ORIGINAL SUBMISSION

645

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GRN 000645

April 1, 2016

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 one paper copy and one eCopy, a generally recognized as safe (GRAS) notification, in accordance with proposed 21 C.F.R. § 170.36 (a), for Novozymes' pullulanase enzyme preparation produced by a genetically modified strain of *Bacillus licheniformis*.

The electronic copy is provided on a virus-free CD, and is an exact copy of the paper submission. Novozymes has determined through scientific procedures that the pullulanase is generally recognized as safe for use in the food industry as a processing aid for the saccharification of liquefied starch and in the brewing and alcohol industries.

Please contact me by direct telephone at 919 494-3187, direct fax at 919 494-3420 or email at jao@novozymes.com if you have any questions or require additional information.

Sincerely,

(b) (6)

Janet Oesterling
Regulatory Affairs Specialist III

Enclosures

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

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

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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 pullulanase 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.*

A pullulanase enzyme preparation produced by a genetically modified *Bacillus licheniformis.*

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

The pullulanase is used as a processing aid in the saccharification of liquefied starch, mainly in the production of high dextrose and high maltose syrups. The enzyme can also be used in the alcohol and brewing industries to increase the amount of fermentable sugars. 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)

Janet Oesterling
Regulatory Affairs Specialist III

04-01-16
Date

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April 1, 2016

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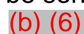

04-01-16

Janet Oesterling — Date
Regulatory Affairs Specialist III

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A Pullulanase Enzyme Preparation Produced by a Genetically Modified *Bacillus licheniformis*

Janet Oesterling, Regulatory Affairs, Novozymes North America, Inc., USA

April 2016

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1. GENERAL INTRODUCTION

The subject of this notification is a pullulanase enzyme preparation produced by submerged fermentation of *Bacillus licheniformis* microorganism carrying a modified gene coding for pullulanase.

The enzyme preparation is to be used in the food industry as a processing aid in the saccharification of liquefied starch, mainly in the production of high dextrose and high maltose syrups. However, the enzyme can also be used in the alcohol and brewing industries to increase the amount of fermentable sugars.

The active enzyme is pullulanase (EC 3.2.1.41, CAS 9075-68-7)

The information provided in the following sections is the basis for our determination of general recognition of safety of this pullulanase 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 (1) (2) (Appendix 1). The production organism, *Bacillus licheniformis,* is discussed in Sections 2 and 7. *Bacillus licheniformis,* is generally considered to be non-pathogenic and non-toxigenic (3) 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. (4)

Bacillus 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 (5). Various enzymes have been produced by *Bacillus licheniformis* and are GRAS substances (GRASP 3G0026 and 0G0363 and GRN 22, 24, 72, 79; 265, 277, 472) (6). In addition, *Bacillus licheniformis* is classified as a Risk Group 1 organism according to the National Institutes of Health Guidelines for Research Involving Recombinant Molecules. (7) Risk Group 1 organisms are those not associated with disease in healthy adult humans.

The *Bacillus licheniformis* recepient 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 (8) (5) (9) (10) (11) (12) (13). 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 *Bacillus licheniformis* production strain, designated HyGe486, was derived via the recipient strain, AEB1763, from a natural isolate of *Bacillus licheniformis* strain DSM 9552. 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 (10). It also meets the criteria for a safe production microorganism as described by Pariza and Foster (2) and later Pariza and Johnson (1) and several expert groups (4) (5) (9) (10) (11) (12) (13).

The pullulanase expression plasmid, pHyGe454, used in the strain construction contains strictly defined chromosomal DNA fragments and synthetic DNA linker sequences. The DNA sequence for the introduced pullulanase gene, *pul256*, is a synthetic gene based on the coding sequence from the pullulanase encoding gene (5' end) of *Bacillus deramificans* (LMGP13056), modified at the 3' end to reflect the coding sequence of pullulanase from *Bacillus acidopullulyticus* (NCIMB11639).

2.2 Recipient Strain

The recipient strain AEB1763 used in the construction of the *Bacillus licheniformis* 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 proteins. The lack of these proteins represents improvements in product purity, safety and stability.

