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GRN 861

April 16, 2019

Dr. Paulette Gaynor
Division of Biotechnology and GRAS Notice Review
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5001 Campus Drive
College Park, MD 20740

Subject: GRAS Notification –
Pullulanase
from *Bacillus deramificans* Expressed in *Bacillus subtilis*
As a Food Processing Aid

Dear Dr. Gaynor,

On behalf of GenScript/Bestzyme, we are submitting a GRAS notification for Pullulanase from *Bacillus deramificans* Expressed in *Bacillus subtilis* as a food processing aid. The enclosed document provides the notice of a claim that a food processing aid, the Pullulanase from *Bacillus deramificans* Expressed in *Bacillus subtilis*, described in the enclosed notification is exempt from the premarket approval requirement of the Federal Food, Drug, and Cosmetic Act because it has been determined to be generally recognized as safe (GRAS), based on scientific procedures, as a food processing aid. We believe that this determination and notification are in compliance with Pursuant to 21 C.F.R. Part 170, subpart E.

We enclose an original copy of this notification and a CD Rom for your review. Please feel free to contact me if additional information or clarification is needed as you proceed with the review. We would appreciate your kind attention to this matter.

Sincerely,

Susan Cho, Ph.D.
Susanscho1@yahoo.com
Agent for GenScript/Bestzyme

Determination of the Generally Recognized As Safe (GRAS) Status of Pullulanase from *Bacillus deramificans* Expressed in *Bacillus subtilis* As a Food Processing Aid

Prepared for GenScript/Bestzyme

GenScript - 860 Centennial Ave, Piscataway, NJ 08854
Bestzyme Biotech Inc. (Bestzyme) - 860 Centennial Ave, Piscataway, NJ 08854

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PART 1. SIGNED STATEMENTS AND A CERTIFICATION

Pursuant to 21 CFR Part 170, subpart E, GenScript and Bestzyme (hereinafter referred to as 'GenScript/Bestzyme') submits a Generally Recognized as Safe (GRAS) notice and claims that the use of pullulanase in food processing, as described in Parts 2 through 7 of this GRAS notice, is not subject to premarket approval requirements of the FD&C Act based on its conclusion that the substance is GRAS under the conditions of its intended use.

1.A. Name and Address of the Notifier

Contact: Dr. Andy Yan
Company: GenScript/Bestzyme
Address: 860 Centennial Ave, Piscataway, NJ 08854

1.B. Common or Trade Name

Pullulanase

1.C. Applicable Conditions of Use of the Notified Substance

1.C.1. Foods in Which the Substance is to be Used

The intended use and use levels are the same or similar to those described in GRN 645. The pullulanase is used for hydrolysis of carbohydrates during processing of starch-containing foods and brewing processes. However, the enzyme used during the food and brewing processes is not expected to exert any unintentional enzymatic activity in the final food due to the following factors: 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, such as filtration and other purification steps, evaporation, and drying.

1.C.2. Levels of Use in Such Foods

Pullulanase is not added to final foodstuffs, but is used as a processing aid during food manufacturing to hydrolyze starch. The intended use and use levels are the same or similar to those described in GRN 645. The enzyme preparation is used at the minimum levels necessary to achieve the desired effect and per requirements for normal production following current good manufacturing practices (cGMP). The following are maximum suggested use levels for food processing applications.
Brewing processes: Up to 10,000 U/kg starch dry matter.
Starch processing: Up to 1,500 U/kg starch dry matter.

1.C.3. Purpose for Which the Substance is Used

The pullulanase will be 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.

1.C.4. Description of the Population Expected to Consume the Substance

The population expected to consume the food produced by pullulanase consists of members of the general population who consume at least one of the food products described above. However, members of the general population may consume foods with no functional

pullulanase activity since the enzyme does not exert a function in the final food/beverage for the reasons described in 1.C.1. Consequently, the presence of residues of food enzymes in the final food does not lead to any effect in or on the final food. The enzyme action is expected to be over in the food product when available to consumers.

1.D. Basis for the GRAS Determination

This GRAS conclusion is based on scientific procedures in accordance with 21 CFR 170.30(a) and 170.30(b).

1.E. Availability of Information

The data and information that are the basis for this GRAS conclusion will be made available to FDA upon request by contacting Susan Cho at NutraSource, Inc. at the address above. The data and information will be made available to FDA in a form in accordance with that requested under 21 CFR 170.225(c)(7)(ii)(A) or 21 CFR 170.225(c)(7)(ii)(B).

1.F. Availability of FOIA Exemption

None of the data and information in Parts 2 through 7 of this GRAS notice are exempt from disclosure under the Freedom of Information Act, 5 U.S.C. §552.

1.G. Certification

GenScript/Bestzyme certifies that, to the best of their knowledge, this GRAS conclusion is based on a complete, representative, and balanced dossier that includes all relevant information, available and obtainable by GenScript/Bestzyme, including any favorable or unfavorable information, and pertinent to the evaluation of the safety and GRAS status of the use of its pullulanase.

