransferase, which follows standard industry practices (12-14). The Quality Management System used in the manufacturing process for the branching glycosyltransferase complies with the requirements of ISO 9001. The enzyme preparation is produced in accordance with current Good Manufacturing Practices, using ingredients that are accepted for general use in foods, and under conditions that ensure a controlled fermentation.

4.1 Raw Materials

The raw materials used in the fermentation and recovery process for the branching glycosyltransferase concentrate are standard ingredients used in the enzyme industry (12-14). 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.

4.2 Fermentation Process

The branching glycosyltransferase is manufactured by submerged fed-batch pure culture fermentation of the genetically modified strain of *Bacillus subtilis* described in Section 3. All equipment is carefully designed, constructed, operated, cleaned, and maintained so as to prevent contamination by foreign microorganisms. During all steps of fermentation, physical and chemical control measures are taken and microbiological analyses are done to ensure absence of foreign microorganisms and confirm strain identity.

4.2.1 Production Organism

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

4.2.2 Criteria for the Rejection of Fermentation Batches

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

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

1. Contamination is observed in 2 or more samples by microscopy

2. Contamination is observed in two successive agar plates at a minimum interval of 6 hours

Any contaminated fermentation is rejected.

4.3 Recovery Process

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

4.3.1 Purification Process

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

1. Pretreatment - pH adjustment

2. Primary Separation - vacuum drum filtration

3. Concentration - ultrafiltration and/or evaporation

4. Pre- and Germ Filtration - for removal of residual production strain organisms and as a general precaution against microbial degradation

5. Preservation and Stabilization of the liquid enzyme concentrate

6. Final concentration – evaporation and/or ultrafiltration if enzyme concentration is too low to reach target yield

4.3.2 Formulation and Standardization Processes

The stabilized concentrate is blended with water, sorbitol and glycerol. The product is standardized according to the product specification.

4.4 Quality Control of Finished Product

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

5. COMPOSITION AND SPECIFICATIONS

5.1 Quantitative Composition

The branching glycosyltransferase preparation is presently available in a formula for use in food applications. The Novozymes A/S trade name for the branching glycosyltransferase preparation is Branchzyme® (Appendix B -Branchzyme® Product Data Sheet). Branchzyme is a liquid formulation with the typical composition shown below:

Enzyme solids (TOS[1])	approx. 4 %
Water	approx. 44 %
Sorbitol	approx. 25 %
Glycerol	approx. 25 %
Methionine	approx. 2 %

Branchzyme has a typical activity of 50 KBEU/g. The Novozymes method used to determine the BEU (Branching Glycosyltransferase Units) is EB-SM-0699.02-D which is an automated assay. The measurement of the branching glycosyltransferase activity is based on the introduction of alpha-1,6-bonds in the substrate amylose resulting in a decrease in absorbance at 660 nm of amylose after reaction with iodine/stop complex. One BEU is the activity which under standard conditions causes a decrease of absorbance of the amylose-iodine complex by 1% per minute.

5.2 Specifications

The branching glycosyltransferase preparation complies with the purity criteria recommended for enzyme preparations as described in *Food Chemicals Codex* (15). In addition, it 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 (16).

[1] TOS = Total Organic Solids, defined as: 100% - water - ash - diluents

6. APPLICATION

6.1 Technological function

The branching glycosyltransferase (EC 2.4.1.18) transfers a segment of a 1,4-alpha-D-glucan chain to a primary hydroxy group in a similar glucan chain thereby increasing the number of branch points in the dextrin. The resulting dextrins have improved physical properties such as higher solubility, lower viscosity and reduced retrogradation.

6.2 Types of foodstuffs

Branchzyme is intended for use as a processing aid in the starch industry to obtain dextrins with improved physical properties such as high solubility, low viscosity and reduced retrogradation which can be used beneficially in producing various foods, e.g., soups, sauces, dried instant food, low fat products and soft drinks.

6.3 Maximum Dosage of the Enzyme Preparation

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

Branchzyme is standardized to 50 KBEU/g (Branching Glycosyltransferase Units/g). Depending on the desired properties of the modified starch the recommended dosage of Branchzyme is 0.04% - 4.0% corresponding to 0.4 – 40.0 kg enzyme preparation per ton of starch dry substance or 20.0 to 2.000 KBEU per kg starch dry substance.

6.4 Enzyme Residues in the Final Food

Branchzyme is used as a processing aid in the starch industry for the production of modified starch and the enzyme is not added directly to food. In the starch industry the branching glycosyltransferase is largely inactivated and removed during the steps and conditions used in starch processing. These steps include inactivation of enzyme activity (by heat or lowering pH), filtration, carbon treatment, ion exchange, evaporation and drying. As a result, any carry-over of active enzyme into the final food preparation will be negligible.

7. SAFETY

The safety assessment of the enzyme preparation for use in food or food processing should include an evaluation of the safety of the enzyme source (production organism), the enzyme, the manufacturing process, and an evaluation of dietary exposure to the enzyme preparation (11, 17-24). Each of these is addressed below.

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 (18-19). Assuming that current good manufacturing practice is followed, toxic contaminants can only come from the enzyme source organism itself. If the organism is non-toxigenic and non-pathogenic, then it is assumed that food or food ingredients produced from the organism, using the current Good Manufacturing Practice (cGMP)

Regulations, is safe to consume (13). Pariza and Foster (18) define a non-toxigenic organism as "one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure" and a non-pathogenic organism as "one that is very unlikely to produce disease under ordinary circumstances." Because the host organism is considered safe due to the genetic modifications being well characterized and specific (see section 3) and the introduced genetic material does not encode and express any known harmful or toxic substances, the genetically modified *Bacillus subtilis* is considered a safe production strain for the branching glycosyltransferase preparation (11, 25). An evaluation of the manufacture of the branching glycosyltransferase embodying the concepts initially outlined by Pariza and Foster in 1983 (18) and further developed by other groups (11, 19-24) demonstrating the safety of *Bacillus subtilis* JA 1343. When assessing the safety of production organisms obtained using r-DNA techniques the following components are evaluated: the host organism, the donor organism, the vectors, and the recombinant strain. Based on the above, it is concluded that the *Bacillus subtilis* used should be considered a safe host organism for expressing the branching glycosyltransferase.

7.1.1 Safety of the Production Organism

The evaluation of the safety of the host organism is very important since all, but a small fraction of the DNA, is that of the host. *Bacillus subtilis*, whose genome has been fully sequenced, is one of industry's workhorses for the production of a wide range of products resulting in one of the worlds' most well-characterized microorganisms (1). *Bacillus subtilis* as a type species is not considered to be a pathogen. The species, in general, is often represented as an example of non-pathogenic micro-organism (26-29). Carbohydrases and proteases from *Bacillus subtilis* have been affirmed as GRAS by FDA (21 CFR §184.1148 and §184.1150), Alpha-Acetolactate decarboxylase enzyme preparation derived from a recombinant *Bacillus subtilis* (21 CFR § 173.115) and Pullulanase from *Bacillus subtilis* (GRN 205).

In addition, *Bacillus subtilis* is a saprophytic microorganism widely distributed in nature and is commonly recovered from water, soil, air, and decomposing plant residues. It is a common contaminant in foods eaten by both man and animals, and has a history of safe use in food enzyme manufacturing (30-32). Strains of *Bacillus subtilis* have been safely used in the manufacture of food grade enzymes for decades and, in the last ten years recombinant *Bacillus subtilis* strains have been safely used in the manufacture of a variety of bio-industrial products such as food-grade enzymes, vitamins, antibiotics, biopolymers, additives and in the production of certain foods such as miso in Japan (from *B. subtilis* var. *natto*). Enzymes derived from B. subtilis include alpha-acetolactate decarboxylase, alpha-amylases, beta-glucanase, glutaminase, maltogenic amylase, pullulanase, protease, and xylanase (3, 19). *Bacillus subtilis* is a well-known and widely used microorganism in recombinant DNA research, and it is classified as a Risk Group 1 organism according to the (U.S.) National Institute of Health (NIH) (33) Risk Group 1 organisms are those not associated with disease in healthy adult humans. Considering *Bacillus subtilis* harmless, NIH exempted sporulation-deficient strains from its Guidelines for Research involving Recombinant Molecules (33). A published paper "On the safety of *Bacillus subtilis* and *Bacillus amyloliquefaciens*: a review" is provided as additional support of the safety of the production strain (29). As a type species, *Bacillus subtilis* is not considered to be a pathogen and is generally represented as an example of non-pathogenic organisms (26). *Bacillus subtilis* also meets the US Environmental Protection Agency (EPA) criteria for nontoxigenicity and nonpathogenicity and it is one of 10 host organisms eligible for Tier I exemption under the EPA regulations (27).

