

GRAS Notice (GRN) No. 472

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ORIGINAL SUBMISSION

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Rethink Tomorrow

April 24, 2013

GRAS Notification Program
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
US Food And Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Dear Sir or Madam,

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' xylanase enzyme preparation produced by a genetically modified strain of *Bacillus licheniformis*. Novozymes has determined through scientific procedures that the xylanase is generally recognized as safe for use in the food industry as a processing aid to modify arabinoxylans in cereals such as wheat, barley, and oats. This is especially useful in the baking industry where it improves dough handling and the characteristics of the final product.

Please contact me by direct telephone at 919 494-3151, direct fax at 919 494-3420 or email at lobg@novozymes.com if you have any questions or require additional information.

Sincerely,

(b) (6)



Lori Gregg
Sr. Regulatory Specialist

Enclosures (3 binders)

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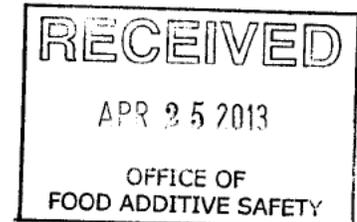
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Sr. Regulatory Specialist

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April 24, 2013

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 xylanase preparations produced by submerged fermentation of a genetically modified *Bacillus licheniformis* 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.*

Xylanase preparations produced by a genetically modified *Bacillus licheniformis* production strain

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

The enzyme preparation is intended for use in the food industry as a processing aid in the baking industry. The xylanase preparation is used to modify arabinoxylans in cereals such as wheat, barley, and oats, thereby improving dough handling and the characteristics of the final bread or food product. 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.

(b) (6)

Lori Gregg
Sr. Regulatory Specialist

Date

4-24-13

Luna No. 2013-06507-01



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**A xylanase preparation produced by a genetically modified
Bacillus licheniformis production strain**

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

April 2013

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1. GENERAL INTRODUCTION

The subject of this notification is a xylanase preparation produced by submerged fermentation of a *Bacillus licheniformis* microorganism expressing a xylanase variant from *B. licheniformis*. The xylanase preparation is used to modify arabinoxylans in cereals such as wheat, barley, and oats, thereby improving dough handling and the characteristics of the final bread.

The active enzyme is endo-1, 4-beta-xylanase (IUB 3.2.1.8, CAS 9025-57-4).

The information provided in the following sections is the basis for our determination of general recognition of safety of this xylanase enzyme preparation. Our safety evaluation in Section 7 includes an evaluation of the production strain, the donor strain, the enzyme, and the manufacturing process, as well as an evaluation of dietary exposure to the preparation.

The safety of the production organism must be the prime consideration in assessing the probable degree of safety of an enzyme preparation intended for use in food (Ref. 1, Ref. 2, and Appendix 1). The production strain is a genetically modified strain of *B. licheniformis* expressing a xylanase variant from *B. licheniformis*. The host organism, *B. licheniformis*, has a long history of safe industrial use for the production of enzymes used in human food and has been routinely used as a host for recombinant enzymes (Ref.3). *Bacillus licheniformis* is generally considered to be non-pathogenic and non-toxicogenic (Ref. 4, Appendix 2) and is often mentioned as an example of a well-characterized and safe production strain with a long history of safe use. It is widely recognized as a harmless contaminant found in many foods (Ref. 5).

B. licheniformis has been used in the fermentation industry for the production of enzymes, antibiotics, and other specialty chemicals and was exempted from EPA review under TSCA (Ref. 6). Various enzymes have been produced by *B. licheniformis* and are GRAS substances (α -amylases – GRASP 3G0026 and 0G0363 and GRN 22, 24, 79; and pullulanase – GRN 72). In addition, *B. licheniformis* is classified as a Risk Group 1 organism according to the National Institutes of Health Guidelines for Research Involving Recombinant Molecules. Risk Group 1 organisms are those not associated with disease in healthy adult humans.

The *B. licheniformis* host strain is derived from a safe strain lineage comprising production strains for more than 10 enzyme preparations which have full toxicological safety studies (i.e. 13-week oral toxicity study in rats, Ames test and chromosomal aberration test or micronucleus assay).

An essential aspect of the safety evaluation of food components derived from genetically modified organisms is the identification and characterization of the inserted genetic material (Ref. 5, 7-11). The methods used to develop the genetically

modified production organism and the specific genetic modifications introduced into the production organism are described in Section 2.

This notification includes information that addresses the safety of the enzyme source, the enzyme component, the manufacturing process and a consideration of dietary exposure which covers all the issues relevant to a safety evaluation of an enzyme preparation. Based on critical review and evaluation of its published and unpublished information, Novozymes concludes through scientific procedures that the subject of this notification, meeting appropriate food grade specifications and produced in accordance with current good manufacturing practices, is GRAS for the intended conditions of use.