2.3 Pullulanase Expression Plasmid

The expression plasmid, pHyGe454, used to transform the *Bacillus licheniformis* recepient strain AEB1763 is based on the well-known *Bacillus* vectors pE194 (14) from Staphylococcus aureus and a standard E. coli vector. No elements of these vectors are left in the production strain. The introduced DNA consists of a fragment of a hybrid *Bacillus* promoter with promoter elements from *Bacillus licheniformis*,

Bacillus amyloliquefaciens and *Bacillus thuringiensis*, the pullulanase (*pul256)* coding sequence. Finally a *Bacillus licheniformis* terminator sequence is inserted to terminate transcription.

The *pul256* gene is synthesized based on sequence data from public databases of *Bacillus deramificans* (LMGP13056) and *Bacillus acidopullulyticus* (NCIMB11639). Following the terminator, a non-coding DNA sequence is inserted to enable targeted integration of the transforming DNA into the genome of the recipient strain. Only the expression cassette with elements between the promoter fragment and the terminator 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, *Bacillus licheniformis* HyGe486, was constructed from the recipient strain AEB1763 through the following steps:

1) Plasmid pHyGe454 was integrated into five specific loci in strain AEB1763 by targeted homologous recombination to these loci using a two-step integration approach. After the initial integration of pHyGe454 at the five target loci, excision of the bacterial backbone occurs by homologous recombination. Thus, only the expression cassette of the *pul256* gene containing the promoter and the transcriptional terminator is left at each target locus.

2) The resulting five copy *pul256* production strain was named HyGe486.

Sequence confirmation of the inserted expression cassettes and the flanking regions at each 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 *Bacillus licheniformis* chromosome and, as such, is poorly mobilizabled 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 genome sequence analysis.

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 (4) is satisfactorily addressed.

3. MANUFACTURING PROCESS

This section describes the manufacturing process for the pullulanase which follows standard industry practices (15) (16) (17). The quality management system used in the manufacturing process for the pullulanase complies with the requirements of ISO 9001. It is produced under a standard manufacturing process as outlined by Aunstrup (16) 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 (18). It also conforms to the General Specifications for Enzyme Preparations Used in Food as proposed by JECFA (19).

3.1 Raw Materials

The raw materials used in the fermentation and recovery process for the pullulanase enzyme concentrate are standard ingredients used in the enzyme industry (15) (16) (17). 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 (if necessary) 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 pullulanase is manufactured by submerged fed-batch pure culture fermentation of the genetically modified strain of *Bacillus 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 stock culture of the production organism, *Bacillus 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 fermenter and the main fermenter before inoculation, at regular intervals during cultivation, and before transfer/harvest. These samples are tested for microbiological contamination by microscopy and by plating on a nutrient agar followed by a 24-48 hour incubation period.

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 designed to separate the desired enzyme from the microbial biomass and partially purify, concentrate, and stabilize the enzyme.

3.3.1 Purification Process

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

1. Pretreatment - pH adjustment and flocculation

2. Primary Separation – vacuum drum filtration or centrifugation

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

6. Preservation and Stabilization of the liquid enzyme concentrate

3.3.2 Formulation and Standardization Processes

The enzyme concentrate is stabilized with sucrose. The product is formulated by preservation with potassium sorbate and sodium benzoate and standardized by the addition of water.

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 pullulanase are given below:

Classification	pullulanase (common and usual name)
IUBMB nomenclature:	pullulan 6-α-glucanohydrolase
EC No.:	3.2.1.41
CAS No.:	9075-68-7
Specificity:	Catalyses the hydrolysis of (1→6)-alpha-D-glucosidic linkages in pullulan, amylopectin and glycogen, and in the alpha- and beta-limit dextrins of amylopectin and glycogen.
Amino acid sequence:	the total nucleotide and amino acid sequences have been determined

5. COMPOSITION AND SPECIFICATIONS

5.1 Quantitative Composition

The pullulanase enzyme concentration is available in a liquid formulation.