7.1.1.1 *Bacillus subtilis* 168

The production strain is derived from a *Bacillus subtilis* strain 168 parent strain. *Bacillus subtilis* strain 168 is a well known and well characterized auxothrophic mutant with a fully sequenced genome (1). Numerous strains widely used in research and industrial applications have been developed from *Bacillus subtilis* 168 originating as far back as 1958 (34). Olempska-Beer et al. list various enzymes produced by

Bacillus subtilis over the years (Appendix A; 3). Page 147 of this review notes the important of well-known wild-type *B. subtilis* strain 168, "the progenitor of many *Bacillus subtilis* strains used as sources of food-processing enzymes."

Bacillus subtilis strain 168 is the parent strain of other Novozymes' production strains expressing an alpha-Acetolactate decarboxylase (2; 21 CFR 173.115), a Maltogenic amylase (GRASP 7G0328, see Appendix A, Table 1, p. 147; 3), and hyaluronic acid (4). Toxicological tests were conducted for the two enzyme preparations and hyaluronic acid with no toxicological effects observed. In addition, both enzyme preparations have been evaluated by JECFA resulting in specifications with an "ADI not specified" (JECFA, 51st meeting, Geneva, 1998; JECFA, 53rd meeting, Rome, 1999).

A database search for potential homologue sequences between *Bacillus subtilis* 168 chromosomal DNA (full sequence) and known sequences coding for *Bacillus cereus*-like toxins showed no matches, strongly indicating that the strain does not have the potential for producing such toxins (35). These findings are supported by publicly available information on *Bacillus subtilis* 168 showing negative results in tests for toxin production (Vero cell test for entero-toxin production and boar sperm motility test for detection of emetic toxins (36).

7.1.2 Safety of the Donor Organism for the Branching Glycosyltransferase Gene

The donor for the branching glycosyltransferase gene is *Rhodothermus obamensis*, JCM 9785. A search of the scientific literature disclosed no evidence connecting strains of *Rhodothermus* with a pathogenic potential or production of secondary metabolites with toxicity against neither humans nor animals.

7.2. Safety of the Enzyme Product

7.2.1 Safety of the Branching glycosyltransferase

A wide variety of enzymes are used in food processing (18-19). Enzyme proteins themselves do not generally raise safety concerns (18, 28, 37). To confirm the safety of the enzyme, safety studies were performed and described in section 7.4. Branching glycosyltransferases have been isolated from a variety of starchy food sources such as maize, wheat, potato, rice, pea and are most likely part of the normal human diet (38).

7.2.2 Absence of Production Organism in the Final Product

The absence of the production organism is an established specification for the commercial product. The production organism does not end up in the food, which satisfies the first step in the safety assessment described by the IFBC (11).

7.2.3 Consideration of the Allergenic Potential of the Enzyme

Enzymes are proteins and proteins can be potential allergens. Only a small percentage of all dietary proteins are allergens, however. Enzyme proteins do not generally raise safety concerns. Enzymes are a special type of protein with a highly specific catalytic function. They are essential to many biochemical reactions in microorganisms, plants, animals, and humans. Due to the specific nature of enzymes only very small amounts are required to achieve the desired effect in food. A wide variety of enzymes have been used safely for centuries in food processing. Enzymes are found in many cells and tissues of plants and animals including those that are consumed by man. They are ubiquitous and, in this respect, common and ordinary. Novozymes is not aware of any allergic reactions caused by the ingestion of Branching glycosyltransferase.

7.2.3.1 Sequence Homology Evaluation

A sequence homology to known allergens has been assessed using procedures suggested in "Report of a joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology", Rome 2001 (39). An assessment of potential homology of sequence fragments of 6 contiguous amino acids to known allergens showed no matches. When searching for identity in the amino acid sequence of the branching glycosyltransferase to known allergens using a window of 80 amino acids and a suitable gap penalty, a 35% match was found to sequences of Asp o 21 (*Aspergillus oryzae* TAKA amylase A [alpha-amylase]). As both *Rhodothermus obamensis* branching glycosyltransferase and *Aspergillus oryzae* TAKA amylase A belong to family 13 glycosylhydrolases (40), it is hardly surprising that some homologous areas can be found. However, there are large differences in the loop regions, and the overall identity is only about 32%.

Also, *Rhodothermus obamensis* branching glycosyltransferase is of bacterial origin, and no sensitization towards a bacterial protein has been reported thus far (41-42). Consequently, oral intake of branching glycosyltransferase enzyme from *Rhodothermus obamensis* is not anticipated to pose any allergenic concern.

As described in section 3, the expression cassette contains a strictly defined gene encoding the branching glycosyltransferase gene, the $P_{amyQ(sc)}/P_{cry3A}$ double tandem promoter and the ribosome binding site of *Bacillus clausii,* the transcription terminator of *Bacillus clausii,* two fragments of the native *amyE* locus from *Bacillus subtilis* and an inactive fragment of pBR322.

Based on the discussion presented above, and more specifically based on the fact that i) there are no known cases of allergic responses to Branching glycosyltransferase in food; ii) known cases of allergic responses to food enzymes are very rare; and iii) exposure to the Branching glycosyltransferase protein is extremely low, it is concluded that the allergy risk due to the ingestion of Branching glycosyltransferase is believed to be negligible.

7.3 Safety of the Manufacturing Process

Branchzyme meets the general and additional requirements for enzyme preparations as outlined in the monograph on Enzyme Preparations in the *Food Chemicals Codex* (1). As described in section 3, the Branching glycosyltransferase preparation is produced in accordance with current GMP, 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 (12-14).

7.4. Safety Studies

This section describes the studies and analyses performed to evaluate the safety of the use of the branching glycosyltransferase.

7.4.1 Description of Test Material

Branchzyme, batch PPY 27209, is the liquid enzyme concentrate used as the test material for the studies conducted below in section 7.4.2. It is a mixture of three test batches that are produced in the same manner as in the production scale (see description given in Section 4), omitting stabilization and standardization.

7.4.2 Summary of Studies

A summary report of the safety studies performed on the branching glycosyltransferase batch PPY 27209 is enclosed in Appendix C.

The following studies were performed and main conclusions of the testing are provided below.

- *In vitro* cytotoxicity test: Neutral Red Uptake in L929 monolayer culture --

Based on in vitro cytotoxicity testing, the Branching glycosyltransferase is non-cytotoxic.

- Repeated-dose (13 weeks) oral (gavage) toxicity in rats

No treatment-related and toxicological relevant changes were observed for the 90-day oral gavage toxicity study in rats. In this study, the no observed adverse effect level (NOAEL) in rats treated orally by daily administration (gavage) for 13 weeks was considered to be the highest dose level administered, which was equivalent to 10 ml undiluted Branching glycosyltransferase (BE), batch PPY 27209/kg bodyweight (bw)/day or 769 mg Total Organic Solids (TOS)/kg bw/day or 940168 BE/kg/bw/day.

- Bacterial Reverse Mutation Assay (AMES Test)

It was concluded that the Branching glycosyltransferase did not induce gene mutations in bacteria in either the absence or the presence of S-9 mix when tested under the conditions of these studies.

- *In vitro* Micronucleus Assay

It was concluded that Branching glycosyltransferase, batch PPY 27209, did not show any evidence of clastogenic or aneugenic activity in cultured human peripheral blood lymphocytes.

7.5 Estimates of Human Consumption and Safety Margin

7.5.1 Estimates of human consumption

As stated in section 6.4, the human exposure to branching glycosyltransferase will be negligible because the enzyme preparation is used as a processing aid in starch production in low amounts and not added directly to food. During the final steps of the industrial starch processing the enzyme will be inactivated and removed from the starch and any carry-over of active enzyme into the final food is expected to be negligible.