2. PRODUCTION MICROORGANISM

2.1 Production Strain

The *B. licheniformis* production strain, designated HyGe329, was derived via the recipient strain BW302 from a natural isolate of *B. licheniformis*, American Type Culture Collection Strain (ATCC) 9789. This ATCC 9789 strain is the ancestor of *B. licheniformis* strains that have been used safely for industrial production of enzymes marketed and sold by Novozymes since 1972. The classification of the ATCC strain as well as this production organism is based on generally accepted taxonomic characteristics (Ref. 12). This genetically modified production organism complies with the OECD (Organization for Economic Co-operation and Development) criteria for GILSP (Good Industrial Large Scale Practice) microorganisms (Ref. 13). It also meets the criteria for a safe production microorganism as described by Pariza and Foster (Ref. 1) and later Pariza and Johnson (Ref. 2) and several expert groups (Ref. 5, 7-11).

The xylanase expression plasmid, pBW120 used in the strain construction contains strictly defined chromosomal DNA fragments and synthetic DNA linker sequences. The specific DNA sequences include i) a gene encoding an engineered variant of the xylanase enzyme; ii) well-characterized non-coding regulatory sequences including a transcription terminator from *B. licheniformis* and a hybrid promoter containing different *Bacillus* promoter regions.

2.2 Recipient Strain

The recipient strain used in the construction of the xylanase production strain, was modified at several chromosomal loci to cause deletion of genes encoding a number of proteases. Also a gene essential for sporulation was deleted, eliminating the ability to sporulate, together with three additional genes encoding unwanted peptides. The lack of these peptides and proteins represents improvements in product safety and stability.

2.3 Xylanase Expression Plasmid

The expression plasmid, pBW120, used to transform the *B. licheniformis* host strain, BW302, is based on the well-known *Bacillus* vector pE194 (Ref. 14) from *Staphylococcus aureus* and a standard *E. coli* vector. No elements of these vectors are left in the production strain. The plasmid contains the expression cassette consisting of a fragment of a hybrid *Bacillus* promoter with promoter elements from *B. licheniformis*, *B. amyloliquefaciens* and *B. thuringiensis*, the xylanase coding sequence and a terminator. This promoter is followed by a chemically synthesized xylanase based on sequence data from a public database. According to Yoon *et. al.*, 1998 (Ref. 15), the gene is derived from a thermophilic soil bacterium *Bacillus sp. KK-1* and codes for a GH8 xylanase (Ref. 16). Based on homology assessment of the GH8 xylanase to public databases showing 98.5% homology to another xylanase from *Bacillus licheniformis*, it is concluded that the strain *Bacillus sp. KK-1* is belonging to the same species, *Bacillus licheniformis*. The xylanase variant, *xyI264*, is a genetically engineered variant of the GH8 xylanase gene with a single amino acid residue difference compared to the wild type sequence. Finally a *B. licheniformis* terminator sequence is inserted to terminate transcription. Following the terminator, an integration fragment is inserted that enables site specific integration on the genome of the recipient strain. Only the expression cassette with elements between the promoter fragment and the integration fragment are present in the final production strain. This has been confirmed by Southern blot analysis and PCR analysis followed by DNA sequencing.

2.4 Construction of the Recombinant Microorganism

The production strain, *B. licheniformis* HyGe329, was constructed from BW302 through the following steps:

- 1) A conjugation donor strain harboring pBW120 was used to mobilize pBW120 into the recipient strain BW302.
- 2) Plasmid pBW120 was integrated into two specific loci in strain BW302 by targeted homologous recombination to these loci using a two-step integration approach. Integration of the expression cassette at these loci allows the expression of the *xyI264* gene from the hybrid promoter and the transcriptional terminator.
- 3) The resulting two-copy *xyI264* strain was named HyGe329.

Sequence confirmation of the inserted expression cassettes and the flanking regions at both of the integration loci was performed in the production strain.

2.5 Stability of the Introduced Genetic Sequences

The presence of the introduced DNA sequences was also determined by Southern hybridization to assess the stability and potential for transfer of genetic material as a component of the safety evaluation of the production microorganism. The transforming DNA is stably integrated into the *B. licheniformis* chromosome and, as such, is poorly mobilizable for genetic transfer to other organisms and is mitotically stable.

2.6 Antibiotic Resistance Gene

No functional antibiotic resistance genes were left in the strain as a result of the genetic modifications. The absence of these genes was verified by Southern blot analysis using the relevant antibiotic resistance gene probes.

2.7 Absence of Production Organism in Product

The absence of the production organism is an established specification for the commercial product. The production organism does not end up in food and therefore the first step in the safety assessment as described by IFBC (Ref. 5) is satisfactorily addressed.

3. MANUFACTURING PROCESS

This section describes the manufacturing process for the xylanase preparation which follows standard industry practices (Ref. 17-19). The quality management system used in the manufacturing process for the xylanase preparation complies with the requirements of ISO 9001. It is produced under a standard manufacturing process as outlined by Aunstrup et al, 1979 and 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. The enzyme preparation complies with the purity criteria recommended for enzyme preparations as described in the Food Chemicals Codex (Ref. 20). It also conforms to the General Specifications for Enzyme Preparations Used in Food as proposed by JECFA (Ref. 21).

3.1 Raw Materials

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The raw materials used in the fermentation and recovery process for the xylanase enzyme concentrate are standard ingredients used in the enzyme industry (Ref. 17-19). 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, the raw materials are sampled by the Quality Control Department and subjected to the appropriate analyses to ensure their conformance to specifications.