Table 1. Typical compositions of the enzyme preparation

Substance	Approximate Percentage
Enzyme Solids (TOS*)	10.4
Water	>50
Sucrose	10–50
Sodium Benzoate	<0.50
Potassium Sorbate	<0.50

* *Total Organic Solids, define as: 100% - water – ash – diluents.

5.2 Specifications

The pullulanase enzyme preparation complies with the recommended purity criteria for enzyme preparations as described in *Food Chemicals Codex* (18). 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 (19).

This is demonstrated by analytical test results of three representative enzyme batches in Table 2 below.

Table 2. Analytical data for three food enzyme batches

Parameter	Specification	PPY38551	PPY38863	PPY40089	Mean
Activity unit	PUN(G)/g	8640	7420	8140	8067
Lead	Not more than 5 mg/kg	0.5	0.5	0.5	0.5
Total Coliforms	Not more than 30/g	<4	<4	<4	<4
Salmonella	Absent in 25g	ND	ND	ND	ND
Escherichia coli	Absent in 25g	ND	ND	ND	ND
Antimicrobial activity	Not detected	ND	ND	ND	ND

6. APPLICATION

6.1 Mode of Action

The active enzyme is a pullulanase (EC 3.2.1.41). Pullulanases hydrolyse 1,6-alpha-D-glucosidic linkages in pullulan and partially hydrolysed amylopectin as well as alpha- and beta-amylase limit dextrins of amylopectin.

These substrates are found in various yeast, animal and plant material, including cereals, such as wheat and barley. Like the substrate the reaction products are natural constituents of cereal-containing foods.

The pullulanase enzyme is used as a processing aid in food manufacturing and is not added directly to final foodstuffs. The typical food processes where this food enzyme is used include brewing, beverage alcohol and starch processing.

The benefits of the action of the food enzyme in brewing, beverage alcohol (distilling) and starch processing are:

- More uniform and predictable production process and brewing yield including the possibility to control the desired level of fermentable sugars at every production
- Improved processing when raw materials low in limit dextranases, like unmalted barley, are applied
- A more efficient process as the brewing time can be shortened
- Increase in the glucose and maltose yield for starch processing

For brewing, the pullulanase is normally applied in mashing where it produces fermentable sugars in synergy with endogenous enzymes, like alpha-amylases, dextranases (same activity as pullulanases) and beta-amylases. The pullulanase can also be applied in fermentation, where the enzyme, together with glucoamylases, degrades dextrins into glucose.

When used in the starch industry, the enzyme preparation is added to the starch slurry after liquefaction. After saccharification the syrup is concentrated by evaporation, filtered and refined by means of activated carbon and, if desired, ion exchange (20) (21) (22) (23). During the evaporation and purification processes of the syrup the enzyme will be inactivated and removed.

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 conditions of use of the pullulanase preparation during food processing do not only depend on the type of application, but also on the food production process of each individual food manufacturer. In order to ensure optimal effectiveness of the enzyme at an acceptable economic cost, the dosage, reaction time, process conditions and processing steps are adjusted.

The recommended use level for the food enzyme:

Brewing processes: Up to 10000 PUN(G)/kg starch dry matter.
Starch processing: Up to 1500 PUN(G)/kg starch dry matter.

6.3 Enzyme Residues in the Final Food

The pullulanase is used for hydrolysis of starch polysaccharides during processing of starch-containing foods. It is used in food manufacturing as a processing aid to ease and optimise the processes and make more efficient use of raw materials. Also, the use of pullulanase ensures a consistent process and minimises potential end product variations.

When using the food enzyme in food and brewing processes the enzyme will not be functional in the final product. The reasons why enzymes do not exert any enzymatic activity in the final food are a combination of various factors, depending on the application and the process conditions used by the individual food producer. These factors include denaturation of the enzymes during processing, depletion of the substrate, lack of water activity, wrong pH, etc.

In some cases the enzymes may no longer be present in the final food, due to the removal during processing i.e. filtration, carbon treatment, ion exchange, evaporation and drying. Therefore, the enzyme does not exert a function in the final food/beverage.