As stated previously in section 6.3, Branchzyme is to be used for production of modified starch in dosages up to a maximum of 4% corresponding to 2.000 KBEU/kg starch dry substance (DS). Branchzyme has an activity of 50 KBEU/g and an approximate content of 4.0% TOS (Total Organic Substances from the fermentation, mainly protein and carbohydrate components).

Because the resulting modified starch may be used for a variety of applications as ingredients in food and/or beverages, the estimation of human consumption is based on the Budget method (43).

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

1. According to the Budget method, a conservative estimate for the food intake is 25 g per kg body weight per day of which processed food is 50% of the food intake.
2. According to the Budget method, a conservative estimate for the beverage (non-milk) intake is 100 ml per kg body weight per day of which processed beverages (soft drinks) is 25% of the intake.
3. According to the budget method, it is assumed that all processed foods and beverages (non-milk) contain modified starch produced using Branchzyme as a processing aid, used at the highest recommended dosage which is 4%.
4. It is further assumed that the above mentioned processed food and beverages contain 3% modified starch.
5. The calculation is made assuming that all TOS remains in the final product.

Based on the "worst case" assumptions given above the amount of TOS per kg body weight (bw) per day is calculated below:

Food Intake:	
Food intake according to Budget method:	25 g food/kg bw/day
50% is processed food:	12.5 g processed food/kg bw/day
Processed food contain 3% modified starch:	375 mg modified starch/kg bw/day
Modified starch contain (max) 4% Branchzyme:	15 mg Branchzyme/kg bw/day
Branchzyme contain 4% TOS:	**0.6 mg TOS/kg bw/day**
Beverage Intake:	
Beverage intake (non-milk) acc. to Budget method:	100 ml/kg bw/day
25% is processed beverages (soft drinks):	25 ml = 25 g processed beverages/kg bw/day
Processed beverages contain 3% modified starch:	750 mg modified starch/kg bw/day
Modified starch contain (max) 4% Branchzyme:	30 mg Branchzyme/kg bw/day
Branchzyme contain 4% TOS:	**1.2 mg TOS/kg bw/day**
Total TOS in food and beverage intake	0.6 mg + 1.2 mg =
(Total Theoretical Maximum Daily Intake):	**1.8 mg TOS/kg bw/day**

According to information reported by an English manufacturer of modified starch[2], the consumption in U.K of modified starch from food sources is estimated to an average of 30.2 mg/day for male adults, 16 – 64 years (the highest intake group). In the group of all-users with the highest intake (male adults) the mean daily intake of modified starch is 150.2 mg/person /day and the 97.5th percentile is 419.6 mg/person/day.

When using the Budget method the estimated average intake of modified starch from food alone is calculated to be 375 mg modified starch/kg bw/day which gives an estimated intake of modified starch for a person weighing 60 kg of 22500 mg/person/day. In our estimate of intake we include also the contribution of modified starch from beverages which is estimated twice the size of the contribution from food, i.e. a total of 67500 mg modified starch/person/day.

This confirms that using the Budget method above for estimating human consumption demonstrates an extremely exaggerated "worst case" calculation.

[2] "Estimated daily intake of modified starches from existing food-uses by the U.K population" (National Starch Food Innovation, England. September 6, 2005. (http://www.food.gov.uk/multimedia/pdfs/annexi.pdf)

7.5.2 Safety margin

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

In the 13 week oral feeding of rats with branching glycosyltransferase it is concluded that dose levels of up to 769 mg total organic solids per kg body weight per day caused no dose-related findings. The NOAEL for branching glycosyltransferase in this species is therefore the highest administered dose level, i.e. 769 mg TOS/kg bw/day.

The estimated human consumption (from an exaggerated "worst case" situation) is 1.8 mg TOS/kg bw/day

The safety margin in a "worst case" situation is thus calculated to be 769/1.8 = **427.2.**

7.6 Results and Conclusion

On the basis of the evaluation described above including a review of the published literature, the history of safe use of *Bacillus subtilis* as a production organism for industrial enzymes, the safe use of the branching glycosyltransferase preparation, and the limited and well defined nature of the genetic modifications, the Branchzyme enzyme preparation produced by *Bacillus subtilis* expressing a branching glycosyltransferase from *Rhodothermus* obame*nsis* can be safely manufactured and used as a processing aid in the starch industry.

8. LIST OF APPENDICES

A. Olempska-Beer, Z.S., Merker, R.I., Ditto, M.D., and DiNovi, M.J. Food-processing enzymes from recombinant microorganisms – a review. *Reg. Tox and Pharm* 45: 144-158, 2006.

B. Branchzyme® Product Data Sheet

C. Summary of Toxicity Data, Branching glycosyltransferase from *Bacillus subtilis*, 2008-16643-02

9. LIST OF REFERENCES

1. F. Kunst, et al. The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*. *Nature* 390:249-256, 1997.

2. Federal Register, Vol. 66, No. 95:27020-27022, May 16, 2001.

3. Olempska-Beer, Z.S., Merker, R.I., Ditto, M.D. & DiNovi, M.J. Food Processing enzymes from recombinant microorganisms – a review. *Reg. Tox & Pharma* 45:144-158, 2006.

4. Widner, B., Behr, R, Von Dollen, S., Tang, M., Heu, T., Sloma, A., Sternberg, D., DeAngelis, P.L., Weigel, P.H., & Brown, S. Hyaluronic acid production in *Bacillus subtilis*. *Applied & Env. Microb* 71(7): 3747-3752, 2005.

5. Gustafsson, C., Govindarajan, S. & Minshull, J. Codon bias and heterologous protein expression. *TRENDS in Biotechnology* 22 (7):346-353, 2004.

6. Takkinen et al. Amino acid sequence of α-amylase from *Bacillus amyloliquefaciens* deduced from the nucleotide sequence of the cloned gene. *J. Biol. Chem.* 258: 1007-1013, 1983.

7. Agaisse and Lereclus. Structural and functional analysis of the promoter region involved in full expression of the *cryIIIA* toxin gene of *Bacillus thuringiensis. Molecular Microbiology*. 13:97-107, 1994.

8. Bolivar, F. et al. Construction and Characterization of a New Cloning Vehicles. II. A Multipurpose Cloning System. *Gene*. 2:95-113, 1977.

9. McKenzie et al. The Nucleotide Sequence of pUB110: Some Salient Features in Relation to Replication and Its Regulation. *Plasmid* 15:93-103, 1986.

10. Horinouchi and Weisblum. Nucleotide Sequence and Functional Map of pC194, a Plasmid That Specifies Inducible Chloramphenicol Resistance. *J. Bact.* 150:815-825, 1982.

11. International Food Biotechnology Council. Biotechnologies and food: Assuring the safety of foods produced by genetic modification. Reg. Toxicol. Pharmacol. 12(3):S1-S196, 1990.

12. Anstrup, K. Production, isolation, and economics of extracellular enzymes *in Applied Biochemistry and Bioengineering*, Vol. 2, Enzyme technology. Wingard, L.B., Katchalski-Katzir,E.& Goldstein, L. (eds.), pp. 28-68, 1979.

13. Aunstrup, K., Andersen, O., Falch, E. A. and Nielsen, T. K.. Production of microbial enzymes *in* Microbial Technology, 2nd ed,. Vol. I. Peppler, H. J. and Perlman, D. (eds.). Chapter 9, pp. 282-309, 1979.

14. Kroschwitz, J. I. Enzyme applications in *Encyclopedia of Chemical Technology*, 4th ed., Vol. 9, pp. 567-620, 1994.

15. Food Chemicals Codex, 6th Edition, National Academy of Sciences, Food and Nutrition Board, Committee on Food Chemicals Codex, National Academy Press, Washington, D.C., p. 289, 2008-2009.

16. JECFA (Joint FAO/WHO Expert Committee on Food Additives), General Specifications and Considerations for Enzyme Preparations Used in Food Processing, Compendium of Food Additive Specifications, FAO FNP (Food and Nutrition Paper) 52, Add. 9, Pages 37-39, FAO, Rome, 2001.