1.H. Name, Position/Title of Responsible Person Who Signs Dossier, and Signature

Name: Andy Yan, Ph.D. Date: 4/16/2019
Title: Senior Director of Business Development

Address correspondence to
Susan S. Cho, Ph.D., NutraSource, Inc.
+1-301-875-6454; susanscho1@yahoo.com
Agent for GenScript/Bestzyme

PART 2. IDENTITY, MANUFACTURING, SPECIFICATIONS, AND TECHNICAL EFFECTS

2.A.1. Identity of the Notified Substance

2.A.1.1. Common Name

The common or usual name for the GRAS ingredient is pullulanase, specifically a pullulanase enzyme preparation produced by a genetically engineered *Bacillus subtilis.*

2.A.1.2. Chemical Names of Main Component

International Union of Biochemistry and Molecular Biology (IUBMB) Enzyme Nomenclature Classification: Pullulanase
IUBN: α-Dextrin endo-1,6-α-glucosidase
IUB No.: EC 3.2.1.41

2.A.1.3. Chemical Abstract Service (CAS) Registry Number

CAS number, 9075-68-7

2.A.1.4. Empirical Formula: Not applicable

2.A.1.5. Structural Formula: Not applicable

2.A.1.6. Molecular Weight: Approximately 92 kD

2.A.1.7. Background

Pullulanase is classified as a debranching enzyme that specifically hydrolyzes α-1,6-glucosidic bonds in starch, pullulan, amylopectin, and glycogen, and in the alpha- and beta-limit dextrins of amylopectin and glycogen. Pullulanase is a naturally occurring enzyme in many microbial species. The National Research Council/National Academy of Sciences reported that pullulanase was first used in 1974 in the United States for production of corn syrup, high fructose corn syrup (HFCS), and dextrose production (NRC/NAS, 1981). Nowadays, pullulanase is routinely applied for starch hydrolysis in the food industry around the world. The enzymes are produced under cGMP using master and working cell banks and safe food production procedures. The GenScript/Bestzyme's enzyme preparation meets the general purity specifications for enzyme preparations as described in the monograph "Enzyme Preparations" of the current edition of the Food Chemicals Codex 11 (FCC 11, 2018).

2.A.2. Potential Toxicants in the Source of the Notified Substance

Filtration steps employed in the manufacturing process are expected to filter out the source microorganism as the vegetative *B. subtilis* is approximately 3-4 μm in length and about 1 μm in width (Sargent, 1975).

<u>Absence of Toxins from Enzyme Preparation</u>

The production microorganism is not known to produce toxins, and the manufacturing process is tightly controlled to prevent microbial contamination from other sources.

Absence of Antibiotics from Enzyme Preparation

No antibiotics are used during the manufacturing of the enzyme preparation; thus, there is no possibility that antibiotics are present in the enzyme preparation.

2.A.3. Particle Size

Not applicable.

2.B. Method of Manufacture

2.B.1 Production Strain

The recipient *Bacillus subtilis*, designated as 8001-YF, was genetically modified by deletion of six genes and a gene cluster that are residential in the parental strain *Bacillus subtilis* BS-YF (CICC 20632). The DNA sequence for the introduced pullulanase gene, GenBank *BD140672*, is a synthetic gene based on the coding sequence from the pullulanase encoding gene (5' end) of *Bacillus deramificans* (NA). *Bacillus subtilis* is classified as a Good Industrial Large Scale Practice (GILSP) microorganism, and it meets the criteria for a safe production microorganism as described by Pariza and Foster (1983). In particular, the *Bacillus subtilis* strain can be engineered to inactivate extracellular proteases, such as subtilisin (AprE) and neutral metalloproteaseE (NprE).

The *Bacillus subtilis* strain was engineered to inactivate proteins that play a role in spore formation, such as sporulation-specific sigma-F factor encoded by the spoIIAC gene. Such genetically engineered *Bacillus subtilis* strains have the advantage of providing improved expression and secretion of the expressed pullulanase enzymes. The deletion of the spB gene cluster further makes the strain resistant to the phage lysis. Different deletion of intact genes with the original chromosomal genes were achieved by single crossover homologous recombination. The corresponding deletion plasmid was transformed into competent *Bacillus* cells. A single transformant selected from plates supplemented with erythromycin at the permissive temperature of 30ºC was streaked onto another erythromycin containing plate, and incubated at the non-permissive temperature of 37ºC for selection of the transformants with the temperature-sensitive plasmid integrated into the host chromosome. To obtain the gene deletion at the designated locus, several colonies selected from plates were transferred into 2YT media and incubated at 30ºC for 5-7 days (fresh 2YT media was exchanged every two days). Erythromycin sensitive *Bacillus* cells were screened by PCR for plasmid excision and allelic gene deletion.

2.B.2. Pullulanase Expression Plasmid

The pullulanase expression plasmid pYF-tsINT-pul was constructed using pYF-tsDE essentially the same way as described above for the gene deletion studies, and the pullulanase expression cassette contained the following components: a synthetic promoter sequence, a synthetic ribosome binding site, a truncated form of the pullulanase gene from *Bacillus deramificans* with the deletion of amino acid residues 1 to 104 from the N-terminus of the native pullulanase gene, and a synthetic termination sequence were inserted into the designated site with two 800-base pair homologous regions of upstream and downstream sequences of the AmyE locus of the chromosome in the host cells. This plasmid was amplified in *E. coli* cells and purified before transforming into the 8001-YF host cells.

The pullulanase expression plasmid pYF-tsINT-pul (Figure 1) is shown as follows.

