GRAS Notice (GRN) No. 476
http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/default.htm
May 30, 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' asparaginase enzyme preparation produced by a genetically modified strain of *Bacillus subtilis*. Novozymes has determined through scientific procedures that the asparaginase is generally recognized as safe for use in the food industry as a processing aid to reduce the formation of acrylamide in bread, potato and cereal based food products such as French fries, potato chips, cookies, crackers, and breakfast cereal. It can also be used in coffee and chocolate products.

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

Sincerely,

Lori Gregg
Sr. Regulatory Specialist

Enclosures (3 binders)
May 30, 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 asparaginase preparations produced by submerged fermentation of a genetically modified Bacillus subtilis 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.

Asparaginase preparation produced by a genetically modified Bacillus subtilis production strain

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

The enzyme preparation is intended for use as a processing aid to reduce formation of acrylamide in bread, potato and cereal based food products such as French fries, potato chips, cookies, crackers, and breakfast cereal. It can also be used in coffee and chocolate products. 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.

Lori Gregg
Sr. Regulatory Specialist
An asparaginase preparation produced by a genetically modified

*Bacillus subtilis* production strain

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

May 2013
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. GENERAL INTRODUCTION</td>
<td>3</td>
</tr>
<tr>
<td>2. PRODUCTION MICROORGANISM</td>
<td>4</td>
</tr>
<tr>
<td>2.1 Production Strain</td>
<td>4</td>
</tr>
<tr>
<td>2.2 Recipient Organism</td>
<td>4</td>
</tr>
<tr>
<td>2.3 Asparaginase Expression Plasmid</td>
<td>5</td>
</tr>
<tr>
<td>2.4 Construction of the recombinant microorganism</td>
<td>5</td>
</tr>
<tr>
<td>2.5 Stability of the Introduced Genetic Sequences</td>
<td>6</td>
</tr>
<tr>
<td>2.6 Antibiotic Resistance Gene</td>
<td>6</td>
</tr>
<tr>
<td>2.7 Absence of the Production Organism in Product</td>
<td>6</td>
</tr>
<tr>
<td>3. MANUFACTURING PROCESS</td>
<td>6</td>
</tr>
<tr>
<td>3.1 Raw Materials</td>
<td>6</td>
</tr>
<tr>
<td>3.2 Fermentation Process</td>
<td>7</td>
</tr>
<tr>
<td>3.3 Recovery Process</td>
<td>7</td>
</tr>
<tr>
<td>3.4 Quality Control of Finished Product</td>
<td>8</td>
</tr>
<tr>
<td>4. ENZYME IDENTITY</td>
<td>8</td>
</tr>
<tr>
<td>5. COMPOSITION AND SPECIFICATIONS</td>
<td>8</td>
</tr>
<tr>
<td>5.1 Quantitative Composition</td>
<td>8</td>
</tr>
<tr>
<td>5.2 Specifications</td>
<td>9</td>
</tr>
<tr>
<td>6. APPLICATION</td>
<td>9</td>
</tr>
<tr>
<td>6.1 Mode of Action</td>
<td>9</td>
</tr>
<tr>
<td>6.2 Use Levels</td>
<td>10</td>
</tr>
<tr>
<td>6.3 Enzyme Residues in the Final Food</td>
<td>10</td>
</tr>
<tr>
<td>7. SAFETY EVALUATION</td>
<td>10</td>
</tr>
<tr>
<td>7.1 Safety of the Production Strain</td>
<td>10</td>
</tr>
<tr>
<td>7.2 Safety of the Donor Organism</td>
<td>13</td>
</tr>
<tr>
<td>7.3 Safety of the Asparaginase Enzyme</td>
<td>13</td>
</tr>
<tr>
<td>7.4 Safety of the Manufacturing Process</td>
<td>15</td>
</tr>
<tr>
<td>7.5 Safety Studies</td>
<td>15</td>
</tr>
<tr>
<td>7.6 Estimates of Human Consumption and Safety Margin</td>
<td>16</td>
</tr>
<tr>
<td>7.7 Results and Conclusion</td>
<td>17</td>
</tr>
<tr>
<td>8. LIST OF APPENDICES</td>
<td>18</td>
</tr>
<tr>
<td>9. LIST OF REFERENCES</td>
<td>19</td>
</tr>
</tbody>
</table>
1. GENERAL INTRODUCTION

The subject of this notification is an asparaginase preparation produced by submerged fermentation of a genetically modified *Bacillus subtilis* microorganism expressing a gene coding for an asparaginase from *Pyrococcus furiosus*.