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 (2) (1). If the organism is non-toxigenic 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 (24). Pariza and Foster (2) define a non-toxigenic organism as “one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure” and a 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 toxigenic (25) (3). 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 (24). *Bacillus licheniformis* has been used in the fermentation industry for the production of enzymes, antibiotics, and other specialty chemicals. Various enzymes are produced by *Bacillus licheniformis* and are considered GRAS substances (α-amylases – GRASP 3G0026 and 0G0363 and GRN 22, 24, 79; pullulanase – GRN 72; and xylanase – GRN 472) (6). In addition, *Bacillus 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 *Bacillus licheniformis* recepient strain is derived from a safe strain lineage comprising production strains for 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), cf. section 7.1.1 below.

An evaluation of the genetically modified production microorganism for the pullulanase, embodying the concepts initially outlined by Pariza and Foster, 1983 (2) and further developed by IFBC in 1990 (24), the EU SCF in 1991 (9), the OECD in 1992 (10), ILSI Europe Novel Food Task Force in 1996 (13), FAO/WHO in 1996 (12), JECFA in 1998 (19) and Pariza and Johnson in 2001 (1) demonstrates the safety of this genetically modified production microorganism strain. The components of this evaluation: the identity of the recepient 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.

The genetic modifications are well characterized 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 pullulanase enzyme.

7.1.1 Safe Strain Lineage

The safety of this *Bacillus licheniformis* production strain was established following published criteria for the assessment of the safe use of microorganisms used in the manufacture of food ingredients (1) (4). The recepient strain, *Bacillus licheniformis* AEB1763, has been thoroughly characterized as shown in sections 2.2. The introduced DNA is well-known and characterized in section 2.3 and 2.4. The procedures used to modify the recepient organism are well defined and commonly used. Therefore, the elements needed to establish a safe strain lineage as defined in Pariza and Johnson, 2001 (1) have been met.

Novozymes' used the decision tree (Appendix 3) in Pariza and Johnson 2001 (1) 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 pullulanase. The enzyme preparation is free of DNA encoding transferable 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 five 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 *Bacillus 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.

Finally, the production strain is derived from a safe lineage. Novozymes has used *Bacillus licheniformis* production strains for over 20 years. An overview of some of the Novozymes products produced by *Bacillus licheniformis* strains and the safety studies conducted on those products is shown below (Table 3).

Table 3. Safe Strain Lineage

Enzyme	EC No.	Predecessor strain[1]	Donor strain	Safety studies[2]
Alpha-amylase (GRASP 0G0363)	3.2.1.1	*Bacillus licheniformis* Si3	*Bacillus stearothermophilus*	Yes
Alpha-amylase (GRN 22)	3.2.1.1	*Bacillus licheniformis* SJ1707	*Bacillus licheniformis*	Yes
Cyclodextrin glucanotransferase	2.4.1.19	*Bacillus licheniformis* SJ1707	*Thermoanaerobacter sp.*	Yes
Alpha-amylase	3.2.1.1	*Bacillus licheniformis* SJ1707	*Bacillus licheniformis*	Yes
Alpha-amylase	3.2.1.1	*Bacillus licheniformis* SJ1904	*Bacillus licheniformis*	Yes
Alpha-amylase	3.2.1.1	*Bacillus licheniformis* MDT223	*Bacillus stearothermophilus*	Yes
Alpha-amylase	3.2.1.1	*Bacillus licheniformis* MDT223	*Bacillus amyloliquefaciens*	Yes
Serine protease	3.4.21.1	*Bacillus licheniformis* MDT223	*Nocardiopsis prasina*	Yes
Alpha-amylase	3.2.1.1	*Bacillus licheniformis* MDT223	*Bacillus licheniformis*	Yes
Xylanase (GRN 472)	3.2.1.8	*Bacillus licheniformis* MDT223	*Bacillus licheniformis*	Yes
Beta-amylase	3.2.1.1	Bacillus licheniformis PP3579	Bacillus flexus	Yes
Beta-galactosidase (GRN 572)	3.2.1.23	Bacillus licheniformis AEB1763	Bifidobacterium bifidum	Yes
Acetolactate decarboxylase (GRN 587)	4.1.1.5	Bacillus licheniformis AEB1763	Bacillus brevis	Yes