17. Organization for Economic Cooperation and Development, Safety Considerations for Biotechnology, 1992.

18. Pariza, M. W. and Foster, E. M. Determining the safety of enzymes used in processing. *J. Food Prot.* 46:453-468, 1983.

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

20. Berkowitz, D. and Maryanski, J.. Implications of biotechnology on international food standards and codes of practice. Joint FAO/WHO Food Standards Programme, Codex Alimentarius Commission, 18th Session, Geneva, July 3-12, 1989.

21. EU Scientific Committee for Food. Guidelines for the presentation of data on food enzymes. Reports of the Scientific Committee for Food, 27th series, 1991.

22. FAO/WHO. Biotechnology and Food Safety, Report of a Joint FAO/WHO Consultation. FAO Food and Nutrition Paper 61. Rome, Italy, 1996.

23. Jonas, D. A., Antignac, E., Antoine, J. M., Classen, H. G., Huggett, A., Knudsen, I., Mahler, J., Ockhuizen, T., Smith, M., Teuber, M., Walker, R. and de Vogel, P.. The safety assessment of novel foods. Guidelines prepared by ILSI Europe Novel Food Task Force. Food Chem. Toxicol. 34:931-940, 1996.

24. Organization for Economic Cooperation and Development, Safety Evaluation of Foods Derived by Modern Biotechnology, 1993.

25. Kessler, D.A., Taylor, M.R., Maryanski, J.H., Flamm, E.L., & Kahl, L.S. The Safety of Foods Developed by Biotechnology. *Science* 256:1747-1749, 1832, 1992.

26. Food and Drug Administration. Statement of Policy for Regulating Biotechnology Products. Federal Register, 51: 23301-23350, June 26, 1986.

27. Environmental Protection Agency. Microbial products of biotechnology: final regulation under the Toxic Substances Control Act; final decision document. Federal Register 62:17910-17958, 1997.

28. Food and Drug Administration. Statement of Policy: Foods Derived From New Plant Varieties. Federal Register 57:22984-23005, 1992.

29. Sietske de Boer, A. and Diderichsen, B. On the safety of *Bacillus subtilis* and *B. amyloliquefaciens*: a review. Appl. Microbiol. Biotechnology 36:1-4, 1991.

30. Organization for Economic Cooperation and Development. Recombinant DNA Safety Considerations, Paris, 1986.

31. Sneath,P. "Endospore-forming Gram Positive Rods and Cocci." Section 13 in Bergey's Manual of Systematic *Bacteriology*. Vol. 2., pages 1113-1114. 1986.

32. WHO. Toxicological Evaluation of some Enzymes, Modified Starches and Certain Other Substances, WHO Food Additives Series No. 1, pp 9-10, 1972.

33. Jensen, B.F. and Norman, B.E. *Bacillus acidopullulyticus* pullulanase: Application and Regulatory Aspects for Use in the Food Industry. *Process Biochemistry* 19(4):129-134, 1984.

34. Spizizen, J. Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonucleate. *Proc. Natl Acad Sci* 44 (10):1072-1078, 1958.

35. Pedersen, P.B., Bjornvad, M.E., Rasmussen, M.D., & Petersen, J.N. Cytotoxic potential of industrial strains of *Bacillus sp.* *Reg Tox& Pharma* 36:155-161, 2002.

36. Report of the scientific committee on animal nutrition on the safety of the enzymatic product BELFEED B1100 ML® for use as feed additive for chickens for fattening. Adopted on 18 April 2002. European Commission, Health & Consumer P rotection Directorate-General, Directorate C – Scientific Opinions.

37. Food and Drug Administration. Lipase Enzyme Preparation From *Rhizopus niveus*: Affirmation of GRAS status as a Direct Food Ingredient. Federal Register 63:24416-24419, 1998.

38. Guan, H.P. & Keeling, P.L. Minireview – Starch biosynthesis: understanding the functions and interactions of multiple isozymes of starch synthase and branching glycosyltransferase. *Trends in Glycoscience and Glycotechnology* 10 (54):307-319, 1998.

39. Evaluation of Allergenicity of Genetically Modified Foods (Report of a Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology 22 25 January 2001), Food and Agriculture Organization of the United Nations (FAO), Rome, 2001.

40. van der Maarel, M., van der Veen, B., Uitdehaag, J., Leemhuis, H. & Dijkhuizen, L. Properties and applications of starch-converting enzymes of the alpha-amylase family. *J. Biotech.* 94:137-155, 2002.

41. Taylor, S.L. Review of the development of methodology for evaluating the human allergenic potential of novel proteins. *Mol. Nutr. Food Res.* 50:604-609, 2006.

42. Pedersen, M.H., Hansen, T.K., Sten, E., Seguro, K., Ohtsuka, T., Morita, A., Bindslev-Jensen, C. & Poulsen, L.K. Evaluation of the potential allergenicity of the enzyme microbial transglutaminase using the 2001 FAO/WHO Decision Tree. *Mol. Nutr. Food Res.* 48:434-440, 2004.

43. ILSI Europe Food Chemical Intake Task Force. An Evaluation of the Budget Method for Screening Food Additive Intake. April 1997.

Appendix A

Pages 000024 - 000038 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

Appendix B

novozymes

Product Data Sheet

Branchzyme®

Valid From 2008-05-16

Product Characteristics:

Declared Enzyme	Branching glycosyltransferase
Declared Activity	50000 BEU/g
Colour	Light brown Colour can vary from batch to batch. Colour intensity is not an indication of enzyme activity.
Physical form	Liquid
Approximate Density (g/ml)	1.20
Viscosity (cPs)	1-25
Stabilisers	Sorbitol Glycerol Methionine
Production organism	Bacillus subtilis
Production Method	Produced by submerged fermentation of a genetically modified micro organism. The enzyme protein, which in itself is not genetically modified, is separated and purified from the production organism.

000040

Product Specification:

	Lower Limit	Upper Limit	Unit
Branching Enzyme Units BEU	50000		/g
pH at 25C	8	10	
Total Viable Count		50000	/g
Coliform Bacteria		30	/g
Enteropathogenic E.Coli	Not Detected		/25 g
Salmonella	Not Detected		/25 g

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 Chemical Codex (FCC).

Packaging: See the standard packaging list for more information.

Recommended Storage:

Best before	When stored as recommended, the product is best used within 3 months from date of delivery.
Storage at customer's warehouse	0-10°C (32°F-50°F)
Storage Conditions	In unbroken packaging - dry and protected from the sun. The product has been formulated for optimal stability. Extended storage or adverse conditions such as higher temperature or higher humidity may lead to a higher dosage requirement.

Safety and Handling Precautions

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

Novozymes A/S
Krogshoejvej 36
2880 Bagsvaerd
Denmark

Tel. +45 4446 0000
Fax +45 4446 9999

For more information, or for more office addresses, visit www novozymes com

Laws, regulations and/or third party rights may prevent customers from importing, using, processing and/or reselling the products described herein in a given manner Without separate, written agreement between the customer and Novozymes to such effect, this document does not constitute a representation or warranty of any kind and is subject to change without further notice

© Novozymes A/S

Appendix C

novozymes®

Toxicology

Date : 28.07 2008
Ref.: FKBi/PBjP
File : 2008-16643-02

SUMMARY OF TOXICITY DATA

Branching enzyme from *Bacillus subtilis*

Author :
Franziska Birkved
Peder Bjarne Pedersen

Issued by :
Toxicology
Novozymes A/S
Krogshøjvej 36
2880 Bagsværd
Denmark

CONTENTS

PAGE

1. Abstract 3

2. Test Substance 3

   2.1 General information on the test material 3
   2.2 Production organism 4
   2.3 Characterization 4

3. Toxicity Data 5

   3.1 General toxicity 5
      3.1.1. *In vitro* cytotoxicity test 5
      3.1.2. Repeated-dose (13-week) oral (gavage) toxicity study in rats 6

   3.2 Mutagenicity 7
      3.2.1. Bacterial Reverse Mutation Assay (Ames Test) 7
      3.2.2. *In vitro* Micronucleus assay 8

4. References 9

   4.1 Studies 9
   4.2 Literature and guidelines 9

Last page 9

1. ABSTRACT

Branching Enzyme (transferase) is a liquid enzyme concentrate produced by a strain of the bacterial species *Bacillus subtilis.*
The present summary covers the toxicological studies relevant for the evaluation of Branching Enzyme when used as a processing aid in the production of food products.