The enzyme is L-asparaginase (EC 3.5.1.1, CAS 9015-68-3). Asparaginase is intended to be used to convert asparagine to aspartic acid in order to reduce the formation of acrylamide during the production of potato and cereal food products such as French fries, potato chips, cookies, crackers, and breakfast cereal as well as coffee and chocolate products. Acrylamide occurs in carbohydrate (reducing sugar)-containing foods prepared by heating above 120°C, i.e. frying, grilling, baking, broiling.

The information provided in the following sections is the basis for our determination of general recognition of safety of this asparaginase enzyme preparation produced by *B. subtilis* expressing a gene encoding an asparaginase from *Pyrococcus furiosus*. Our safety evaluation in Section 7 includes an evaluation of the production strain including an evaluation of safe strain lineage, the introduced DNA, 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 food1,2 (Appendix 1). The production organism for this asparaginase, *Bacillus subtilis*, is discussed in Sections 2 and 7. *Bacillus subtilis* is generally considered to be non-pathogenic and non-toxigenic and is often mentioned as an example of a well-characterized and safe production strain with a long history of safe use.

The production organism is derived from the *Bacillus subtilis* A164 host strain that has been used for over 20 years by Novozymes in the development of strains to be used for manufacturing enzymes used as processing aids in the food industry. In section 7.1.1, we show the basis for a safe strain lineage for this production strain following the procedure outlined in Pariza and Johnson 2001 (Appendix 1).

The evaluation of the safety of the *Bacillus subtilis* host organism is very important since all, but a small fraction of the DNA, is that of the recipient host organism. 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. The genetic modifications used to construct the production microorganism are well defined and are described in Section 2. The recipient *B. subtilis* host strain, PP2982, is derived from the A164 line of host strains used in the construction of production strains for six other Novozymes enzyme products. For each of these production strains a host strain in the *B. subtilis* A164 lineage was used as a host strain for the construction of the final production strain by standard transformation procedures using well-known plasmid vectors and well-characterized DNA sequences.
In each case, the recombinant plasmid DNA was integrated into the *B. subtilis* host strain chromosome by homologous recombination. All of these production strains comply with the OECD (Organization for Economic Co-operation and Development) criteria for GILSP (Good Industrial Large Scale Practice) micro-organisms\(^9\) and meet the criteria for a safe production micro-organism as described by Pariza and Foster\(^2\), Pariza and Johnson\(^1\) and several expert groups\(^3\)-\(^8\). Toxicological testing, including 13 week oral toxicity in rats, Ames test, and human lymphocyte cytogenetic or micronucleus assay, confirming the safety of enzyme preparations derived from these six *B. subtilis* production strains, was done. No toxicological effects were observed for any of the test substances produced by strains derived from host strains in the *B. subtilis* A164 strain lineage. For this closely related *B. subtilis* production strain, we have completed the toxicology studies described in Section 7.5.

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. subtilis* production strain, designated MOL2940, was derived from the recipient strain, PP2982, via strain A164, which is identical to strain ATCC 6051a, the deposited type strain of *Bacillus subtilis*. 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. 10). It also meets the criteria for a safe production microorganism as described by Pariza and Foster\(^2\) and several expert groups\(^3\)-\(^8\).

The asparaginase expression plasmid, pMOL2930 used in the strain construction contains strictly defined chromosomal DNA fragments and synthetic DNA linker sequences. The specific DNA sequences introduced into the production strain include i) a gene encoding the asparaginase of *Pyrococcus furiosus* (*asnPfu*); 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 PP2982 used in the construction of the asparaginase production strain, was obtained from strain A164 by modifying several chromosomal loci to
introduce deletion of genes encoding a number of proteases. Also a gene essential for sporulation was deleted, eliminating the ability to sporulate, together with a gene essential for formation of surfactin. The lack of these peptides and proteins represents improvements in product safety and stability.

2.3 Asparaginase Expression Plasmid

The expression plasmid, pMOL2930, used to transform the *B. subtilis* recipient strain, is based on the well-known *Bacillus* vectors pE194 and pUB110 from *Staphylococcus aureus*. 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. thurigiensis*. This promoter is followed by a chemically synthesized asparaginase gene (*asnPfu*) based on sequence data from a public database. According to Maeder *et al.*, 1999\textsuperscript{10}, the gene codes for an L-asparaginase and is derived from the extremophile *Pyrococcus furiosus* (ATCC 43587) from thermal marine sediments. 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 element 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. subtilis* MOL2940, was constructed from the recipient strain PP2982 through the following steps:

1) A conjugation donor strain harbouring pMOL2930 was used to mobilize pMOL2930 into the recipient strain.