Table 3. Novozymes products derived from *B. licheniformis* strains [1]The predecessor strain shows strains in the GM construction pathway that are in common with the recepient strain AEB1763 lineage. [2]At 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 in (Table 3), 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 recepient 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 xylanases) 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 (1) to evaluate the enzymes derived from *Bacillus 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 *pul256* (pullulanase) gene is synthesized based on sequence data from public databases of *Bacillus deramificans* (LMGP13056) and *Bacillus acidopullulyticus* (NCIMB11639). The introduced DNA does not code for any known harmful or toxic substances.

7.3 Safety of the Pullulanase Enzyme

A wide variety of enzymes are used in food processing (2) (1). Enzyme proteins do not generally raise safety concerns (1) (2). Pullulanase enzyme preparations have been used commerically since the early 1980's (26). And, there are several permitted sources for pullulanase listed in Table V of the Canadian Food and Drug Regulations.

Also, Pariza and Foster (2) note that very few toxic agents have enzymatic properties. The safety of the pullulanase was assessed using the Pariza and Johnson, (2001) decision tree (Appendix 3).

7.3.1 Consideration of the Allergenic Potential of the Pullulanase 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.

- Enzymes in foods are always added in concentrations in the low range of part per millions. 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) (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 pullulanase 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 (28) and modified by Codex Alimentarius Commission, 2009 (29) the pullulanase 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 pullulanase 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 pullulanase and any of the allergens from the databases mentioned above. Also, a search for 100% identity over 8 contiguous amino acids was completed. Again, no significant homology was found. Consequently, oral intake of the pullulanase is not anticipated to pose any food allergenic concern.

7.4 Safety of the Manufacturing Process

The pullulanase enzyme preparation meets the purity criteria for enzyme preparations as outlined in the monograph on Enzyme Preparations in the *Food Chemicals Codex.* 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 (15) (16) (17).

7.5 Safety studies

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

7.5.1 Description of Test Material

The following studies were performed on toxicology test batch PPY38874 with favourable results:

- Reverse Mutation Assay (Ames test)
- *In vitro* Cytotoxicity Test: Neutral Red Uptake
- *In vitro* Micronucleus Test in Cultured Human Lymphocytes

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.

7.6 Estimates of Human Consumption and Safety Margin

The enzyme is largely heat inactivated during the starch processing in which it is applied. The assumption regarding the food enzyme in the final beer is highly exaggerated since the enzyme protein and the other substances resulting from the fermentation are diluted or removed in certain processing steps. Furthermore, beer and beer-like beverages produced with the enzyme are not always produced with the maximum recommended dosage. In the production of beer it is assumed that the totality of the food enzyme used during production will end up in the final beer.

In order to demonstrate a worst case scenerio, the following calculations are made assuming that all of the enzyme activity is retained in the final product. An exaggerated human intake is estimated using the Budget method (30) for the intake associated with starch processing and then using consumption data to estimate the intake associated with beer and other cereal beverages.

<u>Intake associated with starch processing:</u>

Assumptions in the Budget Method

<u>Solid Food</u>: The maximum energy intake over the course of a lifetime is 50 kcal/kg body weight (bw) per day. Fifty kcal corresponds to 25 g food. Therefore, adults ingest 25 g food per kg body weight per day.

Assuming that 50% of the food is processed food, the daily consumption of processed food will be 12.5 g processed foods per kg body weight.

It is further assumed that, on average, all processed food contains 25% starch (or starch-derived) dry matter = 3.12 g starch derived dry matter per kg body weight per day.

<u>Liquids</u>: The maximum intake of liquids (other than milk) is 100 ml/kg body weight (bw) day. Assuming that 25% of the non-milk beverages are processed, the daily consumption will be 25 ml processed beverages per kg body weight.

It is further assumed that all processed beverages contain 13% hydrolysed starch dry matter = 3.25 g hydrolysed starch derived dry matter per kg body weight per day.
It is assumed that the densities of the beverages are ~ 1.