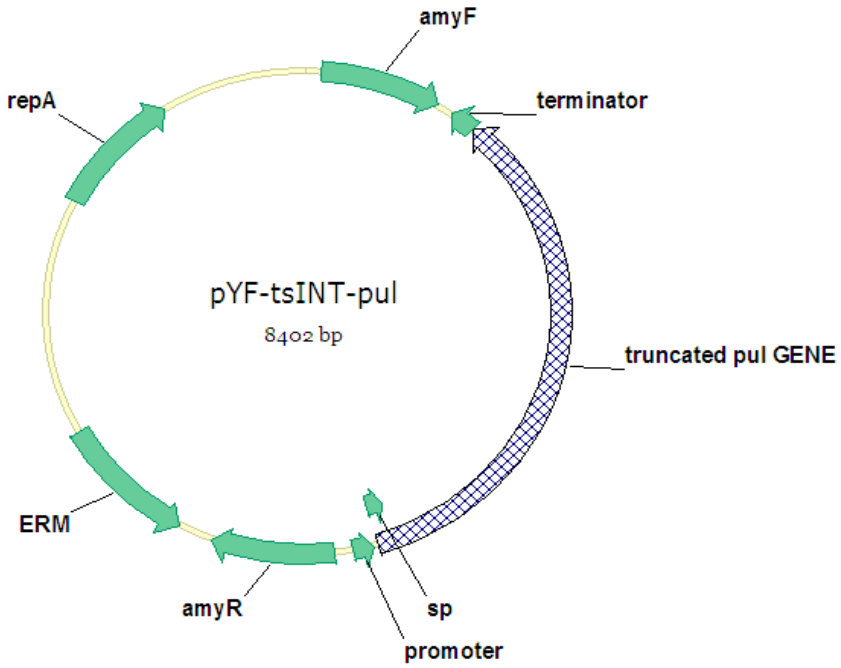


Figure 1. Schematic Drawing of the Pullulanase Expression Plasmid pYF-tsINT-pul

2.B.3. Construction of the Recombinant Microorganism

In order to integrate the expression cassette into the designated AmyE locus of the chromosome, a pullulanase expression cassette was flanked by 800-base pair homologous regions of upstream and downstream sequences of the AmyE locus of the chromosome. A few head to tail native selected bacterial chromosomal DNA fragments and functional synthetic sequences required for controlling the expression of the pullulanase gene were assembled.

The marker-free gene replacement of AmyE with the pullulanase expression cassette was performed essentially the same way, as described above, for the gene deletion studies (Figure 2). Briefly, the genetic exchange of the gene encoding the pullulanase into the AmyE locus on the chromosome of the *Bacillus* cells was done by plasmid-mediated single-crossover homologous recombination. After the integration, only the pullulanase expression cassette flanked by the partial AmyE sequences is inserted at the AmyE loci, and no other components of the plasmid are present in the genome of the recipient after incubation of the cured expression host at 37°C for at least 5 generations. The halo formation on the red-pullulan plates confirmed the successful integration of the pullulanase coding gene into the chromosome of *Bacillus subtilis*. PCR reactions further confirmed that the expression cassette was indeed present at the AmyE locus of the recipient strain.

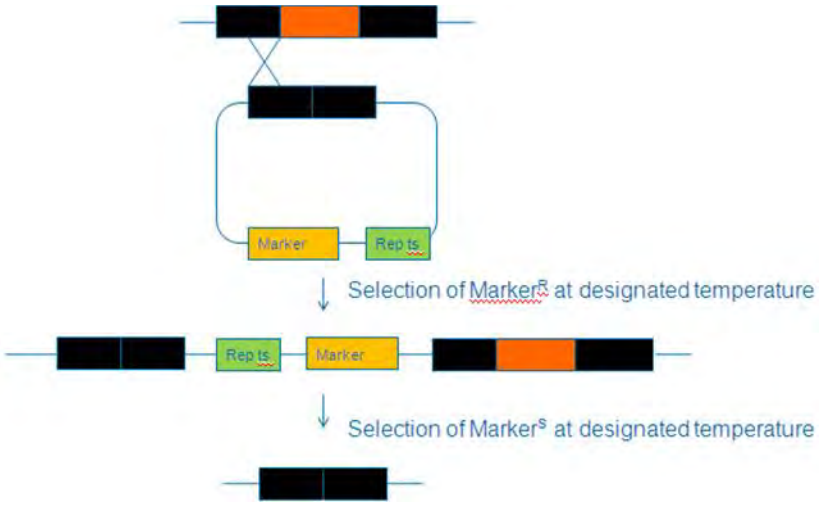


Figure 2. Single Crossover (Marker-free) Integration

2.B.4. Raw Materials

The raw materials used during the manufacturing process are all standard ingredients allowed in the food industry. The QC department of Bestzyme samples the materials on arrival and monitors all the analyses to ensure their qualities. Meanwhile, the quality management system complies with the regulations of ISO 9001. The raw materials conform to Food Chemicals Codex (FCC) 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 the FCC requirements.

Table 1. List of Raw Materials and Their Regulatory Status

Material	CAS No.	Regulatory Status
Maltose syrup	69-79-4	21CFR
Corn syrup	8029-43-4	21CFR §184.1865
Glucose	50-99-7	21CFR §168.120
Yeast	8013-01-2	21CFR §184.1983
Peptone	73049-73-7	21 CFR §184.1553
Potassium dihydrogen phosphate, KH_2PO_4	7778-77-0	21CFR160.110
Magnesium sulphate, $MgSO_4$	7487-88-9	21 CFR §184.1443
Manganese sulphate	10034-96-5	21 CFR §184.1461
Urea	57-13-6	21 CFR §184.1923
Calcium Chloride, $CaCl_2$	7647-14-5	21 CFR §184.1763
Ferrous Sulfate, $FeSO_4$. $7H_2O$	7782-63-0	21CFR§184.1315
Ammonium sulphate, $(NH_4)_2SO_4$	7783-20-2	21 CFR §184.1143
Sodium chloride, NaCl	10035-04-8	21 CFR §184.1193
Citric Acid	77-92-9	21 CFR §184.1033
Cottonseed Flour	68308-37-2	21 CFR §117.5

2.B.5. Fermentation Process

The pullulanase under this notification is prepared by submerged fermentation of genetically engineered *Bacillus subtilis* cells. All fermenters and other equipment used during the manufacturing process were designed and constructed to ensure the absence of contamination of foreign microorganisms during fermentation. Table 1 lists the raw materials used in fermentation and their CAS numbers and regulatory status. The agitation tank is equipped with control valves. When the optimal conditions, such as temperature and pH, are secured, the *B. subtilis* is added into the agitation tank. The tank is equipped with an agitation system to continuously mix the reaction solution.