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

3.2 Fermentation Process

The xylanase preparation is manufactured by submerged fed-batch pure culture fermentation of the genetically modified strain of *B. licheniformis* described in Section 2. All equipment is carefully designed, constructed, operated, cleaned, and maintained so as to prevent contamination by foreign microorganisms. During all steps of fermentation, physical and chemical control measures are taken and microbiological analyses are done to ensure absence of foreign microorganisms and confirm strain identity.

3.2.1 Production Organism

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

3.2.2 Criteria for the Rejection of Fermentation Batches

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

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

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

Any contaminated fermentation is rejected.

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3.3 Recovery Process

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

3.3.1 Purification Process

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

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

3.3.2 Formulation and Standardization Processes

The stabilized concentrate is blended with water and glycerol and preserved with sodium benzoate and potassium sorbate. The product is standardized according to the product specification.

3.4 Quality Control of Finished Product

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

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4. ENZYME IDENTITY

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

Classification	Xylanase
IUB nomenclature:	endo-1, 4-beta-xylanase
IUB No.:	3.2.1.8
CAS No.:	9025-57-4
Specificity:	hydrolyses the xylosidic linkages in the arabinoxylan backbone resulting in a depolymerization of the arabinoxylans into smaller oligosaccharides.
Amino acid sequence:	the total nucleotide and amino acid sequences have been determined

5. COMPOSITION AND SPECIFICATIONS

The xylanase enzyme preparation is presently available in a granulated formula for use in food applications.

5.1 Quantitative Composition

The xylanase preparation has the following typical composition:

Xylanase (endo-1, 4-)	approx.	4 %
Wheat Flour	approx.	90 %
Sodium Chloride	approx.	6 %

5.2 Specifications

The xylanase preparation complies with the purity criteria recommended for enzyme preparations as described in Food Chemicals Codex (Ref. 20). 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 (Ref. 21).

6. APPLICATION

6.1 Mode of Action

The active enzyme is endo-1, 4-beta-xylanase (IUB 3.2.1.8), which hydrolyses xylosidic linkages in the arabinoxylan backbone resulting in a depolymerization of the arabinoxylans into smaller oligosaccharides.

Arabinoxylans are highly branched xylans that are characteristic for the outer cell walls and endosperm of cereals such as wheat, barley, rye, and oat. Wheat flour contains 2-4% arabinoxylans, which play an essential role in bread making.

The xylanase preparation is used to modify the arabinoxylans, resulting in improved dough handling and characteristics of the final bread.

6.2 Use Levels

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

The xylanase preparation is standardized to a xylanase activity of 2350 NXU/g. For the baking applications, the recommended dosage is up to 11.75 NXU/kg flour, corresponding to 0.5 g of xylanase enzyme preparation per 100 kg flour.

6.3 Enzyme Residues in the Final Food

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

7. SAFETY EVALUATION

7.1 Safety of the Production Strain

The safety of the production organism must be the prime consideration in assessing the degree of safety of an enzyme preparation intended for use in food (Ref. 1, 2). If the organism is non-toxicogenic and non-pathogenic, then it is assumed that food or food ingredients produced from the organism, using current Good Manufacturing Practices, is safe to consume (Ref. 5). Pariza and Foster (Ref. 1) define a non-toxicogenic organism as "one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure" and a nonpathogenic organism as "one that is very unlikely to produce disease under ordinary circumstances". *B. licheniformis* is not a human pathogen and it is not toxicogenic (Ref. 3, 4). *B. licheniformis* has a long history of safe industrial use for the production of enzymes used in human food. It is widely recognized as a harmless contaminant found in many foods (Ref. 5). *B. licheniformis* has been used in the fermentation industry for the production of enzymes, antibiotics, and other specialty chemicals. Various enzymes are produced by *B. licheniformis* and are considered GRAS substances (α -amylases – GRASP 3G0026 and 0G0363 and GRN

22, 24, 79; and pullulanase – GRN 72). In addition, *B. licheniformis* is classified as a Risk Group 1 organism according to the National Institutes of Health Guidelines for Research Involving Recombinant Molecules. Risk Group 1 organisms are those not associated with disease in healthy adult humans.

An evaluation of the genetically modified *B. licheniformis* production microorganism according to the concepts initially outlined by Pariza and Foster (Ref. 1) and further developed by IFBC in 1990 (Ref. 5), the EU SCF in 1991 (Ref.7), the OECD in 1993 (Ref. 8), FAO/WHO in 1996 (Ref.10), ILSI Europe Novel Food Task Force in 1996 (Ref. 11), and updated by Pariza and Johnson in 2001 (Ref. 2) demonstrates the safety of this genetically modified production microorganism strain. The components of this evaluation: the identity of the host strain, a description of the introduced DNA sequences, the sources and functions of the introduced genetic material, an outline of the genetic construction of the production strain, and some characteristics of the production strain and the enzyme derived from it are given in Sections 2 and 3.

The *B. licheniformis* host strain is derived from a safe strain lineage comprising production strains for more than ten enzyme preparations which have full toxicological safety studies (i.e. 13 week oral toxicity study in rats, Ames test and chromosomal aberration test or micronucleus assay), cf. section 7.1.1 below.