TMDI calculation: Starch

The pullulanase registration batches have a mean activity of 8067 PUN(G)/g and an approximate content of 10.4% TOS (Total Organic Substances from the fermentation, mainly protein and carbohydrate components). This corresponds to an activity/mg TOS ratio of 77.6 PUN(G)/mg TOS.

Solid and Liquid Food: The highest dosage given for solid food is 1500 PUN(G)/kg starch based raw material which corresponds to 19.3 mg TOS.

Based on this 3.12 gram starch derived dry matter in solid food + 3.25 gram starch derived dry matter in liquids will maximally contain:

19.3 mg TOS per kg / 1000 g per kg x (3.12 + 3.25) g = 0.123 mg TOS

The theoretical maximum daily intake (TMDI) of consumers of the food enzyme based on the starch contribution is therefore:

0.123 mg TOS/kg body weight/day.

Intake associated with beer and other cereal based beverage processes:

In order to demonstrate a worst case calculation, an exaggerated human intake for beer and beer-like beverages was used. This intake calculation is based on a mean and the 90th percentile consumption of alcoholic beverages in the United States using NHANES Survey 2003-2012 combined 2-day consumption data (31). For a 60 kg person. Thus, using the highest mean intake of beer and the lowest weight average represents the "worst case" scenario.

Based on this, 15.8 g of beer and beer-like beverage is consumed kg of body weight per day.

Typical values for the starch content of malt and barley is 65% (32). As a rule of thumb 1 kg of grits will be used for the production of 6 kg of beer. Therefore, an intake per kg bw per day of 15.8 g "Beer and beer-like beverage" corresponds to

15.8 g beer/kg bw/day ÷ 6 g beer/g grits = 2.63 g grits/kg bw/day x 0.65 g starch/per g grits = 1.71g starch/kg bw/day.

TMDI calculation: Brewing and Cereal Based Beverage

The pullulanase registration batchs have an activity of 8067 PUN(G)/g and an approximate content of 10.4% TOS (Total Organic Substances from the fermentation, mainly protein and carbohydrate components). This corresponds to an activity/mg TOS ratio of 77.6 PUN(G)/mg TOS.

The dosage for the pullulanase in brewing processes and other cereal based beverage processes is up to 10000 PUN(G)/kg starch.

The corresponding TOS is calculated as: 129 mg TOS/kg starch.

Based on this, 1.71g starch/kg bw/day results in a consumption of:

129 mg TOS/kg starch x 0.00171 kg starch/kg bw/day = 0.221g starch/kg bw/day

The TMDI contribution from beverage alcohol is assumed to be zero due to the distilling process.

Total TMDI Calculation:

0.123 mg TOS/kg bw/day + 0.221 mg TOS/kg bw/day = 0.344 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 concluded in 13 week oral rat feeding studies conducted on Novozymes enzyme preparations is normally the highest dosage that can be practically applied, i.e. limited by the concentration (TOS%) of the test batch and typically in the range of 500 to 1500 mg TOS per kg bodyweight per day. For the safety evaluation of this enzyme, a NOAEL level of (at least) 500 mg TOS/kg bw/day is assumed and this value is used for the Safety Margin calculation. See Table 4 below.

Table 4. NOAEL Calculation

NOAEL (mg TOS/kg bw/day)	500
Total TMDI in both starch and beer/cereal based beverages (mg TOS/kg bw/day)	0.344
Safety margin	1453

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 *Bacillus licheniformis,* and the limited and well defined nature of the genetic modifications, the pullulanase enzyme preparation can be safely used as a processing aid in the starch, brewing and alcohol industry.

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Applied Microbiology and Biotechnology

January 1994, Volume 40, Issue 5, pp 595-598

On the industrial use of Bacillus licheniformis: a review

Anne Sietske de Boer, Fergus Priest, Børge Diderichsen

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

This pullulanase 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
   If yes, go to 2.

   The production strain *Bacillus licheniformis* designated HyGe486, was derived via the recipient strain AEB1763 from a natural isolate of *Bacillus licheniformis* strain DSM 9552.