All studies were carried out in accordance with current EU and OECD guidelines and in compliance with the OECD principles of Good Laboratory Practice (GLP). The studies were carried out at Novozymes in Denmark, Covance in UK and TNO in Netherlands during the period July 2007 to February 2008.

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

Cytotoxicity of Branching Enzyme was tested in the Neutral Red Uptake applying the mouse fibroblast cell line L929 as test system. Branching Enzyme tested up to 30 mg/ml media was not detected to be cytotoxic in the present set-up and it can be concluded that Branching Enzyme is non-cytotoxic *in vitro* in the L929 cell line at the given concentration range.

Branching Enzyme showed no mutagenic activity in a bacterial reverse mutation assay and there were no indication of a clastogenic or aneugenic potential when assessed in an *in vitro* micronucleus assay in human lymphocytes.

Oral toxicity was tested by in a 90-day oral gavage toxicity study in rats. No treatment-related and toxicological relevant changes were observed for the investigated parameters. In this study the no observed adverse effect level (NOAEL) in rats treated orally by gavage for 13 weeks was considered to be the highest dose level administered, equivalent to 10 ml undiluted Branching Enzyme, batch PPY 27209 /kg bodyweight(bw)/day or 769 mg Total Organic Solids (TOS)/kg bw/day or 940168 BE/kg bw/day.

2. TEST SUBSTANCE

2.1 General information on the test material

The Branching Enzyme (BE) is a bacterial enzyme originating from the extreme thermophilic species *Rhodothermus obamensis.* It is expressed in a strain of *Bacillus subtilis* most commonly used as recipient for production of enzymes at Novozymes.

Branching Enzyme is a transferase used to modify starch by transferring a segment of a 1,4-alpha-D-glucan chain to a primary hydroxyl group of a main chain thereby increasing the number of 1,6-alpha-D-glucosidic linkages. It is classified by the E.C. number 2.4.1.18.

Branching Enzyme, batch PPY 27209, is the liquid enzyme concentrate used as the test article in the present toxicological program. It is a mixture of three identically produced fermentation sub-batches. The batch is recovered by purification/concentration of the fermented culture broth according to the same procedures as are used for the production of commercial Branching Enzyme preparations, except that formulation/standardization is omitted.

2.2 Production organism

This genetically modified production strain of *Bacillus subtilis* 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 known not to encode or express any harmful or toxic substances. The strain is free of any antibiotic resistance marker. The development of the production strain was evaluated at every step to assess incorporation of the desired functional genetic information and to ensure no unintended sequences were incorporated.

Bacillus subtilis is generally regarded as non-pathogenic and non-toxigenic and the potential risk associated with the use of this microorganism in fermentation facilities is very low. Industrial strains belonging to this species have a long history of safe use. It has been used for many years in the production of enzymes, and in the last decade as recombinant organism for production of bio-industrial products.

The production strain can thus be considered a safe, non-toxigenic micro-organism.

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

2.3 Characterization

This batch has been analysed for chemical and microbial content, and all the analytical results comply with the recommended purity specifications for food grade enzymes.
The test material used for the studies is characterized as follows:

Table 1. Characterization of Branching Enzyme (batch PPY 27209)

	Batch PPY 27209
Activity BE/g	89200
Water (KF)	89.3 % w/w
Dry matter	10.7 % w/w
Ash (600°C)	3.4 % w/w
Total Organic Solids (TOS[1])	7.3 % w/w
Specific gravity (g/ml)	1.054

[1] %TOS = 100% ÷ % water ÷ % ash.

The enzyme is measured as BE/g. The measurement of the Branching Enzyme activity is based on the introduction of alpha 1,6-bonds in the substrate amylose resulting in a decrease in absorbance at 660 nm of amylose after reaction with iodine/stop complex.1 BE is the activity which under standard conditions causes a decrease of absorbance of the amylose-iodine complex by 1% per minute.

3. TOXICITY DATA

3.1 General toxicity

3.1.1. *In vitro* cytotoxicity test: Neutral Red Uptake in L929 monolayer culture

The purpose of this study was to screen for the cytotoxic potential of Branching Enzyme, batch PPY 27209.
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 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 bovine serum (FBS). 96-well microplates were added 150 µl (5 x 10^5 cells per ml) cell culture per well. Plates were incubated for 24 hours at 37°C, establishing a near confluent monolayer.
The following concentrations were selected for the sample for an accurate determination of cytotoxicity in this test model: 30, 10, 3, 1 and 0.3 mg of each test substance as received per ml growth medium (EMEM 10% FBS).
The positive control consisted of 120, 100, 80 µg SDS per ml growth medium. The time of exposure was 24 hours at 37° C.
The positive control, SDS, gave on the plate a NRU_{50} value (94 µg/ml) which fell within two standard deviations of the historical mean (98 ± 8.0 µg/ml) and it was thereby meeting the acceptance criteria of a valid test.
The concentration of the test substance 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_{50}). The NRU_{50} for Branching Enzyme, batch PPY 27209 was estimated to be >30 mg/ml.

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.

In conclusion, the results from this study indicated that the sample of Branching Enzyme, batch PPY 27209 is non-cytotoxic *in vitro* in the present Neutral Red Uptake assay applying the mouse fibroblast cell line L929 as test system.

3.1.2. Repeated-dose (13-week) oral (gavage) toxicity in rats

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

Procedure
Three groups each of ten male and ten female rats received Branching Enzyme, batch PPY 27209 at dose levels of up to 769 mg Total Organic Solids (TOS) per kg body weight (bw) per day (equivalent to 940168 BE /kg bw/day) for thirteen weeks. The dose volume was 10 ml/kg bw/day. A similarly constituted group received the vehicle (tap water) at the same volume dosage and served as the negative control.
Clinical observations, neurobehavioral testing, growth, food and water consumption, ophthalmoscopy, hematological and clinical chemistry parameters, gross examination at necropsy, organ weights and microscopic examination of various organs and tissues were used as criteria for disclosing possible harmful effects.
Analysis of achieved concentration was performed on samples taken once during weeks 1, 6 and 13. Achieved concentration was evaluated by measurement of enzyme activity, BE/g.

Results
No mortality or treatment-related clinical signs were observed during the study. The neurobehavioral observations and motor activity assessment did not indicate any neurotoxic potential. Body weights were similar among the groups throughout the study. Food and water consumption were similar among the groups throughout the study. Ophthalmoscopic examination did not show any treatment-related changes. Hematological and clinical chemistry did not show treatment-related changes.
Slightly lower absolute brain weight and slightly higher relative liver weight in high-dose males were not accompanied by histopathological changes. Therefore these changes were considered minor and not toxicological relevant.
Gross examination at necropsy and microscopic examination did not reveal any treatment-related changes.
The results from the content check analysis showed that the Branching Enzyme activities (BE/g) in the dose solutions from week 1, 6, and 13 were close to the intended enzyme activity for all dose solutions.
No significant difference was found between week 1, 6, and 13.
No significant differences were found between Group 4 (undiluted ~ highest dose level) and the reference of batch PPY 27209.

In conclusion, no treatment-related and toxicological relevant changes were observed for the investigated parameters in the 90-day oral gavage toxicity study in rats. In this study the no observed adverse effect level (NOAEL) in rats treated orally by gavage for 13 weeks was considered to be the highest dose level administered, equivalent to 10 ml undiluted Branching Enzyme, batch PPY 27209 /kg bodyweight(bw)/day or 769 mg Total Organic Solids (TOS)/kg bw/day or 940168 BE/kg bw/day.

3.2 Mutagenicity

3.2.1. Bacterial Reverse Mutation Assay (Ames Test)

Branching Enzyme, batch PPY 27209 was examined for mutagenic activity in the bacterial reverse mutation assay using the histidine-requiring strains TA1535, TA100, TA1537, TA98 of *Salmonella typhimurium* and the tryptophan-requiring strains *Escherichia coli* WP2uvrApKM101.

The study was conducted in accordance with OECD Guideline for testing of chemicals, No. 471: Bacterial Reverse Mutation Assay" (July 1997) concerning the general specifications of the test. However the exposure of test bacteria in liquid culture ("treat and plate"), as it was applied in this study, is not specifically described in any guidelines.