2.B.6. Recovery and Purification Process

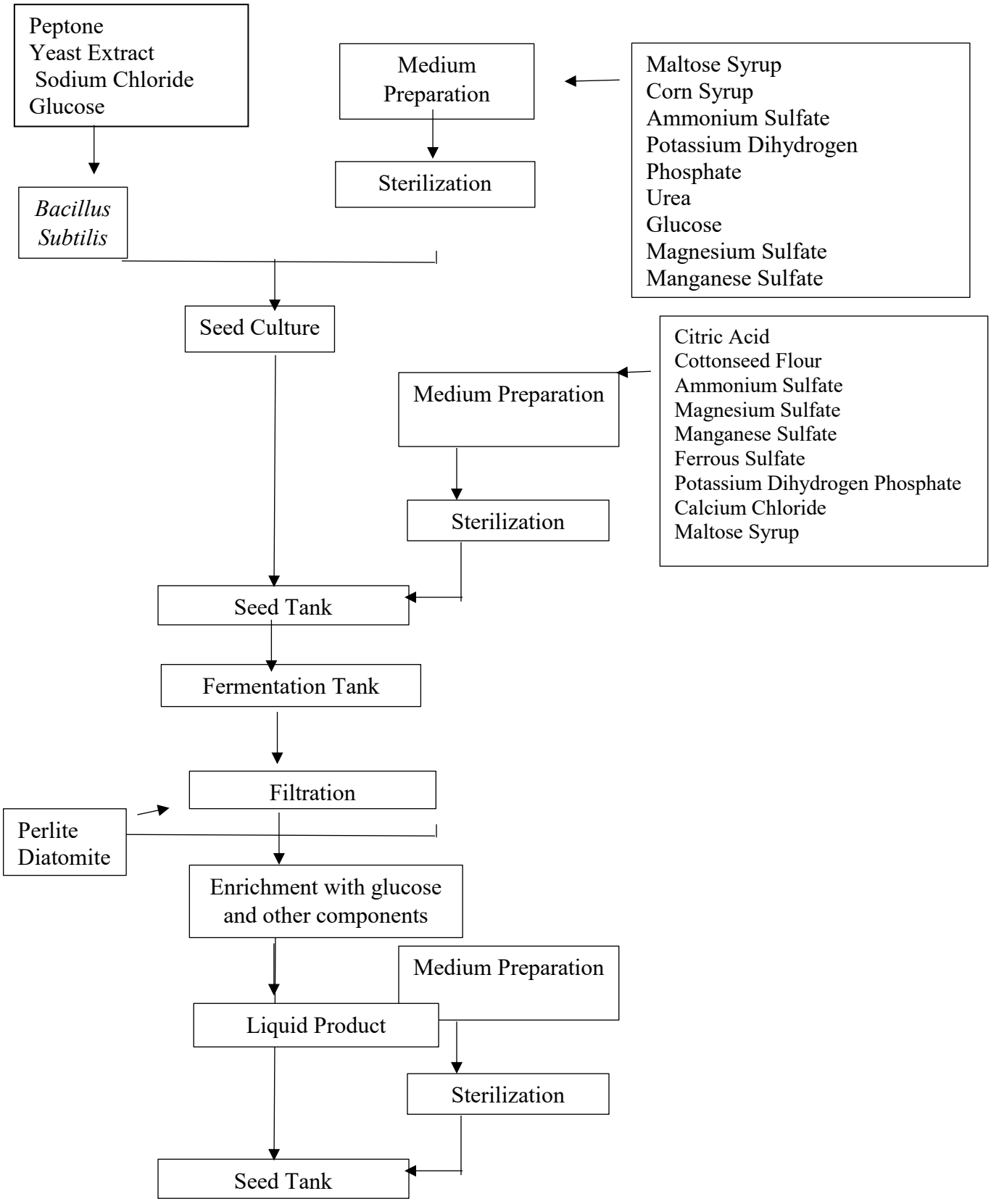
The recovery process starts immediately after the completion of the fermentation process that consists of pretreatment with flocculant, filtration, concentration, and formulation. The final product is mixed with glucose, sodium benzoate, and potassium sorbate, and standardized according to the product specification.

2.B.7. Quality Assurance Procedure

The quality of the product is monitored according to the designated product specification. Periodically, samples from the fermenter are taken out to analyze under the microscope to confirm the stain identity by staining, and the absence of foreign microorganisms in the fermenter is regularly tested under the microscope by plating on a nutrient agar plate for overnight incubation at 37°C. The fermentation is declared "contaminated" if one of the following conditions are fulfilled: 1) contamination is observed in 2 or more samples by microscopy and 2) contamination is observed in two successive agar plates at a minimum interval of 8 hours. Any contamination observed will be rejected.

Pullulanase is manufactured under cGMP using common food industry materials and processes. GenScript/Bestzyme observes the principles of Hazard Analysis and Critical Control Point (HACCP)-controlled manufacturing process and rigorously tests its final production batches to verify adherence to quality control specifications. All processing aids used in the manufacturing process are food grade. Process tanks and lines are cleaned with sodium hydroxide and hydrogen peroxide following standard procedures common to the food industry.

Figure 3. Flow Chart of the Production Process



2.C. Composition and Specifications of GenScript/Bestzyme's Pullulanase

Tables 2 and 3 show the composition and specifications of GenScript/Bestzyme's pullulanase, respectively. Table 4 presents the analytical values for three non-consecutive lots of pullulanase. The pullulanase enzyme preparation complies with the recommended purity criteria for enzyme preparations as described in *Food Chemicals Codex*. 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 (JECFA, 2006). The enzyme preparation, that is the subject of this notification, does not contain a potential food allergen (such as soy) from the fermentation media.