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
      Bacillus 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. Various enzymes have been produced by *Bacillus licheniformis* and are GRAS substances (GRASP 3G0026 and 0G0363 and GRN 22, 24, 72, 79; 265, 277, 472). In addition, *Bacillus licheniformis* is classified as a Risk Group 1 organism according to the National Institutes of Health Guidelines for Research Involving Recombinant Molecules
      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**)

   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. If no go to 3d.

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 several selected loci of the *Bacillus licheniformis* chromosome. Sequence confirmation was performed in the production strain.
If yes go to 5. If no, go to 6.

5. Is the production strain sufficiently well characterized so that one may reasonably conclude that unintended pleiotropic effects which may result in the synthesis of toxins or other unsafe metabolites will not arise due to the genetic modification method that was employed?
YES
If yes go to 6. If no go to 7.

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

The *Bacillus licheniformis* strain, HyGe486, used in the construction of this pullulanase production strain has previously been used as a host strain in several production strains for Novozymes enzyme products. These production strains were constructed by standard transformation procedures using well-known plasmid vectors and well characterized DNA sequences that were integrated into the *Bacillus licheniformis* strain chromosome by homologous recombination.

The safety of this production strain was established following published criteria for the assessment of the safe use of microorganisms used in the manufacture of food ingredients (1) (2). The host strain, has been thoroughly characterized as

shown in Sections 2.2. The introduced DNA is well-known and characterized in Section 2.3 and 2.4 and the introduced genetic material does not encode and express any known harmful or toxic substances.

If yes the test article is ACCEPTED.

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SUMMARY OF TOXICITY DATA

Pullulanase, batch PPY38874, from *Bacillus licheniformis*

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1. ABSTRACT

The below series of toxicological studies were undertaken to evaluate the safety of Pullulanase, batch PPY38874.

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), Envigo (UK) and Covance (UK) during the period October 2015 to March 2016.

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

- Pullulanase, batch PPY38874, was tested in a Neutral Red Uptake assay applying the BALB/c 3T3 cell line as test system and observations were in line with previous observations for pullulanases.

- Pullulanase, batch PPY38874, did not induce gene mutations in the Ames test, in the absence or presence of a rat liver metabolic activation system (S-9).

- Pullulanase, batch PPY38874, did not induce biologically relevant increases in micronuclei *in vitro*, in cultured human peripheral blood lymphocytes following treatment in the absence and presence of a rat liver metabolic activation system (S-9).

Based on the present toxicity data it can be concluded that Pullulanase, represented by batch PPY38874, exhibits no significant toxicological effects under the experimental conditions described.

2. TEST SUBSTANCE

Pullulanase, also known as debranching enzyme, is a pullulan 6-glucano-hydrolase (E.C. 3.2.1.41) that degrades α-1,6 bonds in pullulan, amylopectin, and glucagon.

2.1 Characterization

The toxbatch Pullulanase, batch PPY38874, was used for the conduct of all the toxicological studies. The characterization data of the toxbatch is presented in Table 1.

Table 1. Characterization data of Pullalanse, batch PPY38874

Activity	5330 PUN(G)/g
N-Total (% w/w)	0.99
Water (KF) (% w/w)	88.9
Dry matter (% w/w)	11.1
Ash (% w/w)	1.5
Total Organic Solids (TOS[1]) (% w/w)	9.6
Specific gravity (g/mL)	1.042

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

3. MUTAGENICITY

3.1 *In Vitro* Cytotoxicity Test: Neutral Red Uptake in BALB/c 3T3 Cell Culture

The aim of this study was to evaluate the cytotoxicity of Pullulanase, Batch PPY38874, usinga Neutral Red Uptake (NRU) assay in 3T3 cells.

The growth of 3T3 cells treated with a range of concentrations of the test item was compared with vehicle control cultures after 48 hours exposure both visually and using neutral red uptake.