2) Plasmid pMOL2930 was integrated into three specific loci in strain PP2982 by targeted homologous recombination to these loci using a two-step integration approach. Integration of the expression cassettes at these loci allows the expression of the asparaginase gene *asnPfu* from the hybrid promoter and the transcriptional terminator.

3) The resulting three-copy asparaginase strain was named MOL2940.

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. subtilis chromosome and, as such, is poorly mobilizable for genetic transfer to other organisms and is mitotically stable.

2.6 Antibiotic Resistance Gene

The introduced DNA does not contain any genes encoding antibiotic resistance. The absence of these genes in the production strain 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 is satisfactorily addressed.

3. MANUFACTURING PROCESS

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

3.1 Raw Materials

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

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

The asparaginase is manufactured by submerged fed-batch pure culture fermentation of the genetically modified strain of *B. subtilis* 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. 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.

3.2.2 Criteria for the Rejection of Fermentation Batches

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

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

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

Any contaminated fermentation is rejected.

3.3 Recovery Process

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

3.3.1 Purification Process

The enzyme is recovered from the culture broth by a series of operations:
3.3.2 Formulation and Standardization Processes

The enzyme concentrate is stabilized with sodium chloride. The liquid product is formulated by preservation with sodium benzoate and potassium sorbate and standardized by addition of water and sorbitol. For the granulated product, dextrin is added to the liquid concentrate, which is then granulated and standardized with corn flour.

3.4 Quality Control of Finished Product

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

4. ENZYME IDENTITY

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

<table>
<thead>
<tr>
<th>Classification</th>
<th>Asparaginase</th>
</tr>
</thead>
<tbody>
<tr>
<td>IUB nomenclature:</td>
<td>L-Asparagine amidohydrolase</td>
</tr>
<tr>
<td>IUB No.:</td>
<td>3.5.1.1</td>
</tr>
<tr>
<td>CAS No.:</td>
<td>9015-68-3</td>
</tr>
<tr>
<td>Specificity:</td>
<td>hydrolyzes aspargine to aspartic acid</td>
</tr>
<tr>
<td>Amino acid sequence:</td>
<td>the total nucleotide and amino acid sequences have been determined</td>
</tr>
</tbody>
</table>

5. COMPOSITION AND SPECIFICATIONS

The asparaginase enzyme preparation is presently available in both a liquid and granulated formula for use in food applications.

5.1 Quantitative Composition

The enzyme concentrate is formulated into a granulated and a liquid commercial product.
The typical composition for the granulated product is:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme concentrate</td>
<td>0.8%</td>
</tr>
<tr>
<td>Corn flour</td>
<td>92%</td>
</tr>
<tr>
<td>Water</td>
<td>4%</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>3%</td>
</tr>
<tr>
<td>Dextrin</td>
<td>0.3%</td>
</tr>
</tbody>
</table>

The typical composition for the liquid product is:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme concentrate</td>
<td>0.8%</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>47%</td>
</tr>
<tr>
<td>Water</td>
<td>47%</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5%</td>
</tr>
<tr>
<td>Sodium benzoate</td>
<td>0.3%</td>
</tr>
<tr>
<td>Potassium sorbate</td>
<td>0.2%</td>
</tr>
</tbody>
</table>

5.2 Specifications

The asparaginase enzyme preparation complies with the recommended purity criteria for enzyme preparations as described in Food Chemicals Codex\textsuperscript{14}. 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\textsuperscript{15}.

6. APPLICATION

6.1 Mode of Action

The thermostable asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) enzyme hydrolyzes the amino acid asparagine to aspartic acid by hydrolyzing the amide in asparagine to the corresponding acid (=aspartic acid). The thermostable asparaginase shows very little if any activity on glutamine and it has no activity on other amino acids. It has no activity on asparagine residues in peptides or proteins.

Asparaginases are produced by a variety of microbes, including both bacteria and fungi\textsuperscript{16}. In many of these microbes, two asparaginases are found, a cytoplasmic form and a periplasmic or extra-cellular form. In the few cases where they have been compared, the extra-cellular asparaginases have a higher affinity for asparagines\textsuperscript{17-18}. Several asparaginases from bacteria are well characterized\textsuperscript{19}.