As outlined in section 2.3, the product gene is not isolated from a donor strain, but fully synthesized based on sequence data from a public database. The product gene has been altered to allow for a single amino acid difference in the mature amino acid sequence compared to the wild type xylanase.

The genetic modifications are well characterised and specific utilizing well-known plasmids for the vector constructs, and the introduced genetic material does not encode and express any known harmful or toxic substances. The production strain is considered a safe strain for the production of the xylanase enzyme.

7.1.1 Safe Strain Lineage

The safety of this *B. licheniformis* production strain was established following published criteria for the assessment of the safe use of microorganisms used in the manufacture of food ingredients (Ref. 2, 5). The recipient strain, *B. licheniformis* BW302, has been thoroughly characterized as shown in section 2.2. The introduced DNA is well-known and characterized in sections 2.3 and 2.4. The procedures used to modify the recipient organism are well defined and commonly used. Therefore, the elements needed to establish a safe strain lineage as defined in Pariza and Johnson, 2001 (Ref. 2) have been met.

Novozymes used the decision tree (Appendix 3) in Pariza and Johnson 2001 (Ref.2) as a basis for our safety assessment. The production strain is genetically modified by rDNA techniques as discussed in section 2. The expressed enzyme product is a xylanase. The enzyme preparation is free of DNA encoding transferable antibiotic

resistance genes. The introduced DNA is well characterized and safe for the construction of microorganisms to be used in the production of food grade products. The DNA is stably integrated into the chromosome at two specific sites in the chromosome and the incorporated DNA is known not to encode or express any harmful or toxic substances. Novozymes has extensive experience working with *B. licheniformis* production strains and has developed expertise in identifying and characterizing these strains in order to prevent contamination and ensure continuing acceptable, economic yields of a functional enzyme product. Research scientists, fermentation engineers, chemical operators, and quality control technicians follow standard aseptic microbiological procedures as well as specific Novozymes protocols for monitoring the biological activity, growth, and physiological characteristics of the production organism during strain improvement programs and during large scale industrial fermentations. In addition, the final commercial enzyme product must perform reproducibly, meet Novozymes technical service department requirements, and consistently meet the needs of customers in the food industry. All of these periodic and continuous monitoring activities serve not only to guarantee customer satisfaction with Novozymes enzyme products, but also indicate that no unexpected secondary effects of the genetic modifications have been observed. Furthermore, the information included in this GRN has been reviewed by Novozymes Regulatory Affairs staff for suitability.

For the xylanase preparation which is the subject of this GRN, we have conducted a 13 week oral rat feeding study, an Ames test and an *in vitro* micronucleus assay as discussed in section 7.5.

Finally the production strain is derived from a safe lineage. Novozymes has used *B. licheniformis* production strains for over 20 years. An overview of some of the Novozymes products produced by *B. licheniformis* strains and the safety studies conducted on those products is shown below (Table 1).

Table 1

Enzyme	IUB no	Predecessor strain ¹	Donor strain	Safety studies ²
Alpha-amylase (GRASP 3G0026)	3.2.1.1	<i>Bacillus licheniformis</i> (ATCC 9789)	none	Yes
Alpha-amylase (GRASP 0G0363)	3.2.1.1	<i>Bacillus licheniformis</i> DN2717	<i>Bacillus stearothermophilus</i>	Yes
Alpha-amylase (GRN 22)	3.2.1.1	<i>Bacillus licheniformis</i> SJ1707	<i>Bacillus licheniformis</i>	Yes
Alpha-amylase (GRN 24)	3.2.1.1	<i>Bacillus licheniformis</i> SJ1707	<i>Bacillus stearothermophilus</i>	Yes
Cyclodextrin glucanotransferase	2.4.1.19	<i>Bacillus licheniformis</i> SJ1707	<i>Thermoanaerobacter sp.</i>	Yes
Alpha-amylase	3.2.1.1	<i>Bacillus licheniformis</i> SJ1904	<i>Bacillus licheniformis</i>	Yes
Alpha-amylase	3.2.1.1	<i>Bacillus licheniformis</i> MDT223	<i>Bacillus stearothermophilus</i>	Yes
Protease	3.4.21.1	<i>Bacillus licheniformis</i> MDT223	<i>Nocardiosis prasina</i>	Yes
Alpha-amylase	3.2.1.1	<i>Bacillus licheniformis</i> MDT223	<i>Bacillus amyloliquefaciens</i>	Yes
Alpha-amylase	3.2.1.1	<i>Bacillus licheniformis</i> MDT223	<i>Bacillus licheniformis</i>	Yes

Table 1. Novozymes products derived from *B. licheniformis* strains ¹The predecessor strain shows strains in the GM construction pathway that are in common with the BW302 strain lineage. ²At least the following: *in vitro* test for gene mutations in bacteria (Ames); *in vitro* test for chromosomal aberration or *in vitro* micronucleus assay; 13 week sub chronic oral toxicity study in rats.

As shown (Table 1), safety studies have been performed for the same enzyme in different strains in the lineage, supporting the fact that the genetic modifications performed in the *B. licheniformis* strain lineage of the host strain BW302 do not result in safety concerns. Additionally, no safety issues are observed when different products that are produced in the same strain (e.g., amylases and proteases) are investigated, demonstrating that the safety of the strains in the lineage is not product-dependent.