In order to avoid the risk of artifacts due to an amino acid feeding effect, a "treat and plate" assay was applied. Bacterial cultures were exposed to Branching Enzyme, 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 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 (S-9 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 Branching Enzyme, 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/ml.

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.

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.

Branching Enzyme was not toxic to any of the test strains applied.
No treatments of any of the bacterial strains with Branching Enzyme in the treat and plate assay resulted in any dose related and reproducible increase in revertant numbers compared to the solvent control. Our criteria for a positive or equivocal response were not met in these experiments.

It was concluded, that Branching Enzyme 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* Micronucleus Assay

The clastogenic and the aneugenic activity of Branching Enzyme, batch PPY 27209, was assessed by evaluation of its effects on the frequency of micronuclei in cultured human peripheral blood lymphocytes applying the cytokinesis-block methodology.

The study was conducted in compliance with OECD draft guideline: 'Genetic Toxicology: OECD Guideline for the testing of chemicals. Draft proposal for a new Guideline 487: *In vitro* micronucleus test (2004) and accepted scientific/regulatory principles described in current guidelines for clastogenicity testing *in vitro*

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 1254. Heparinised whole blood cultures from female donors were established. Mitotic division of the lymphocytes was stimulated, by adding phytohaemagglutinin (PHA) to the cultures for 24 hours in first experiment and 48 hours in second experiment before treatment.
Sets of duplicate cultures were treated with the solvent (sterile purified water), test chemical or positive controls.
In both experiments treatment in the absence of S-9 was for 20 hours followed by a 28-hour recovery period prior to harvest. Treatment in the presence of S-9 was for 3 hours (pulse treatment) followed by a 45-hour recovery period prior to harvest.
By the addition of the actin inhibitor cytochalasin-B about 28 hours prior to harvest cytokinesis (cell division) was blocked resulting in the formation of binucleate cells (the cytokinesis-block methodology).
Treatments with Branching Enzyme covered a broad range of doses, separated by narrow intervals, where the highest dose level used was 5000 µg/ml. Cells were harvested by repeated centrifugation, hypotonic treatment and fixation. Slides were prepared and cells were stained with Giemsa.
Micronuclei were counted in binucleate cells at three consecutive dose levels, selected by evaluating the effect of Branching Enzyme on the replication index (RI) as a measure for cytotoxicity.
A total of 1000 cells per dose level (500 from each replicate culture) from the selected Branching Enzyme treatments and solvent controls were scored blind for micronuclei in binucleated cells. The proportion of cells with micronuclei for each treatment condition were compared with the proportion in solvent controls by using Fisher's exact test.

The proportion of binucleate cells with micronuclei 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 micronuclei, thus demonstrating the sensitivity of the test procedure and the metabolic activity of the S-9 mix employed.

In both Experiments treatment of cells with Branching Enzyme up to 5000 µg/ml in the absence and presence of metabolic activation (S-9) resulted in frequencies of micronucleated binucleate cells (MNBN), which were similar

to those observed in concurrent vehicle control cultures for all concentrations analysed.

It was concluded, that Branching enzyme, batch PPY 27209, did not show any evidence of clastogenic or aneugenic activity in cultured human peripheral blood lymphocytes.

4. REFERENCES

4.1 Studies

BE Branching Enzyme, batch PPY 27209 *In Vitro* Cytotoxicity Test: Neutral Red Uptake in L929 Monolayer Culture. NZ Study No.: 20078068. Novozymes July 2007. File no. 2007-32555-01.

Repeated-dose (13-week) oral (gavage) toxicity study with Branching Enzyme, PPY 27209 in rats. TNO Study Code: 7510/02. March 2008. Novozymes Reference No.: 20076033.

Branching Enzyme: Induction of micronuclei in Cultured Human Peripheral Blood Lymphocytes. NZ Study No.: 20076058. Covance Study No. 1974/71. Covance, March 2008. File No.: 2008-13740-01

Branching Enzyme, PPY 27209: Test for Mutagenic Activity with Strains of *Salmonella typhimurium* and *Escherichia coli.* NZ Study No.: 20078073. Novozymes, January 2008. File No.: 2008-01386-01

4.2 Literature and Guidelines

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

OECD principles of Good Laboratory Practice (GLP) (as revised in 1997), ENV/MC/CHEM(98)17. OECD, Paris.

SUBMISSION END

Reference List for Industry Submission, GRN 000274

Pages	*Author*	*Title*	*Publish Date*	*Publisher*	*BIB_Info*
000024 - 000038	Olempska-Beer, Zofia S.; Merker, Robert I.; Ditto, Mary D.; DiNovi, Michael J.	Food-processing enzymes from recombinant microorganisms -- a review	2006	Regulatory Toxicology and Pharmacology	Volume 45, pgs 144-158

NA- Not applicable

3-6-09 Correspondence from Notifier

West-Barnette, Shayla

From: DCBE (Denise Bernstein) [DCBe@novozymes.com]
Sent: Friday, March 06, 2009 3:33 PM
To: West-Barnette, Shayla
Subject: Follow-up re: GRN 274
Attachments: Response to FDA_GRN 00274 _090306.pdf

Dear Dr. West-Barnette -

As a follow-up to our phone conversation of February 25, 2009, I am providing additional information concerning the characterization of our host microorganism; updated IUBMB and JECFA references; and additional commentary concerning the sequence homology evaluation and consideration of the allergenic potential of enzymes including the enzyme described in GRN 00274.

We look forward to receiving FDA's letter of no objection in the near future.

Best Regards
Denise Bernstein
Staff Specialist

Novozymes North America Inc.
PO BOX 576
77 Perry Chapel Church Road
Franklinton NC 27525 United States
Phone: +1 9194943152
E-mail: dcbe@novozymes.com

Novozymes North America, Inc. (reg. no 13-2639630). Registered address: CT Corporation System, 111 8th Avenue, New York, NY 10011, United States of America
This e-mail (including any attachments) is for the intended addressee(s) only and may contain confidential and/or proprietary information protected by law. You are hereby notified that any unauthorized reading, disclosure, copying or distribution of this e-mail or use of information herein is strictly prohibited. If you are not an intended recipient you should delete this e-mail immediately. Thank you.

From: West-Barnette, Shayla [mailto:Shayla.WestBarnette@fda.hhs.gov]
Sent: Friday, February 20, 2009 12:04 PM
To: DCBE (Denise Bernstein)
Subject: RE: Phone Conference About GRN 274

Ms Bernstein,

Thank you for agreeing to speak with us regarding GRN 274. In preparation for next week's discussion about this submission, I would like to share the topics which the review team would like to discuss with you:

1) your discussion of the potential allergenicity of the enzyme
2) your discussion of the production microbe (no safety issues concerning this, just a matter of the inclusion of a few details)

3) a few suggestions for additional information that you may consider including in the GRN

Also, I wanted to confirm that for the conference, I can reach you and your participants at (919) 494-

3152. If you will have participants from an off-site location, please let me know and I can provide you with call-in instructions so that everyone can reach the line to participate. I look forward to speaking with you on Wednesday. If I can be of further assistance with this GRN, please let me know.

Regards,

Shayla

From: DCBE (Denise Bernstein) [mailto:DCBe@novozymes.com]
Sent: Wednesday, February 18, 2009 11:45 AM
To: West-Barnette, Shayla
Subject: RE: Phone Conference About GRN 274

Dr. West-Barnette -

Wednesday, February 25, 10:30-11:30, is an acceptable telephone conference date and time.

I look forward to receiving your comments in the near future.

Best Regards
Denise Bernstein
Staff Specialist

Novozymes North America Inc.
PO BOX 576
77 Perry Chapel Church Road
Franklinton NC 27525 United States
Phone: +1 9194943152
E-mail: dcbe@novozymes.com

Novozymes North America, Inc. (reg. no.:13-2639630). Registered address: CT Corporation System, 111 8th Avenue, New York, NY 10011, United States of America
This e-mail (including any attachments) is for the intended addressee(s) only and may contain confidential and/or proprietary information protected by law. You are hereby notified that any unauthorized reading, disclosure, copying or distribution of this e-mail or use of information herein is strictly prohibited. If you are not an intended recipient you should delete this e-mail immediately. Thank you.