This asparaginase is to be used to reduce acrylamide in various food applications. Due to the thermostability of this enzyme, it works well at higher processing temperatures. Acrylamide is formed as a reaction product from asparagine and reducing sugars when food products are baked or fried at temperatures above...
120°C. Both asparagine and reducing sugars are commonly found in many food raw materials. By using asparaginase, the asparagine content will be reduced and hereby also the acrylamide in the final product. Typical dough based applications include: biscuits, crackers, crisp bread, tortilla chips, fabricated potato chips, pretzels, bread, etc. The enzyme can also be used for direct treatment of cut potatoes for production of French fries, sliced potato chips, potato flakes and potato granules. Because this asparaginase works well at high temperatures it can be used in the manufacture of breakfast cereals, pre-treatment of green coffee beans and other applications where food is processed at high temperatures.

6.2 Use Levels

The enzyme preparation is used at minimum levels necessary to achieve the required asparagine/acrylamide reduction and according to requirements for normal production following cGMP.

In general, the recommended use level is up to 15,000 TASU/kg dry matter.

6.3 Enzyme Residues in the Final Food

The enzyme treatment is taking place upstream and the enzyme is largely heat inactivated during subsequent heat treatment steps, i.e. extrusion and toasting of breakfast cereals, frying of potato based snacks or chips, and roasting of coffee beans.

7. SAFETY EVALUATION

7.1 Safety of the Production Strain

The safety of the production organism must be the prime consideration in assessing the probable degree of safety of an enzyme preparation intended for use in food\textsuperscript{1,2}. If the organism is nontoxigenic and nonpathogenic, then it is assumed that food or food ingredients produced from the organism, using current Good Manufacturing Practices, is safe to consume\textsuperscript{3}. Pariza and Foster (1983)\textsuperscript{2} define a nontoxigenic organism as "one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure" and a nonpathogenic organism as "one that is very unlikely to produce disease under ordinary circumstances". \textit{B. subtilis} meets these criteria for nontoxigenicity and nonpathogenicity.

\textit{B. subtilis} as a type species is not considered to be a pathogen\textsuperscript{20}. The species in general is often represented as an example of non-pathogenic micro-organisms\textsuperscript{21}. Because \textit{B. subtilis} meets the US Environmental Protection Agency (EPA) criteria for
nontoxigenicity and nonpathogenicity, it is one of 10 host organisms eligible for Tier I exemption under the EPA regulations\textsuperscript{22}.

\textit{B. subtilis} is widely distributed in nature, is a common contaminant in foods eaten by both man and animals, and has a history of safe use in food enzyme manufacturing\textsuperscript{23-25}. Carbohydrases and proteases from \textit{B. subtilis} are affirmed as GRAS by the US FDA and are covered in the regulations under 21 CFR 184.1148 and 21 CFR 184.1150. \textit{Bacillus subtilis} is described as the production organism for different enzymes in GRAS notifications 20, 114, 205, 274 and 406.

The host organism used for expressing the asparaginase enzyme was derived from \textit{B. subtilis}, which is a well-known and widely used microorganism in recombinant DNA research. The A164 line of \textit{Bacillus subtilis} host strains have been used for the construction of Novozymes production strains for many years. The (US) National Institute of Health has exempted sporulation-deficient strains of \textit{B. subtilis} from its Guidelines for Research involving Recombinant Molecules because it considers \textit{B. subtilis} to be harmless.

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

Because the genetic modifications are well characterized and specific, and the incorporated DNA does not encode and express any known harmful or toxic substances, the asparaginase enzyme preparation derived from the genetically modified \textit{B. subtilis} is considered safe\textsuperscript{3,26}.

A published paper “On the safety of \textit{Bacillus subtilis} and \textit{B. amyloliquefaciens}: a review” is enclosed in Appendix 2.

7.1.1 Safe Strain Lineage

The safety of this \textit{B. subtilis} production strain was established following published criteria for the assessment of the safe use of microorganisms used in the manufacture of food ingredients\textsuperscript{1,3}. The host strain, \textit{B. subtilis} PP2982, has been thoroughly characterized as shown in sections 2.2. The introduced DNA is well-known and characterized in section 2.3. The procedures used to modify the host organism are well defined and commonly used. Therefore, the elements needed to
establish a safe strain lineage as defined in Pariza and Johnson, 2001 have been met.

Novozymes' used the decision tree (Appendix 3) in Pariza and Johnson 2001 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 an asparaginase (See section 7.3). The enzyme preparation is free of transferable coding antibiotic resistance gene DNA. 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 three specific sites and the incorporated DNA is known not to encode or express any harmful or toxic substances. Novozymes has extensive experience working with B. subtilis 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.