Novozymes has repeatedly used the procedures outlined by Pariza and Johnson 2001 (Ref. 2) to evaluate the enzymes derived from *B. licheniformis* production strains. Therefore, following the evaluation outlined in this section this production strain is considered to be derived from a safe lineage and is safe for use in the production of enzyme preparations for use in food.

7.2 Safety of the Donor Organism

The donor for the xylanase enzyme is *Bacillus sp.KK-1*. The product gene is not isolated from a donor strain but chemically synthesized, based on sequence data from a public database. See section 7.1 on the safety of *B. licheniformis*.

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7.3 Safety of the Xylanase Enzyme

A wide variety of enzymes are used in food processing (Ref. 1, 2). Enzyme proteins do not generally raise safety concerns (Ref. 1, 2, 22, 23). Exceptions could include enzymes that produce substances that are not ordinarily digested and metabolized or that produce toxic substances (Ref.24). Pariza and Foster (Ref. 1) note that very few toxic agents have enzymatic properties. The safety of the xylanase was assessed using the Pariza and Johnson (2001) decision tree (Appendix 3).

As indicated in section 4, the subject of this GRAS notification is a xylanase EC 3.2.1.8. Enzymes including xylanases have a long history of use in food (Ref. 1, 2) and animal feed (Ref 25). Xylanases have been used extensively for more than 25 years in various industrial food applications such as starch processing, manufacturing of alcohol, brewing and baking products (Ref. 26).

7.3.1 Consideration of the Allergenic Potential of the Xylanase Enzyme

The ingestion of a food enzyme protein is not considered a concern for 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 majority of proteins are not food 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*, 2006, Ref. 27). The investigation comprised enzymes produced by wild-type and genetically modified strains as well as wild-type enzymes and Protein Engineered variants and comprised 400 patients with a diagnosed allergy to inhalation allergens, food allergens, bee or wasp. It was concluded from

this study that ingestion of food enzymes in general is not likely to be a concern with regard to food allergy.

In order to further evaluate the possibility that the xylanase will cross-react with known allergens and induce a reaction in an already sensitized individual, a sequence homology to known food allergens was assessed. Following the guidelines developed by FAO/WHO, 2001 (Ref. 28) and modified by Codex Alimentarius Commission, 2009 (Ref. 29) the xylanase was compared to allergens from the FARRP allergen protein database (<http://allergenonline.org>) as well as the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee (<http://www.allergen.org>). A search for more than 35% identity in the amino acid sequence of the expressed protein using a window of 80 amino acids and a suitable gap penalty showed no matches. Alignment of the xylanase to each of the allergens and identity of hits with more than 35% identity over the full length of the alignment was analyzed. No significant homology was found between the xylanase and any of the allergens from the databases mentioned above. Consequently, oral intake of the xylanase is not anticipated to pose any food allergenic concern.

7.4 Safety of the Manufacturing Process

The xylanase meets the purity specifications for enzyme preparations as outlined in the monograph on Enzyme Preparations in the Food Chemicals Codex. As described in Section 3, the xylanase preparation is produced in accordance with current good manufacturing practices, using ingredients that are acceptable for general use in foods, and under conditions that ensure a controlled fermentation. These methods are based on generally available and accepted methods used for production of microbial enzymes (Ref. 17-19).

7.5 Safety studies

This section describes the studies and analysis performed to evaluate the safety of the use of the xylanase object of this GRN.

7.5.1 Description of Test Material

The toxicological testing of the xylanase was conducted on a batch of xylanase concentrate (batch (b) (4)) which was produced according to the description given in section 3. The test batch was a xylanase enzyme concentrate without addition of additives or other standardization or stabilization ingredients.

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7.5.2 Studies

The following studies were performed on test batch (b) (4) with favourable results:

- 13 Week Oral Toxicity, daily dosing, rats
- Reverse Mutation Assay (Ames test)
- *in vitro* micronucleus assay

These tests are summarized in Appendix 4.

7.6 Estimates of Human Consumption and Safety Margin

The enzyme is largely heat inactivated during the baking process. However, in order to illustrate a “worst case” situation the following calculations are made assuming that all enzyme activity is retained in the baking product.

The xylanase preparation has an activity of 2350 NXU/g and an approximate content of 4% TOS (Total Organic Substances from the fermentation, mainly protein and carbohydrate components).

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

United Kingdom

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

Denmark

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

USA

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

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

- a) The calculation is made assuming that all TOS remains in the baking product. The xylanase preparation contains 4 % TOS.
- b) It is assumed that all baking products are produced using the xylanase preparation as a processing aid, used at the highest recommended dosage.

The maximum recommended dosage of the xylanase preparation is up to 11.75 NXU/kg flour, corresponding to 5 mg per kg flour. The xylanase preparation contains 4 % TOS. Using a standard recipe, 1 kg flour results in 1.4 kg bread, giving a theoretical content of 0.143 mg TOS/kg bread.

Based on the highest average daily intake of baking products (158 g), the daily intake per person corresponds to $0.143 \times 0.158 = 0.023$ mg TOS per day.

For an average person weighing 60 kg this corresponds to 0.376×10^{-3} mg / kg body weight per day.