Table 2. Composition of GenScript/Bestzyme's Pullulanase

Component	Content
Enzyme Solids (TOS*), %	4.2
Activity, U/mg TOS	53.8
Water, %	>50
Glucose, %	20-40
Sodium Benzoate, %	<0.50
Potassium Sorbate, %	<0.50

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

Table 3. Specifications for GenScript/Bestzyme's Pullulanase

Parameter	Specification	Method of Analysis
Activity unit, U/g	>2,000	GB 1886.174-2016
Lead, ppm	NMT 1	GB 1886.174-2016
Arsenic, ppm	NMT 1	GB 1886.174-2016
TPC	NMT 5,000 cfu/g	GB 1886.174-2016
Total Coliforms	NMT 10/g	GB 1886.174-2016
Salmonella	Absent in 25g	GB 1886.174-2016
Escherichia coli	Absent in 25g	GB 1886.174-2016
Antimicrobial activity	Not detected	GB 1886.174-2016

Table 4. Summary of Analytical Values for GenScript/Bestzyme's Pullulanase

Parameter	B011170901	B011171120	B011180311	Mean
Activity unit, U/g	2270	2230	2280	2260
Lead, ppm	< 0.1	< 0.1	< 0.1	< 0.1
Arsenic, ppm	< 0.5	< 0.5	< 0.5	< 0.5
TPC	< 300	< 300	< 300	< 300
Total Coliforms	<3	<3	<3	<3
Salmonella	ND	ND	ND	ND
Escherichia coli	ND	ND	ND	ND
Antimicrobial activity	ND	ND	ND	ND

2.D. Intended Technical Effects

The pullulanase enzyme is used as a processing aid in food manufacturing and is not added directly to the final foodstuffs. In particular, pullulanase hydrolyzes the 1,6 alpha-glucosidic bonds of starch and other starch-related substance. The typical food processes, where pullulanase is used, include brewing, beverage alcohol, and starch processing.

PART 3. DIETARY EXPOSURE

3.A. Estimated Dietary Intakes (EDIs) of Pullulanase Under the Intended Use

Since the intended use and use levels are the same or similar to those described in GRN 645, EDIs were calculated using the same method described in GRN 645.

In GRN 645, an assumption was made that all the enzyme product is retained in the final food product to provide a worst-case scenario for the calculation of the possible daily human exposure. This assumption regarding the food enzyme in the final food product is highly exaggerated since the enzyme protein and other substances resulting from the processing are diluted or removed after achieving the desirable technical effects. In the production of beer, it is assumed that the pullulanase used during the production will remain in the final beer. However, beer and beer-like beverages produced with the enzyme are not always produced with the maximum suggested dosage. In GRN 645, exaggerated human intakes were estimated using the Budget method (ILSI, 1997) for the intake associated with starch processing and then using the consumption data to estimate the intake associated with beer and other cereal beverages (Table 5). Since the intended use and use levels are the same or similar to those described in GRN 645, EDIs were calculated using the same method described in GRN 645. Thus, the method used for calculating EDIs will be briefly discussed in this GRAS notice with no details.

The enzyme preparation, net content of water, may be represented by its total solids level. Total solids include ash constituents that can be considered inert relative to the safety issues. Non-ash components of the product are measured as total organic solids (TOS), which include residues from the fermentation and metabolic end products from the production microorganism. The TOS include the enzyme protein, the functional agent of the preparation. Enzyme residues are presented as the estimated TOS per unit of enzyme in the commercial product.

The theoretical maximum daily intake of consumers of the food enzyme based on the contribution by both starch and brewing and cereal based beverage is 0.496 mg TOS/kg bw/day. Calculation methods are briefly discussed as follows:

Calculation Methods

Determination of enzyme U/mg TOS: The pullulanase batches have a mean activity of 2,260 U/g and an approximate content of 4.2% TOS. Thus, a corresponding activity/mg TOS ratio is 53.8 U/mg TOS.

Intake Associate with Starch Processing

Solid food based on starch contribution: 3.12 g starch derived dry matter/kg bw/day (adopted from GRN 645).

Liquid food based on starch contribution: 3.25 g hydrolyzed starch derived dry matter/kg bw/day assuming that the densities of the beverages are ~ 1 (adopted from GRN 645).

Solid and liquid food: The highest dosage given for solid food is 1,500 U/kg starch based raw material which corresponds to 27.88 mg TOS.

The combination of 3.12 g starch derived dry matter in solid food and 3.25 g starch derived dry matter in liquids is expected to contain the maximum of 0.178 mg TOS based on the following formula: 27.88 mg TOS per kg / 1000 g per kg x (3.12 + 3.25) g = 0.178 mg TOS.

Intake associated with beer and other cereal based beverage processes: 1.71g starch/kg bw/day (adopted from GRN 645).

The dosage for the pullulanase in brewing processes and other cereal based beverage processes is up to 10,000 U/kg starch. The corresponding TOS is calculated as 185.9 mg TOS/kg starch (derived from the following formula: 10,000 U/kg starch / 53.8 U/mg TOS).

Consumption of 1.71 g starch/kg bw/day is expected to result in 0.318 g TOS/kg bw/day based on the following formula: 185.9 mg TOS/kg starch x 0.00171 kg starch/kg bw/day = 0.318 mg TOS/kg bw/day. The theoretical maximum daily intake contribution from beverage alcohol is assumed to be zero due to the distilling process.

As presented in Table 5, total theoretical maximum daily intake is expected to be 0.496 mg TOS/kg bw/day based on the following formula: 0.178 mg TOS/kg bw/day + 0.318 mg TOS/kg bw/day =0.496 mg TOS/kg bw/day. This level corresponds to a total pullulanase activity of 26.7 U/kg BW/day.