Finally, the production strain is derived from a safe lineage. Novozymes has used B. subtilis production strains for over 20 years. Safety studies including 13 week oral toxicity in rats, Ames test, and human lymphocyte cytogenetic assay, have been performed on enzyme preparations from these production strains. No toxicological effects were ever observed for any of the test substances produced by these B. subtilis production strains.

The recipient host strain for this asparaginase, B. subtilis PP2982, is derived from the A164 line of host strains used in the construction of production strains for six other Novozymes' production strains. Safety studies including 13 week oral toxicity in rats, Ames test, and human lymphocyte cytogenetic assay or micronucleus assay, confirming the safety of enzyme preparations derived from these six B. subtilis production strains, was done. No toxicological effects were observed for any of the test substances thus supporting the view, that strains derived from B. subtilis A164 strain lineage can be used safely for the production of food enzymes. The same well known procedures have been used to construct this B. subtilis production strain. Therefore, we would not expect to see any negative results in similar toxicological studies.
For the asparaginase that is the subject of this notification, we have conducted a 13 week oral toxicity study in rats, an Ames test, an In vitro Micronucleus Test, and an In vitro cytotoxicity test (as discussed in section 7.5). The conclusion of these tests is that the test preparation is considered non-cytotoxic and does not induce gene mutations in bacteria under the conditions of the study. In the 13 week oral toxicity study, the test substance was well tolerated and did not cause any toxicologically significant changes at any dose level.

Novozymes has used the procedures outlined by Pariza and Johnson 2001¹ to evaluate the enzymes derived from *B. subtilis* 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 asparaginase enzyme is *Pyrococcus furiosus*. However, as noted above, the gene was synthesized to mimic the asparaginase gene from *Pyrococcus furiosus*. By synthesizing the asparaginase gene, it is ensured that no genetic material (target gene or other DNA) from the donor organism is found in the production strain.

### 7.3 Safety of the Asparaginase Enzyme

A wide variety of enzymes are used in food processing¹. Enzyme proteins do not generally raise safety concerns²,²⁰,²⁷. Exceptions could include enzymes that produce substances that are not ordinarily digested and metabolized or that produce toxic substances²⁸. Pariza and Foster² note that very few toxic agents have enzymatic properties. The safety of the asparaginase was assessed using the Pariza and Johnson (2001) decision tree (Appendix 3).

As indicated in section 4, the subject of this GRAS notification is asparaginase, EC 3.5.1.1. Because asparaginases have been isolated from a variety of sources, animal cells, plant cells and microbial cells²⁹, they are most likely part of the normal human diet. Asparaginases have been used in cancer research as anti-tumor agents over the last 20 years³⁰. Some asparaginases have been used clinically in the treatment of leukemia especially in children³⁰,³¹,³².

Asparaginase enzyme preparations are the subject of GRAS Notifications No. 201, 214, and 428.
7.3.1 Consideration of the Allergenic Potential of the 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 the 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)\textsuperscript{33}. 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 asparaginase 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\textsuperscript{34} and modified by Codex Alimentarius Commission, 2009\textsuperscript{35}, the asparaginase 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 asparaginase 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 asparaginase and any of the allergens from the databases mentioned above. Consequently, oral intake of the asparaginase is not anticipated to pose any food allergenic concern.

7.4 Safety of the Manufacturing Process

The enzyme preparation meets the purity criteria for enzyme preparations as outlined in the monograph on Enzyme Preparations in the *Food Chemicals Codex*\textsuperscript{14}. As described in Section 3, the enzyme 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\textsuperscript{11-13}.

7.5 Safety studies

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

7.5.1 Description of Test Material

The toxicological testing of the asparaginase was conducted on a batch of asparaginase concentrate (batch PPV 33595) which was produced according to the description given in section 3. The test batch was an asparaginase enzyme concentrate without addition of additives or other standardization or stabilization ingredients.

7.5.2 Studies

The following studies were performed:

- Test for mutagenic activity (Ames Test)
- *In vitro* Cytotoxicity Test: Neutral Red Uptake applying the BALB/c3T3 cells
- *In vitro* Micronucleus Test in Cultured Human Lymphocytes
- 13 weeks oral toxicity study in rats

These tests are described in Appendix 4. The conclusion of these tests is that the test preparation is considered non-cytotoxic and does not induce gene mutations in bacteria under the conditions of the study. In the 13 week oral toxicity study, the test substance was well tolerated and did not cause any toxicologically significant changes at any dose level.
7.6 Estimates of Human Consumption and Safety Margin

The enzyme is largely heat inactivated during the food manufacturing processes in which it is applied. However, in order to illustrate a "worst case" situation the following calculations are made assuming that all enzyme activity is retained in the final product.