7.6.2 Safety margin

The safety margin is calculated as dose level with no adverse effect (NOAEL) divided by the estimated human consumption. The NOAEL dose level in the 13 weeks oral toxicity study in rats was 10 ml/kg/day corresponding to 1020 mg TOS/kg/day (Appendix 4).

The estimated human consumption is 0.376×10^{-3} mg TOS/kg/day

The safety margin can thus be calculated to be: $1020 / 0.376 \times 10^{-3} = 2.7 \times 10^6$

7.7 Results and Conclusion

On the basis of the evaluation contained in Section 7, a review of the published literature, the history of use of *B. licheniformis* and the limited and well defined nature of the genetic modifications, the xylanase preparation can be safely manufactured and used for its intended use in food.

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8. LIST OF APPENDICES

1. 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.
2. de Boer AS, Priest F, Diderichsen B. On the Industrial Use of *Bacillus licheniformis*: a review. Appl. Microbiol. Biotechnol. 40, 595-598, 1994.
3. Pariza and Johnson Decision Tree analysis
4. Summary of Toxicity Studies

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Pages 000027-000040 have been removed in accordance with copyright laws. The removed reference is:

Michael W. Pariza, *Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing: Update for a New Century*, *Regulatory Toxicology and Pharmacology* 33, 173-186 (2001)

Pages 000042-000045 have been removed in accordance with copyright laws. The removed reference is:

De Boer AS, *On the industrial use of Bacillus licheniformis: a review*, Appl Microbiol Biotechnol (1994) 40: 595-598

Appendix 3- Pariza & Johnson Decision Tree analysis of a xylanase produced by a genetically modified strain of *Bacillus licheniformis*

This modified xylanase produced by a genetically modified strain of *Bacillus licheniformis* was evaluated according to the decision tree published in Pariza and Johnson, 2001. The result of the evaluation is presented below.

Decision Tree

1. Is the production strain genetically modified?

YES

The production strain *Bacillus licheniformis* HyGe329 is derived via the recipient BW302 from a natural isolate of *B. licheniformis*.

If yes, go to 2.

2. Is the production strain modified using rDNA techniques?

YES

If yes, go to 3.

3. Issues relating to the introduced DNA are addressed in 3a-3e.

- a. Does the expressed enzyme product which is encoded by the introduced DNA have a history of safe use in food?

Yes

Xylanases have been used extensively in various industrial food applications for more than 25 years. The xylanase which is the subject of this GRN is substantially equivalent to wild type xylanases due to only one amino acid change. The xylanase is produced by a production strain that is from a safe strain lineage with an extended history of safe use.

If yes go to 3c. If no, go to 3b.

- b. Is the NOAEL for the test article in the appropriate short-term oral studies sufficiently high to ensure safety? (Not required since 3a is YES however the answer here is also YES)

YES

For the xylanase preparation which is the subject of this GRN, we have conducted a 13 week oral rat feeding study, an Ames test and an in vitro micronucleus assay. The NOAEL obtained from the 13 week oral rat feeding study provides for a very high safety margin, thus confirming the safety for its intended use.

- c. Is the test article free of transferable antibiotic resistance gene DNA?

YES

No functional antibiotic resistance genes were left in the strain as a result of the genetic modifications. The absence of these genes was verified.

If yes go to 3e.

- e. Is all other introduced DNA well characterized and free of attributes that would render it unsafe for constructing microorganisms to be used to produce food products?

YES

The genetic modifications are well characterized and specific and the incorporated DNA does not encode and express any known harmful or toxic substances.

If yes, go to 4.

4. Is the introduced DNA randomly integrated into the chromosome?

NO

Site specific integration of the DNA was achieved at two selected loci of the *B. licheniformis* chromosome. Sequence confirmation was performed in the production strain.

If no, go to 6.

6. Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure?

YES

The production strain is a *B.licheniformis* derived via the recipient *B. licheniformis* BW302 which is from a natural isolate. Novozymes has safely used *B. licheniformis* production strains derived from this isolate for industrial production of enzymes since 1972 (including an enzyme preparation that is the subject of the GRAS affirmation petition 3G0026 submitted by Novo Nordisk and affirmed by FDA in 1983).

If yes, the test article is **ACCEPTED**.

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Toxicology

Date : 27 February 2013
File : 2013-03354-01
Ref.: BTR/PBjP

SUMMARY OF TOXICITY DATA

Xylanase, (b) (4) from *Bacillus licheniformis*

Authors:
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Issued by:
Novozymes A/S
Krogshoejvej 36

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1. ABSTRACT

The below series of toxicological studies were undertaken to evaluate the safety of Xylanase, batch (b) (4).

All studies were carried out in accordance with current OECD guidelines and in compliance with the OECD principles of Good Laboratory Practice (GLP). The studies were performed at Novozymes A/S, Denmark, (b) (4), England and (b) (4) S, Denmark during the period May 2012 to February 2013.

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

- Xylanase, (b) (4) did not induce gene mutations in the Ames test, neither in the presence or absence of S-9 mix.
- Xylanase, (b) (4) did not show any clastogenic activity, neither in the presence or absence of S-9 mix, when tested in the *in vitro* micronucleus assay.
- In a 13 weeks oral toxicity study in rats Xylanase, (b) (4) was well tolerated and did not cause any toxicologically significant changes at any dose level.