Table 5. Theoretical Maximum Daily Intake Under the Intended Use

Under the Intended Use	The theoretical maximum daily intake	
	mg TOS/kg bw/day	Unit of Enzyme/kg bw/day
Intake associated with starch processing:	0.178	9.6
Intake associated with beer and other cereal based beverage processes	0.318	17.1
Total	0.496	26.7

For the safety evaluation of this enzyme, a NOAEL level of 1,285 mg TOS/kg bw/day or 42,649 U/kg bw/day determined from a 13-week oral rat feeding study conducted on pullulanase (GRN 205; FDA, 2006) was used for the safety margin calculation (details are described in Part 6.B.2.1 or pages 23-26 of this notice). The subject of the 13-week oral toxicity study was pullulanase from *Bacillus deramificans* expressed in *Bacillus subtilis.* After considering the safety margin of 100, the safe intake level is estimated to be 12.85 mg TOS/kg bw/day. The intended use levels are approximately one twenty-fifth (approximately 3.8%) of the safe intake.

Alternatively, the theoretical maximum daily intake can be compared with the NOAEL expressed in enzyme unit/kg bw/day. The theoretical maximum daily intake of 26.7 U/kg bw/day is far less than the NOAEL of 42,649 U/kg bw/day (Please note: This enzyme preparation tested in a 13 week oral toxicity study had 33.19 U/mg TOS. Total pullulanase U present in 1,285 mg TOS is calculated from the equation of 1,285 mg TOS x 33.19 U/mg TOS=42,649 U/kg bw/day). After considering the safety margin of 100, the safe intake level is estimated to be 426.5 U pullulanase/kg bw/day. The intended use level (26.7 pullulanse U/kg bw/day) is approximately one fifteenth (approximately 6.3%) of the safe intake level.

However, these EDIs are highly amplified since the presence of the enzyme residues in the final food does not lead to any effect in or on the final food. It is due to the facts that the enzymes will be denatured or removed after achieving the desired technical effects and that the enzyme action will be over before the food product is available for consumers.

3.B. Food Sources of Pullulanase
No applicable

3.C. EDIs of Pullulanase from Diet
No applicable

3.D. Total EDIs of Pullulanase from Diet and Under the Intended Use
Same as 3.A.

3.E. EDIs of Other Nutrients Under the Intended Use
Not applicable

Summary of Consumption Data

The intended use and use levels are the same or similar to those described in GRN 645. The theoretical maximum daily intake of consumers of the food enzyme based on the contribution by both starch and brewing and cereal based beverage is 0.496 mg TOS/kg bw/day (corresponding to 26.7 U pullulanse/kg bw/day) even if all the enzyme residue is present in the final food product. From a 13-week oral rat feeding study conducted on pullulanase from *Bacillus deramificans* expressed in *Bacillus subtilis,* the NOAEL was determined to be 1,285 mg TOS/kg bw/day or 42,649 U/kg bw/day (GRN 205). After considering the safety margin of 100, the intended use levels are less than one fifteenth (3.8 to 6.3%) of the safe intake levels estimated from a 13-week oral toxicity of pullulanase in rats.

The EDIs were based on the assumption that all the enzyme product is retained in the final food product to provide a worst-case scenario for the calculation of the possible daily human exposure. However, these EDIs are highly amplified since the presence of the enzyme residues in the final food is not expected to have any effect in or on the final food, and the enzyme action is expected to be over before the food product is available for consumers.

PART 4. SELF LIMITING LEVELS OF USE

The use of the pullulanase enzyme preparation to produce GOS is limited by the level that can economically be added to maximize the enzyme efficiency in the production of the ingredient.

PART 5. HISTORY OF CONSUMPTION

Nontoxigenic and nonpathogenic strains of *B. subtilis* are widely available and have been safely used as components of food products. Typical examples include *B. subtilis* present in the Japanese food natto, a fermented soy bean food product. Carbohydrase and protease enzyme preparations derived from *B. subtilis* were in common use in food prior to January 1, 1958.

PART 6. NARRATIVE

6.A. Current Regulatory Status

The Enzyme, Pullulanase

The safety of pullulanase are well acknowledged by authorities. Self-affirmed GRAS for carbohydrase and proteases from *Bacillus subtilis* has been accepted by the U.S. FDA and these enzymes are covered in the regulations under 21 CFR 184.1148 and 21 CFR 184.1150.

The enzyme, pullulanase, has a history of safe use in food production and, even more specifically, in the production of starch and alcohol and cereal based beverages. FDA has issued several "no questions" letters about the GRAS status of the following sources of pullulanase (Table 6): GRN 645 (FDA, 2016a), GRN 205 (FDA, 2006), GRN 72 (FDA, 2001), and GRN 20 (FDA, 1999).

Table 6. Regulatory Status of Pullulanase Produced by Genetically Engineered Microorganisms

GRN	Production microorganism	Source Microorganism	Company	Closure Date
645	*B. licheniformis* HyGe486 (via AEB1763 strain)	*B. deramificans* LMGP13056 or *B. acidopullulyticus* NCIMB11639	Novozymes North America	11/1/2016
205	*B. subtilis* MDT96	*B. acidopullulyticus*		12/4/2006
72	*B.licheniformis*	*B. deramificans*	Genencor International Inc.	6/12/2001
20	*B. subtilis* B1-109	*B. naganoensis*	Enzyme Bio-Systems Ltd	9/30/1999

Bacillus subtilis, the Production Microorganism

B. subtilis has been safely used in the production of many carbohydrases and proteases. *Bacillus subtilis* is described as the production organism for different enzymes in many GRAS notifications. Relevant GRAS notices are summarized in Table 7 (GRN 114- FDA, 2003; GRN 274- FDA, 2009; GRN 476- FDA, 2014; GRN 579- FDA, 2015a, GRN 592- FDA, 2015b; GRN 649- FDA, 2016b; GRN 714- FDA 2018a; GRN 746- FDA, 2018b).