From: West-Barnette, Shayla [mailto:Shayla.WestBarnette@fda.hhs.gov]
Sent: Wednesday, February 18, 2009 11:20 AM
To: DCBE (Denise Bernstein)
Subject: Phone Conference About GRN 274

Ms Bernstein,
Thank you for your quick response to my request for a telephone conference about GRN 274 (A branching glycosyltransferase produced by *Bacillus subtilis* expressing the *Rhodothermus obamensis* branching glycosyltransferase gene). Per our conversation earlier, the conference will take place on Wednesday, February 25, from 10:30 am to 11:30 am. Please note that the meeting may not take the entire hour. If you find that another meeting date/time would be better for you, please let me know and I will accommodate.

So that our discussion will be as productive as possible, I will forward you the comments that the review team would like to discuss. I'll be sure to send it by the end of this week. Again, thank you and if I can be of any further assistance with this notice, please feel free to contact me.

Regards,

Shayla West-Barnette, Ph.D.
Consumer Safety Officer
Center for Food Safety and Applied Nutrition
Food and Drug Administration
(301) 436-1262 (office)
Shayla.WestBarnette@fda.hhs.gov

RECEIVED
MAR 10 2009
BY (b)(6)

AM

novozymes®
Rethink Tomorrow

March 6, 2009

VIA FACSIMILE/CONFIRMATION BY MAIL

Shayla West-Barnette, Ph.D.
Consumer Safety Officer
Division of Biotechnology and GRAS Notice Review
Center for Food Safety and Applied Nutrition
Food and Drug Administration
College Park, MD 20740

Re: GRAS Notice No. GRN 000274 -
A branching glycosyltransferase produced by *Bacillus subtilis* expressing the *Rhodothermus obamensis* branching glycosyltransferase gene

Dear Dr. West-Barnette:

I thank you and your colleagues for your early review of our GRAS notification. In response to our telephone conference of February 25, 2009, I am providing additional information based on you and your colleagues review of the above-referenced GRN concerning 1) the Host Microorganism; 2) an updated IUBMB reference; 3) an updated JECFA reference; and 4) additional commentary concerning the sequence homology evaluation and consideration of the allergenic potential of the enzyme.

1. Characterization of Host Microorganism

The host strain, designated JA1343, is a geneticaly modified derivative of *Bacillus subtilis* derived from *Bacillus subtilis* 168 (Bacillus Genetic Stock Center 1A1, Columbus, Ohio), an auxothrophic mutant for which the genome has been fully sequenced (Kunst F, Ogasawara N, Moszer I, Yoshikawa H, Danchin A., et. al. 1997. The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*. *Nature*, 390, p. 249-256.

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. Unless separate agreements exist, the contents of this document are subject to change without further notice.

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

Tel:919-494-3000 ***Fax: 919-494-3420*** ***www.novozymes.com***

The following comprises the taxonomy of the host strain:

Name:	*Bacillus subtilis*
Class:	*Firmibacteria*
Order:	*Bacillaceae*
Genus:	*Bacillus*
Species:	*subtilis*

The *Bacillus subtilis* 168 was genetically modified by replacement of four resident genes with inactive/deleted versions. These genes comprise those encoding sporulation factor sigma F, neutral protease, alkaline protease and amylase (designated *sigF*, *nprE*, *aprE* and *amyE)*. The resulting strain, JA1343 is therefore sporulation negative, protease deficient and amylase negative.

The classification of the host strain has been confirmed by Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ).

2. Revised IUBMB Reference (page 3, section 2.1 of GRN)

Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB). Enzyme nomenclature -- Recommendations of the NC-IUBMB on the nomenclature and classification of enzyme-catalyzed reactions can be found at: http://www.chem.qmul.ac.uk/iubmb/enzyme/. Also published in *Enzyme Nomenclature 1992* [Academic Press, San Diego, California, ISBN 0-12-227164-5 (hardback), 0-12-227165-3 (paperback)] with Supplement 1 (1993), Supplement 2 (1994), Supplement 3 (1995), Supplement 4 (1997) and Supplement 5 (in *Eur. J. Biochem.* 1994, 223, 1-5; *Eur. J. Biochem.* 1995, 232, 1-6; *Eur. J. Biochem.* 1996, 237, 1-5; *Eur. J. Biochem.* 1997, 250; 1-6, and *Eur. J. Biochem.* 1999, 264, 610-650; respectively); subsequent supplements are available on the IUBMB website cited above.

3. Updated JECFA Reference (reference no. 16 in GRN)

JECFA, 2006. General Specifications and Considerations for Enzyme Preparations Used in Food Processing as proposed by the Joint FAO/WHO Expert Committee on Food Additives in Combined Compendium of Food Additive Specifications, available online at: http://www.fao.org/ag/agn/jecfa-additives/search.html?lang=en

4. Addendum to sections 7.2.3 and 7.2.3.1 concerning enzymes and food allergenicity

Using the WHO/FAO recommended JECFA allergen analysis of *Rhodothermus obamensis* branching glycosyltransferase for the 80 amino acid window search, a match was found in Asp o 21 (*Aspergillus oryzae* TAKA amylase A) when using the strict threshold of 35% identity (Reference 39 of GRN - Evaluation of Allergenicity of Genetically Modified Foods (Report of a Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology 22 25 January 2001), Food and Agriculture Organization of the United Nations (FAO), Rome 2001; www.who.int/foodsafety/publications/biotech/ec.jan2001/en/index.html). As indicated on page 12 of the GRN, both *Rhodothermus obamensis* branching glycosyltransferase and *Aspergillus oryzae* TAKA amylase A belong to family 13 glycosyl hydrolases, thus it is hardly surprising that some homologous areas can be found. However, there are large differences in the loop regions, and the overall identity is only about 32%.

Furthermore, only four cases (each with one person) of potential food allergy to Asp o 21 alpha-amylase have been reported in the literature (44-47), whereas three were linked to occupational sensitization. The few reported cases were described with very little detail and, in most cases, never confirmed by a proper food allergy diagnosis, which, on one hand, requires a skin prick test and specific serum immunoglobulin E (IgE) to confirm an IgE-mediated sensitization, and more importantly a double blind, placebo controlled food challenge (DBPCFC) to confirm food allergy (48-49). Sensitization in itself is not a disease and many people are asymptomatically sensitized to one or more proteins and may never develop food allergy. In addition, *Rhodothermus obamensis* branching enzyme is of bacterial origin, and no sensitization towards a bacterial protein has been reported so far (50, 51).

Further to the discussion presented above, in general, enzymes intended for use in foods (that is ingestion of enzymes) are not considered to be a concern in relation to food allergy. This is based on the following considerations:

- Enzymes have a long history of safe use in food, with no indication of adverse effects or reactions.

- The vast majority of proteins are not allergens (only 0.3% of all identified proteins are listed as allergens). A wide variety of enzyme classes and structures are naturally present in plant and animal based foods, and based on previous experience, food enzymes are not homologues to known allergens, which make it very unlikely that a new enzyme would be a food allergen.

- Exposure to enzymes via food is always very low, and food allergens are generally recognized to be abundant in their allergenic food source.

    - First, enzymes in foods are always added in concentrations in the low range of part per millions.

    - Second, the enzyme is typically removed or denatured during food processing, and denatured protein has been shown to be very susceptible to digestion in the gastro-intestinal system. Moreover, a wide range of naturally occurring food enzymes have been shown to be very labile in the gastro-intestinal system even in native unprocessed form.