Based on the present toxicity data it can be concluded that Xylanase, represented by batch (b) (4), exhibits no toxicological effects under the experimental conditions described.

2. TEST SUBSTANCE

Xylanase is a liquid enzyme concentrate containing an Endo-1,4-beta-xylanase (E.C. number 3.2.1.8) which degrades starch (amylopectin and amylose) by hydrolysis of α -1,4-glycosidic linkages, forming dextrans and maltose.

2.1 Production organism

Xylanase is produced by a strain of *Bacillus licheniformis*, containing a genetically engineered variant of the GH8 xylanase gene obtained from a *Bacillus sp.* strain. This genetically modified production strain meets the criteria for a safe production microorganism. It is constructed by common transformation procedures using well-known plasmid vectors with strictly defined and well-characterized DNA sequences that are not known to encode or express any harmful or toxic substances. The strain is free of any antibiotic resistance marker. The development of the production strain was evaluated at every step to assess incorporation of the desired functional genetic information and to ensure that no unintended sequences were incorporated.

Bacillus licheniformis has long history of safe use. This species has been used for decades in the production of enzymes, and in the last decade as recombinant organism for production of a variety of bio-industrial products.

Bacillus licheniformis is generally regarded as non-pathogenic and non-toxicogenic. Further investigations have revealed that the strain lineage to which the production strain belongs do not produce any known *Bacillus* toxins.

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

2.2 Characterization

The toxbatch (b) (4) was used for the conduct of all the toxicological studies. The characterization of the toxbatch is presented in Table 1.

Table 1. Characterization data of Xylanase, batch (b) (4) 2

Batch number	(b) (4) 2
Activity	3670 GH8XU/g
Water (KF) (% w/w)	88.3
Dry matter (% w/w)	11.7
Ash (% w/w)	2.0
Total Organic Solids (TOS ¹)	9.7%
Specific gravity (g/mL)	1.052

¹ % TOS is calculated as 100% - % water - % ash - % diluents.

3. MUTAGENICITY

3.1 Bacterial Reverse Mutation assay (Ames test)

Xylanase, (b) (4) was examined for mutagenic activity in the bacterial reverse mutation assay using *Salmonella typhimurium* strain TA1535, TA100, TA1537, and TA98 and *Escherichia coli* WP2uvrA. The study was carried out according to the OECD test guideline 471 (adopted in 1997) and in compliance with GLP.

Crude enzyme preparations, like the present batch of Xylanase, contain the free amino acids histidine and tryptophan, most often in an amount, which exceeds the critical concentration for incorporation in the direct standard assay.

To overcome this problem all strains were exposed to Xylanase in liquid culture ("treat and plate assay").

Two independent experiments were performed, with and without the inclusion of metabolic activation (S-9 mix). In each experiment cultures of bacteria were exposed to six doses of the test substance (5000, 2500, 1250, 625, 313, and 156 µg dry matter/mL) in a phosphate buffered nutrient broth for 3 hours. After incubation, the test substance was removed by centrifugation prior to plating.

No treatments of any of the bacterial strains with the test substance resulted in dose related and reproducible increases in revertant numbers that exceeded a doubling in the mean number of revertants per plate compared to the appropriate solvent control either in the presence or absence of S-9 mix.

The results obtained with the diagnostic mutagens and the solvent control demonstrated the sensitivity of the tests and the efficacy of the S-9 mix metabolic activation system.

It was concluded that Xylanase, (b) (4) did not induce gene mutations in bacteria either in the presence or absence of metabolic activation when tested under the conditions employed in this study.

3.2 *In vitro* Micronucleus assay

In order to assess the clastogenic and the aneugenic activity of Xylanase, (b) (4) its effects on the frequency of micronuclei was investigated in cultured human peripheral blood lymphocytes applying the cytokinesis-block methodology.

The study was conducted according to GLP, in compliance with the OECD draft guideline: Genetic Toxicology: OECD Guideline for the testing of chemicals. Guideline 487: *In vitro* micronucleus test (2009).

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

Sets of duplicate cultures were treated with the solvent (purified water), test substance or appropriate positive controls. Treatments with the test substance covered a broad range of doses, separated by narrow intervals. The highest concentrations used was 5000 µg/mL (expressed in terms of the test substance as supplied), which is the highest dose level recommended in the guidelines for *in vitro* cytogenetic assays.

Cell cultures were exposed to the test substance for 3 hours in the presence and absence of metabolic activation (S-9 mix) and harvested 24 hours after the beginning of treatment (3+21 hour treatment). Additionally, a continuous 24-hour treatment without S-9 mix was included with harvesting 24 hours after removal of the test substance (24+24 hour treatment). The cultures were treated with cytochalasin-B after removal of the test substance. Three concentrations, covering an appropriate range of cytotoxicity, were selected for scoring of micronuclei by evaluating the effect of the test substance on the replication Index (RI). 2000 cells per concentration (1000 cells from each replicate culture) were scored.

The proportion of binucleate cells with micronuclei in all cultures of the vehicle 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.