Table 7. Regulatory Status of Other Enzymes Produced by *B. subtilis*

GRN	Enzyme	Production Organism, the name of *B. subtilis* strain	Source Microorganism	Closure Date
746	Maltogenic amylase	RF12029	*Geobacillus stearothermophilus*	6/13/2018
714	Subtilisin	BG3600-1425-3D	*B. amyloliquefaciens ATCC 23844*	2/06/2018
649	β-galactosidase	NA	*B. circulans* ATCC31382	11/28/2016
592	β-glucanase	CF 624B-1	*B. subtilis* BG125	10/07/2015
579	Lactase	JL47	*Bifidobacterium bifidum* BIF917	11/05/2015
476	Asparaginase	MOL2940		2/03/2014
274	Branching glycosyltransferase	JA 1343	*R. obamensis*	6/25/2009
114	Pectate lyase	IFO 3134	*B. subtilis* IFO 3134	1/27/2003

NA=not avialble.

Bacillus deramificans, the Source Microorganism

Bacillus deramificans has been recognized as a safe source microorganism for pullulanase (GRN 645, FDA 2016a; filed by Novozymes North America, Inc., closure date, 11/1/2016; GRN 72, FDA, 2001; filed by Genencor International Inc., closure date, 6/12/2001). Toxicological tests have been conducted on a pullulanase preparation from *Bacillus deramificans* including bacterial reverse mutation test, human lymphocytes *in vitro* micronucleus test, and *in vitro* cytogenecity test (GRN 645, FDA 2016a). No toxicological adverse effect was observed.

6.B. Review of Safety Data

6.B.1. Identification of Production Microorganism

Ribosomal RNA sequence, especially 16S ribosomal RNA, is the best single target for defining phylogenetic relationships among bacteria. This genetic information provides a phylogenetic framework and basis for modern microbial taxonomy (Ludwig and Klenk, 2001). For the delineation of microorganisms at species level, 97% of 16S ribosomal RNA similarity is commonly applied as the conservative threshold in microbial phylogeny. The 16S ribosomal RNA of *Bacillus subtilis* NCBI reference sequence (Accession Number: NC_000964.3) is rrnA-16S and located from position 30279 to 31832 in the whole genome. After whole genome sequencing by next generation sequencing (NGS), the reads could be mapped to and cover 100% region of the rrnA-16S gene with 100% similarity. Thus, the species of the test strain was identified as *Bacillus subtilis*.

Bacillus subtilis was identified by next generation sequencing (NGS). Illumina paired end sequencing (i.e., PE150) was selected to sequence the whole genome of this bacteria, in which TruSeq DNA LT Sample Prep Kit was used to construct the NGS library, and Miseq platform was used as the NGS sequencer. The reference genome of *Bacillus subtilis subsp. subtilis str. 168* (NCBI Reference Sequence: NC_000964.3, length 4,215,606 bp) was downloaded from

NCBI. The reads generated by NGS was mapped to this reference genome. The average sequencing depth of the whole genome was 295.78, and 99.94% of the whole genome DNA was covered. Meanwhile, the test strain has completely identical sequences with the rrnA-16S gene of the *Bacillus subtilis* NCBI reference sequence (Accession Number: NC_000964.3), indicating that the test strain belongs to *Bacillus subtilis*.

Trimmomatic v0.38 was utilized for the trimming tasks of raw illumina paired-end data. The qualified reads were stored as fastq files (Supplementary file: R1.fastq.gz and R2.fastq.gz). BWA v0.7.17 was used to do read mappings, and Samtools version 1.9 was used to do depth calculation. The complete reference genome (NC_000964.3) of *Bacillus subtilis subsp. subtilis str. 168* was stored in the supplementary file: Bacillus_subtilis.fasta (fasta format) and Bacillus_subtilis.gb (GenBank format), and the calculated depth mapping to NC_000964.3 was summarized in Depth.txt, in which the first column was the name of the genome, the second column was the base position, and the third column was the depth. Freebayes v1.0.2 was used for variant calling, and no variant was detected at the rrnA-16S gene of the *Bacillus subtilis* NCBI reference sequence (Accession Number: NC_000964.3). Therefore, the test strain has completely identical 16S ribosomal RNA with *Bacillus subtilis* (NCBI reference sequence accession number: NC_000964.3).

6.B.2. Safety of the Pullulanase Enzyme

Pullulanase enzyme preparations have been commercially used since the early 1980s (Jensen, 1984). A wide variety of enzymes are used in food processing, and enzyme proteins do not generally raise safety concerns (Pariza and Foster, 2003; Pariza and Josnson, 2001). Pariza and Foster (2003) noted that very few toxic agents have enzymatic properties. From the investigation on possible allergenicity of 19 different commercial enzymes used in the food industry, Bindslev-Jensen et al. (2006) concluded that ingestion of food enzymes, in general, is not likely to be a concern with regard to food allergy.

A recent review by Ladics and Sewalt (2018) also summarizes the safety of food industrial enzymes including pullulanase as follows:

1) Enzymes, in general, don't produce acute toxicity, dermal sensitization, genotoxicity, or repeated dose oral toxicity.
2) Several hundred mutagenicity studies have been conducted on bacterial and mammalian cells using a variety of enzymes. No positive findings were observed.
3) Over 225 90-day studies have reported no adverse findings, including in the bone marrow. The data showing no adverse effects for enzyme preparations also confirms that the microbial metabolites and fermentation materials lack toxicity as well.
4) Exposure to enzyme products is also minimal as recommended use levels are low, generally <0.1% (wt/wt). The weight-of-evidence indicates that there are no concerns for oral toxicity of enzymes, in general, nor genotoxicity.
5) Therefore, continued routine practice of performing genotoxicity and 90-day studies on enzyme preparations as a part of the approval requirements is questionable, and establishing general health-based guidance values for enzymes may be considered.

Based on these toxicological data and the history of safe use, it is concluded that the pullulanase is safe for its intended use as a processing aid in various food applications.

6.B.2.1. Animal Toxicity Studies of Pullulanase

As shown in Table 8, animal toxicity studies of various pullulanase preparations found no adverse effects, regardless of the *Bacillus* species employed as the source or production microorganisms.