The asparaginase has an activity of 6000 TASU/g and an approximate content of 0.8% TOS (Total Organic Substances from the fermentation, mainly protein and carbohydrate components). This corresponds to an activity/TOS ratio of 750 TASU/mg TOS.

In order to demonstrate a worst case calculation, an exaggerated human intake is estimated using the Budget method to estimate the intake associated with breakfast cereals, snacks and chips, and using consumption data to estimate the intake associated with coffee.

**Intake associated with breakfast cereals, snacks and chips**

a) According to the Budget method, a conservative estimate for the food intake is 25 g per kg body weight per day (g/kg bw/day) of which processed food is 50% of the food intake or 12.5 g/kg bw/day. Processed foods is considered = breakfast cereals, snacks and chips.

b) It is assumed that all processed foods are produced using the asparaginase as a processing aid, used at the highest recommended dosage.

c) The calculation is made assuming that all TOS remains in the final product.

The maximum recommended dosage is 15000 TASU/kg dry matter. The asparaginase has an activity/TOS ratio of 750 TASU/mg TOS, giving an overestimate of 20 mg TOS/kg processed food.

Based on the estimate for processed food intake of 12.5 g/kg bw/day, the intake of the asparaginase corresponds to 20 x 0.0125 = 0.25 mg TOS/kg bw/day.

**Intake associated with coffee**

According to the annual coffee report of the German coffee federation and customer information, 10 kg of roasted coffee per person per year represents a high-end consumption of coffee. Additional information on coffee consumption from the internet confirms that 10 kg represents a high-end consumption of coffee. For a 60 kg person, the coffee intake is thus 10 x 1000 / (60 x 365) = 0.46 g coffee/kg bw/day.

The maximum recommended dosage is 15000 TASU/kg dry matter. The asparaginase has an activity/TOS ratio of 750 TASU/mg TOS, giving an overestimate of 20 mg TOS/kg coffee.

Based on the above estimated coffee intake of 0.46 g/kg bw/day, the intake of the asparaginase corresponds to 20 x 0.00046 = 0.01 mg TOS/kg bw/day.
**Total estimate of Human Consumption**
Estimated Intake = 0.25 + 0.01 = 0.26 mg TOS/kg bw/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 conducted on asparaginase, batch PPV33595, was the highest dosage possible, 1207 mg TOS/kg bw/day.

The estimated human consumption is 0.26 mg TOS/kg bw/day

The safety margin can thus be calculated to be 1207/0.26 or approximately 4600.

7.7 Results and Conclusion

On the basis of the evaluation contained in Section 7, a review of the published literature, the history of safe use of *B. subtilis*, and the limited and well defined nature of the genetic modifications, the asparaginase enzyme preparation can be safely manufactured and used as a processing aid to reduce acrylamide levels in food.
8. LIST OF APPENDICES


3. Pariza and Johnson Decision Tree Analysis

9. LIST OF REFERENCES


Pages 000027-000040 and 000042-000045 have been removed in accordance with copyright laws. The removed references can be found on page 000021.
Appendix 3- Pariza & Johnson Decision Tree analysis of an asparaginase produced by a genetically modified strain of Bacillus subtilis

This asparaginase produced by a genetically modified strain of Bacillus subtilis 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 subtilis MOL2940 is derived from the recipient PP2982 via strain A164. This A164 line of host strains has been used in the construction of production strains for six other Novozymes products.
   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
      Asparaginases have been isolated from a variety of sources including plant, animal and microbial cells. Asparaginase enzyme preparations are the subject of GRN Nos. 201, 214 and 428.
      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 asparaginase 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
The introduced DNA does not contain any genes encoding antibiotic resistance. 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 three specific loci of the B. subtilis 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 B. subtilis production strain, designated MOL2940, was derived from the recipient strain, PP2982, via strain A164, which is identical to strain ATCC 6051a, the deposited type strain of Bacillus subtilis. Novozymes has used B. subtilis production strains for over 20 years. The A164 line of host strains has been used in the construction of production strains for six other Novozymes products. Safety studies including 13 week oral toxicity in rats, Ames test, and human lymphocyte cytogenetic assay, have been performed on enzyme preparations from these production strains. No toxicological effects were ever observed for any of the test substances produced by these B. subtilis production strains.