Pullulanase was toxic at approximately 50% viability when compared with the vehicle control at the highest five concentrations, 2 – 30 mg/mL, and less toxic at the lowest three concentrations, 0.1 – 1 mg/mL, according to the neutral red uptake results. A visual assessment of the cell monolayers showed that the test item produced around 10% confluency at the highest four concentrations, 20% confluency at the fifth concentration, 30% confluency at the sixth concentration and 40% confluency at the lower two concentrations The vehicle control produced approximately 50% confluency.

The IC50 value of the positive control, sodium lauryl sulphate, was calculated to be 94.69 µg/mL which lay within the historical control range of this laboratory.

It was concluded that Pullulanase, batch PPY38874, demonstrated cytotoxicity at approximately 50% viability compared to the vehicle control in the concentration range 2 – 30 mg/mL, with less toxicity observed at the lower concentrations of 0.1 – 1 mg/mL.

3.2 Bacterial Reverse Mutation assay (Ames test)

Pullulanase, batch PPY38874, 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 Pullulanase, 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 Pullulanase in liquid culture known as “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 toxicity of the test substance to the bacteria was observed. 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 Pullulanase, batch PPY38874, 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.

3.3 *In vitro* Micronucleus Test In Cultured Human Lymphocytes

Pullulanase, batch PPY38874, was tested in an *in vitro* micronucleus assay using duplicate human lymphocyte cultures prepared from the pooled blood of two male donors in a single experiment. Treatments covering a broad range of concentrations, separated by narrow intervals, were performed both in the absence and presence of metabolic activation (S-9) from Aroclor 1254-induced rats. The test article was formulated in water for irrigation (purified water) and the highest concentration tested in the Micronucleus Experiment, 5000 µg TOS/mL (an acceptable maximum concentration for *in vitro* micronucleus studies according to current regulatory guidelines) was determined following a preliminary cytotoxicity Range-Finder Experiment.

All positive control compounds induced statistically significant increases in the proportion of cells with micronuclei. All acceptance criteria were considered met and the study was herefore accepted as valid.

Pulse 3+21 hour treatment of cells with Pullulanase, batch PPY38874 in the absence and presence of S-9 resulted in frequencies of MNBN cells which were similar to and not significantly ($p \leq 0.05$) higher than those observed in concurrent vehicle controlsfor all concentrations analysed. The MNBN cell frequency of all Pullulanase, batchPPY38874 treated cultures fell within the normal ranges.

Following 24+24 hour treatment in the absence of S-9 a small but statistically significant increase in MNBN cells was observed at the highest concentration analysed (5000 µg TOS/mL, inducing 52% cytotoxicity, and where post treatment precipitate was observed). However, this increase was marginal and restricted to just one of the two replicate cultures (with a MNBN cell frequency that did not exceed the observed historical vehicle control range). A marginal increase in MNBN cell frequency (0.65%) above normal values was observed in a single culture at 4000 µg TOS/mL, inducing 42% cytotoxicity, this increase was not statistically significant. The MNBN cell values of all other Pullulanase, batch PPY38874 treated cultures (all concentrations) fell within normal values. Although the linear trend testindicated a statistically significant result, there was no real evidence of a concentration related effect on MNBN cell frequency.

Overall, this isolated statistical increase occurring at a high concentration of 5000 µg TOS/mL inducing high cytotoxicity was not considered of biological importance. These findings are consistent with recent guidance on the interpretation and hazard identification of *in vitro* genotoxicity data.

It is concluded that Pullulanase, batch PPY38874, tested up to 5000 µg TOS/mL did not induce biologically relevant increases in micronuclei in cultured human peripheral blood lymphocytes following treatment in the absence and presence of a rat liver metabolic activation system (S-9).

4. REFERENCES

4.1 Study reports

Envigo: Study No.: XW84SV. Novozymes Reference No.: 20156091: Pullulanase, PPY38874: 3T3 Neutral Red Uptake test. (March 2016). LUNA file: 2016-04343.

Novozymes A/S: Study No.: 20158054. Pullulanase, batch PPY38874: Test for mutagenic activity with strains of *Salmonella typhimurium* and *Escherichia coli.* (February 2016). LUNA file: 2016-00781.

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SUBMISSION END