Treatment of the cells with the test substance resulted in frequencies of micronucleated binuclear cells (MNBN cells), which were similar to and not significantly ($p \leq 0.05$) higher than those observed in concurrent vehicle controls for all concentrations analysed.

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It was concluded that Xylanase, (b) (4) did not induce micronuclei in cultured human peripheral blood lymphocytes either in the absence or presence of S-9 mix under the experimental conditions employed for this study.

4. GENERAL TOXICITY

4.1 A 90-Day Gavage Toxicity Study in Rats

The objective of this study conducted at CiToxLAB Scantox A/S, was to assess the systemic toxicity of Xylanase, (b) (4), when administered daily by oral treatment (gavage) to rats for 90/91 days.

The study was conducted in 40 male and 40 female SPF Sprague Dawley rats of the Ntac:SD strain, approximately 5 weeks old. The animals were randomly allocated to four groups: Group 1 (Vehicle (Ion-exchanged water), 0 mg TOS/kg bw and 0 GH8XU/kg), Group 2 (102 mg TOS/kg bw and 3861 GH8XU/kg), Group 3 (336.7 mg TOS/ kg bw and 12741 GH8XU/kg) and Group 4 (1020 mg TOS/kg bw and 38608 GH8XU/kg). Treatment was performed by oral treatment (gavage) once daily for 90/91 days with a dose volume of 10 mL/kg.

Clinical signs were recorded daily and once weekly detailed clinical observations outside the cage were performed. Body weight and food consumption were recorded once weekly, while water consumption was recorded twice weekly. Pre-treatment and before termination, the animals were examined with respect to motor activity (open field test) and reactivity to different types of stimuli. Blood and urine samples were collected from all animals before termination of treatment in Week 13 for evaluation of clinical chemistry (blood and urine) and haematology (blood) parameters. At termination of the study, the animals were euthanised and subjected to a full macroscopic examination and selected organs were weighed, fixed and examined histopathologically.

No treatment related signs were recorded at the clinical examination (clinical observations, open field and stimuli tests and ophthalmoscopy), on body weight gain, on food or water consumption. The test item had no treatment related effects on the clinical chemistry, haematology, and coagulation parameters or on the urinalysis and urine microscopy. At necropsy, no microscopic or macroscopic treatment related findings were observed.

In conclusion, 90/91-days of daily oral (gavage) treatment of rats with Xylanase, (b) (4), at dose levels of up to 1,020 mg TOS/kg bw/day or 38608 GH8XU/kg bw/day administered in a dose volume of 10 mL/kg bw/day did not cause any test item related changes. The NOAEL (No Observed Adverse Effect Level) for both females and males for Xylanase, (b) (4), was 1,020 mg TOS/kg bw/day corresponding to 38608 GH8XU/kg bw/day.

5. REFERENCES

5.1 Study reports

Novozymes A/S: Study No.: 20128049. Xylanase, (b) (4): Test for mutagenic activity with strains of *Salmonella typhimurium* and *Escherichia coli*. (October 2012). LUNA file: 2012-13362.

(b) (4) Laboratories: Study No.: 8261061. Novozymes Reference No.: 20126017: Xylanase, (b) (4) : Induction of micronuclei in cultured human peripheral blood lymphocytes. (July 2012). LUNA file: 2013-02893.

(b) (4) : Study No.: 74852. Novozymes Reference No.: 20126010: Xylanase, (b) (4) A 90-Day Gavage Toxicity Study in Rats. (February 2013). LUNA file: 2013-02669.

Ramos-Valle, Moraima

From: LOBG (Lori Gregg) <LOBG@novozymes.com>
Sent: Wednesday, May 08, 2013 3:11 PM
To: Ramos-Valle, Moraima
Subject: amendment to GRAS exemption claim for Novozymes' xylanase
Attachments: exemp claim.pdf

Dear Moraima,

As requested, I have amended the wording for the proposed 170.36 (c)(1)(v) in the GRAS exemption claim for our xylanase enzyme preparation. Please find the amended exemption claim attached.

If you have any questions or need additional information please don't hesitate to contact me.

Best Regards
Lori Gregg
Sr.Regulatory Specialist

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May 8, 2013

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 xylanase preparations produced by submerged fermentation of a genetically modified *Bacillus licheniformis* 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.*

Xylanase preparations produced by a genetically modified *Bacillus licheniformis* production strain

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

The enzyme preparation is intended for use in the food industry as a processing aid in the baking industry. The xylanase preparation is used to modify arabinoxylans in cereals such as wheat, barley, and oats, thereby improving dough handling and the characteristics of the final bread or food product. 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.*

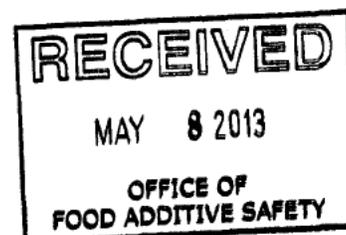
A notification package providing a summary of the information which supports this GRAS determination is enclosed with this letter. The package includes a safety evaluation of the production strain, the enzyme, and the manufacturing process, as well as an evaluation of dietary exposure. Complete data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

(b) (6)

Lori Gregg
Sr. Regulatory Specialist

5-8-2013
Date

000058



SUBMISSION END

000059