If yes, the test article is ACCEPTED.
SUMMARY OF TOXICITY DATA

Asparaginase PPV33595

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CONTENTS

1. SUMMARY ............................................................................................................. 3
2. TEST SUBSTANCE ................................................................................................. 4
   2.1 Production organism ......................................................................................... 4
   2.2 Characterization ............................................................................................... 4
3. CYTOTOXICITY ..................................................................................................... 4
   3.1 In Vitro Cytotoxicity Test: Neutral Red Uptake in L929 Monolayer Culture ........... 4
4. MUTAGENICITY .................................................................................................... 4
   4.1 Test for mutagenic activity with strains of Salmonella typhimurium and
       Escherichia coli ................................................................................................. 4
   4.2 In vitro Micronucleus Test in Cultured Human Lymphocytes ................................ 5
5. IN VIVO TOXICITY ............................................................................................... 5
   5.1 13 Weeks Oral Toxicity Study in Rats ............................................................... 5
6. REFERENCES ......................................................................................................... 7

Last page ...................................................................................................................... 7
1. SUMMARY

The below series of toxicological tests were undertaken to evaluate the toxicological profile of Asparaginase PPV33595.

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 carried out and finalised at CiTox/Scantox, DK, Huntingdon Life Sciences or in-house at Novozymes A/S during the period March 2012 to January 2013.

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

- Asparaginase PPV33595 was considered non-cytotoxic in vitro in a Neutral Red Uptake assay applying the BALB/c 3T3 cells as test system.

- The Ames test showed no mutagenic activity of Asparaginase PPV33595, neither in the absence or presence of S9.

- Asparaginase PPV33595 did not cause an increase in the induction of micronuclei in cultured human lymphocytes in this in vitro test using human lymphocytes either in the presence or in the absence of S-9 mix.

- In a 13 weeks oral toxicity study in rats Asparaginase PPV33595 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 Asparaginase PPV33595 exhibits no toxicological effects under the experimental conditions described.
2. TEST SUBSTANCE

2.1 Production organism

The production organism is a non-pathogenic Bacillus subtilis microorganism carrying the genes coding for asparaginase. Three genes of asnP23MHU (coding for the asparaginase) was introduced into the chromosome of the recipient B. subtilis. The host strain is derived from strain A164 by modifying several chromosomal loci to introduce deletion of genes encoding a number of proteases. Also a gene essential for sporulation was deleted, eliminating the ability to sporulate, together with a gene essential for formation of surfactin. The lack of these peptides and proteins represents improvements in product safety and stability.

2.2 Characterization

The test substances were the 3 sub-batches (HQFR9, HQFR10 and HQFR11) and the toxbatch PPV33595 mixed from the three sub-batches of Asparaginase. The toxbatch PPV33595 was used for the conduct of all the toxicological studies. The characterisation of the toxbatch is presented in table 1.

Table 1. The composition of the Asparaginase toxbatch used during the studies reviewed.

<table>
<thead>
<tr>
<th>Batch number</th>
<th>PPV33595</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activity</td>
<td>55200 TASU/g</td>
</tr>
<tr>
<td>Water (KF) (% w/w)</td>
<td>86.9</td>
</tr>
<tr>
<td>Dry matter (% w/w)</td>
<td>13.1</td>
</tr>
<tr>
<td>Ash (% w/w)</td>
<td>1.7</td>
</tr>
<tr>
<td>Total Organic Solids (TOS(^1))</td>
<td>11.4</td>
</tr>
<tr>
<td>Specific gravity (g/mL)</td>
<td>1.059</td>
</tr>
</tbody>
</table>

\(^1\) TOS is calculated as 100% - % water - % ash - % diluents.

3. CYTOTOXICITY

3.1 In Vitro Cytotoxicity Test: Neutral Red Uptake in BALB/c 3T3 cell culture.

Asparaginase, batch PPV33595, was examined for cytotoxic potential in an in vitro bioassay, the Neutral Red Uptake assay using BALB/c 3T3 cells in 96-well microplates.

The time of exposure for the test substance and positive control was 48 hours in total. 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 (NRU50). The NRU50 value for Asparaginase, batch PPV33595 was calculated from the linear part of the curve (10 to 1 mg Asparaginase/mL) and determined to be 5.72 mg/mL. Conclusion: The NRU50 value for Asparaginase, batch PPV33595 is 5.72 mg/mL in the present in vitro Neutral Red Uptake assay applying the BALB/c 3T3 cell line as test system.