The above statements are further supported by the publication: "Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry" – Bindslev-Jensen *et al.*, Food and Chemical Toxicology (52). This study involved a large investigation on the possible allergenicity of 19 different commercial enzymes used in the food industry. The investigation comprised enzymes produced by wild-type or genetically-modified strains as well as wild-type enzymes and protein engineered variants. Four hundred patients with a diagnosed allergy to inhalation allergens, food allergens, bee or wasp allergens were included, and all were skin prick tested with the 19 enzymes, which included a number of alpha-amylases from bacterial and fungal sources, lipases, proteases and lactase to name a few. Thirteen positive skin prick test results were obtained and these reactions were further investigated by *in vitro* basophil histamine release using blood from the patients with the positive skin tests. These patients were furthermore subjected to double blind, placebo controlled food challenge (DBPCFC) with the enzymes they were skin positive to, but no reactions were seen except one reaction to the placebo. The DBPCFC tests were performed according to the most recently recommended guidelines (49), and to add an extra safety factor all enzymes were native (unprocessed) and the doses used in the challenge tests were exaggerated compared to normal daily intake. Since no allergy could be diagnosed by DBPCFC the positive skin prick test results were of no clinical relevance. It was concluded from this study that ingestion of food enzymes, in general, is not considered to be a concern with regard to food allergy.

Also, some concern has been expressed in the past whether commercial enzymes may be potent sensitizers via the gastrointestinal route and hence possible food allergens. This seems not to be the case as only very few cases of allergy to ingested commercial enzymes have been reported, and most could be linked to an occupational sensitization obtained from working in a place where a high concentration of the enzyme was found in air/dust-samples, before granulation and other safety measures were installed to eliminate this risk years ago.

000055.001

Most concern has been with regard to the baking enzyme fungal alpha-amylase, where a total of four potential food allergy cases have been reported over several decades. To further investigate the possible food allergenicity of fungal alpha-amylase, Skamstrup-Hansen *et al.* tested 18 patients occupationally sensitized to fungal alpha-amylase and suffering from respiratory symptoms (53). All eighteen underwent a DBPCFC with 100 g bread containing twice the normal amount of enzyme and bread with no enzyme as placebo. Reactions were seen to both active and placebo in equal number, so no food allergy specifically related to alpha-amylase could be confirmed. Furthermore, 950 sera were collected upon employment at Novo Nordisk A/S and tested for specific serum IgE to alpha-amylase to look for sensitization in the general population, and none of these sera were positive. Skamstrup-Hansen *et al.* concluded from this study that food allergy to fungal alpha-amylase must be extremely rare.

Based on the foregoing, it is concluded that due to the continued use of very low doses of enzymes in food production processes and the long history of safe use of enzymes over several decades, there is no unacceptable risk for the general consumer regarding the development of a food allergy to a commercial enzyme including the use of branching glycosyltransferase in the production of starch products.

References:

44. Losada, E., Hinojosa, M., Quirce, S., Sanchez-Cano, M., Moneo, I. 1992. Occupational asthma caused by alpha-amylase inhalation: clinical and immunologic findings and bronchial response patterns. *J. Allergy Clin. Immunol.* 89:118-125.

45. Kanny, G., Moneret-Vautrin, D.A. 1995. Alpha-amylase contained in bread can induce food allergy. *J. Allergy Clin. Immunol.*, 95:132-133.

46. Baur, X., Czuppon, A.B. 1995. Allergic reaction after eating alpha-amylase (Asp o 2)-containing bread. A case report. *Allergy*. 50:85-87.

47. Moreno-Ancillo, A., Dominguez-Noche, C., Gil-Adrados, A.C., Cosmes, P.M. 2004. Bread eating induced oral angioedema due to alpha-amyase allergy. *J. Investig. Allergol. Clin. Immuno*l., 14:346-347.

48. Taylor,S.L., Hefle,S.L., Bindslev-Jensen,C., Atkins,F.M. *et al.* 2004. A consensus protocol for the determination of the threshold doses for allergenic foods: how much is too much?, *Clin. Exp. Allergy*, *34*, 689-695.

49. Bindslev-Jensen,C., Ballmer-Weber,B.K., Bengtsson,U., Blanco,C. *et al.* 2004. Standardization of food challenges in patients with immediate reactions to foods--position paper from the European Academy of Allergology and Clinical Immunology, *Allergy*, *59*, 690-697.

50. Taylor, S.L. 2006. Review of the development of methodology for evaluating the human allergenic potential of novel proteins. *Mol. Nutr. Foods Res*. 50:604-609.

51. Pedersen, M.H., Hansen, T.K., Sten, E., Seguro, K., et al. 2004. Evaluation of the potential allergenticity of the enzyme microbial transglutaminase using the 2001 FAO/WHO Decision Tree, *Mol. Nutr. Food Res*. 48:434-440.

52. Bindslev-Jensen *et al.* 2006. Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry. *Food and Chemical Toxicology* 44:1909-1915.

53. Skampstrup-Hansen, K., Vestergaard, H., Petersen, L.N., Bindslev-Jensen, C., Poulsen, L.K. 1999. Food allergy to fungal alpha-amylase in occupationally sensitized individuals. *Allergy*. 54[s52]:64-65.

Again, thank you for your constructive review of our GRAS notification. Should you require any additional information, please contact me by phone (919-494-3152) or by email (DCBe@novozymes.com).

Sincerely,

(b)(6)

Denise Bernstein
Staff Specialist

6-8-09 Correspondence from Notifier b

West-Barnette, Shayla

From: DCBE (Denise Bernstein) [DCBe@novozymes.com]
Sent: Monday, June 08, 2009 11:06 AM
To: West-Barnette, Shayla
Subject: RE: Question Regarding GRN 274

Dear Dr. West-Barnette –

Thank you for the opportunity to respond to your request clarifying the genes used in the selection, chromosomal integration, and amplification of the expression cassette containing the branching glycosyltransferase enzyme gene.

The gene-replacement plasmid used in the construction of the production strain contained the ampicillin resistance marker and origin of replication from pBR322, a neomycin resistance marker from pUB110, a chloramphenicol resistance marker from pC194, and two fragments of the *amyE* gene from *Bacillus subtilis*. Both of these *amyE* fragments flank the chloramphenicol resistance marker, the P_{scBAN}/P_{cry3A} triple tandem promoter, and the *aprH* coding region. The *aprH* coding region may be replaced by another coding region (such as *BEK*) to facilitate the introduction of an expression cassettes into the *amyE* locus of *Bacillus subtilis*. Integration of this plasmid in the strain of interest occurs by a double cross-over at the *amyE* locus. As a result of this integration event only the expression cassette and the chloramphenicol resistance marker integrate in the strain; the genes which confer resistance to neomycin and ampicillin, as well as all of the pBR322-derived sequences, are lost during the integration process.

At a final stage of construction of the production organism indicated in section 3.2 of the GRAS notification, the chloramphenicol resistance gene (*cat*) was deleted in order to make the *BEK* production strain marker free. Thus, the DNA introduced into the resultant *Bacillus subtilis* production strain does not contain any antibiotic resistance genes.

We hope this information is sufficient and are looking forward to receiving the FDA letter.

Best Regards
Denise Bernstein

Novozymes North America Inc.
PO BOX 576
77 Perry Chapel Church Road
Franklinton NC 27525 United States
Phone: +1 9194943152
E-mail: dcbe@novozymes.com

Novozymes North America, Inc. (reg. no :13-2639630). Registered address: CT Corporation System, 111 8th Avenue, New York, NY 10011, United States of America
This e-mail (including any attachments) is for the intended addressee(s) only and may contain confidential and/or proprietary information protected by law. You are hereby notified that any unauthorized reading, disclosure, copying or distribution of this e-mail or use of information herein is strictly prohibited. If you are not an intended recipient you should delete this e-mail immediately. Thank you.

From: West-Barnette, Shayla [mailto:Shayla.WestBarnette@fda.hhs.gov]
Sent: Friday, June 05, 2009 2:15 PM
To: DCBE (Denise Bernstein)
Subject: Question Regarding GRN 274

Ms Bernstein,

I am preparing the agency's response letter for GRN 274 (subject: branching glycosyltransferase

enzyme), and needed some information from you so that I can complete the document. At your earliest convenience, could you tell me what gene (ex. chloramphenicol resistance gene or other) is used for the selection, chromosomal integration, and amplification of the expression cassette which contains the branching glycosyltransferase enzyme gene? This information must be included in our letter, and I'm not completely clear on this after looking through the notice and the amendment. If you have any questions, please feel free to contact me.

Regards,

Shayla West-Barnette, Ph.D.
Consumer Safety Officer
Center for Food Safety and Applied Nutrition
Food and Drug Administration
(301) 436-1262
Shayla.WestBarnette@fda.hhs.gov