4. MUTAGENICITY

4.1 Test for Mutagenic Activity with Strains of Salmonella typhimurium and Escherichia coli

Asparaginase was examined for mutagenic activity in four histidine-dependent strains of Salmonella typhimurium, strain TA98, TA100, TA1535 and TA1537 and the tryptophan-dependent strain Escherichia coli WP2uvrApKM101 using a “treat and plate” procedure.
The study was carried out with and without the metabolic activation system S9 - a liver preparation from male rats, pre-treated with Aroclor 1254, and the co-factors required for mixed function oxidase activity (S9 mix). Each test with each strain was conducted on two separate occasions. In all experiments bacteria were exposed to 6 doses separated with bi-sections. The highest dose level applied was 5 mg (dry matter) per ml as the highest concentration.

No toxicity of the test substance to the bacteria was observed.

No treatment of any of the Salmonella and E.coli strains with Asparaginase PPV33595 resulted in any increases in revertant numbers that meet the criteria for a positive or equivocal response.

It was concluded that Asparaginase PPV33595 did not induce gene mutations in bacteria in either the absence or presence of S9, when tested under the conditions employed in this study.

4.2 In vitro Micronucleus Test In Cultured Human Lymphocytes

Asparaginase, PPV33595 was tested in an in vitro cytogenetics assay using cultured human lymphocytes stimulated to divide by addition of phytohaemagglutinin (PHA) 48 hours prior to treatment. The lymphocytes were treated for 3 hours in both absence and presence of metabolic activation (S9 mix) and for 20 hours in the absence of S9 mix. In this study, blood taken from healthy male non-smoking donors was pooled and diluted with tissue culture medium. Five test concentrations were assessed for determination of induction of micronuclei. The highest Asparaginase, PPV33595 concentration selected (2.5 % v/v) was that which did not alter the osmolality of the medium by more than 50 mOsm/kg relative to the vehicle controls. Concentrations of Asparaginase, PPV33595 selected for micronucleus analysis were 0.16, 0.31, 0.63, 1.25 and 2.5% v/v.

In both the absence and presence of S9 mix following 3-hour treatment, and in the absence of S9 mix following 20 hour treatment, Asparaginase, PPV33595 did not cause any statistically significant increases in the number of binucleate cells containing micronuclei when compared with the vehicle controls.

The positive control compounds (mitomycin C, colchicine and cyclophosphamide) caused significant increases in the number of binucleate cells containing micronuclei demonstrating the efficacy of the test system.

It is concluded that Asparaginase, PPV33595 administered for 3 hours at concentrations of up to 2.5% v/v, in both the absence and presence of S9 mix, and for 20 hours in the absence of S9 mix only, did not show evidence of causing an increase in the induction of micronuclei in cultured human lymphocytes.

5. IN VIVO TOXICITY

5.1 13 Weeks Oral Toxicity Study in Rats

The objective of this study was to assess the systemic toxic potential of Asparaginase PPV33595 when administered daily by oral treatment (gavage) to rats for 13 weeks. Three groups, each comprising ten males and ten females, received Asparaginase, PPV33595 at doses of 1.0, 3.3 or 10.0 mL/kg/day corresponding to 0.121 g TOS/kg bw or 58457 TASU/kg in Group 2, 0.398 g TOS/kg bw or 192907 TASU/kg in Group 3 and 1.207 g TOS/kg bw or 584568 TASU/kg in Group 4. A similarly constituted Control group received the vehicle (purified water obtained by reverse osmosis) at the same volume-dosage.

During the study, clinical condition, detailed physical and arena observations, sensory reactivity, grip strength, motor activity, bodyweight, food and water consumption, ophthalmic examination, haematology, blood chemistry, organ weight, macropathology and histopathology investigations were undertaken.

No treatment related signs were recorded at the clinical examination (clinical observations, 0 0 0 0 5 4)
open field and stimuli tests and ophthalmoscopy), on body weight gain, on food or water consumption or on clinical pathology parameters. At necropsy, no microscopic or macroscopic treatment related findings were observed.

In conclusion, 90 days of oral (gavage) treatment of rats with Asparaginase, PPV33595, at dose levels of up to 1.207g TOS/kg bw or 584568 TASU/kg administered at a dose volume of 10 ml/kg did not cause any treatment related changes. The NOAEL (No Observed Adverse Effect Level) for both female and male animals for Asparaginase, PPV33595, is 1.207g TOS/kg bw corresponding to 584568 TASU/kg bw.
6. REFERENCES