Table 8. Summary of Animal Toxicity Studies of Various Pullulanase Enzyme Preparations

Species	Pullulanase source	Dose	Duration	LD_{50} or NOAEL	Reference
The study of GenScript/Bestzyme's Pullulanase (from *B. subtilis* via *B. deramificans*)					
Rat	From *Bacillus subtilis* via *Bacillus deramificans*	0, 10, 30, or 60 mL/kg bw	Single dose; 14-day observation	LD_{50}>>>60 mL/kg bw (or 151,800 U/kg bw)	Gao, 2018b. Appendix B
Study of Another Pullulanase Preparation from *B. subtilis* via *B. deramificans*					
Rat, Wistar	From *B. subtilis* via *B. deramificans*	0, 1.0, 3.0, or 10.0 mL/kg/d pullulanase (equivalent to 128.5, 387, or 1,285 mg TOS/kg/d, respectively	13 wk	NOAEL=10.0 mL/kg/day or 1,285 mg TOS/kg bw/d, corresponding to 42,649 NPUN (pullul-anase unit Novo) /kg bw/d	GRN 205*, Stamped pages 47 to 49
Study of Pullulanases Manufactured by Other Combinations of Bacillus Species					
Young adult albino rats (Crl:CD® (SD)BR strain)	From *B. subtilis* B1-163/pEB301	5 g/kg bw of pullulanase or vehicle control by oral gavage	Single dose, 14 d observation	LD_{50}>>>5 g/kg bw	GRN 20, stamped pages 94, 128, and 129
Crl:CD® (SD)VAF/ Plus rats		0, 0.625, or 1.25% of diet	4 wk	NOAEL = 914 - 1,313 mg/kg/d for males, and 995 - 1,226 mg/kg/d for females	GRN 20, stamped pages 94-95 and 130 to 134
Crl:CD BR rats	From *Bacillus licheniformis via B. deramificans*	0, 0.2, 1.0, and 5.0% of diet	4 wk	NOAEL = 5% of the diet	Modder-man and Foley, 1995
Crl:CD BR rats		0, 0.1, 0.5, 1.0, 3.0, and 5.0% of diet	2 wk	NOAEL = 5% of the diet	

*The subject of GRN 205 was pullulanase from *Bacillus acidopullulyticus* expressed in *Bacillus subtilis*. However, in this GRAS notice, the safety data on the pullulanase from *Bacillus deramificans* (source microorganism) expressed in *Bacillus subtilis* (production microorganism) were presented to support the safety of pullulanase expressed in *B. subtilis*.

A Study of GenScript/Bestzyme's Pullulanase (from *Bacillus subtilis* via *Bacillus deramificans*)

The subject of the present GRAS notice is pullulanase from *Bacillus deramificans* expressed in *Bacillus subtilis*. The aim of this study was to evaluate the acute toxicity of GenScript/Bestzyme's pullulanase after oral administration in rats. The test substance was administered to young rats by oral gavage at doses of 0 (control), 10, 30, or 60 mL/kg body weight (bw) (5 males and 5 females per group). The pullulanase preparation had 2,530 U/mL. Animals were observed for 14 days to monitor changes in clinical signs (i.e., changes in eyes, mucous membranes, or behavior patterns; loss of fur or scabbing), body weight, and food consumption. At the end of the study, animals were sacrificed, and major organs (such as liver, kidneys, spleen, heart, and lungs) were examined macroscopically and microscopically for the highest dose group. No animal died during the 14-day observation period, and no clinical signs of abnormality were observed at any dose levels. Furthermore, no significant differences in mean body weight, food consumption, and organ weights were found among the groups. No treatment-related abnormalities were observed in the macroscopic examinations of the organs. In summary, this study found that the lethal dose (LD_{50}) of GenScript/Bestzyme's pullulanase was far above 60 mL/kg bw (or 151,800 U/kg bw), the highest dose tested. Details are described in Appendix B. The unpublished status of this study will not impact the safety evaluation by other experts since the data from an acute toxicity study of a similar preparation have been available through GRN 20.

A Study of Another Pullulanase Preparation from *B. subtilis* via *B. deramificans*

In GRN 205, the data from a 13-week oral toxicity study of pullulanase from *Bacillus deramificans* expressed in *Bacillus subtilis* were presented to support the safety of pullulanase expressed in *Bacillus subtilis* (GRN 205, stamped pages 47 to 49; FDA, 2006). The subject of the present GRAS notice is pullulanase derived from the same species of source and production microorganisms as that described in GRN 205, i.e., from *Bacillus deramificans* expressed in *Bacillus subtilis*. Therefore, this notice incorporates, by reference, the toxicity study discussed in the GRN 205, and will not discuss a previously reviewed reference in detail.

In this 13-week oral toxicity study, Mol:SPRD rats received 1.0, 3.0, or 10.0 mL/kg bw/day pullulanase (equivalent to 128.5, 387, or 1,285 mg TOS/kg bw/d, respectively) or vehicle control. Pullulanase, up to 10.0 mL/kg bw/day, resulted in no significant toxic effects. The pullulanase activity used in this study was measured to be 3,950 NPUN (new pullulanase unit Novo)/g with an amount of 11.9% Total Organic Solids (TOS = 100% - % water - % ash - % any diluents). The NOAEL was determined to be 10.0 mL/kg bw/day or 1,285 mg TOS/kg bw/ for the pullulanase from *Bacillus deramificans* expressed in *Bacillus subtilis*. This level corresponds to 42,649 New Pullulanase Unit Novo (NPUN)/kg bw/day based on the following facts: the enzyme activity was measured to be 3950 NPUN/g with an amount of 11.9% TOS. The activity/mg TOS ratio was 33.19 NPUN/mg TOS. The NOAEL, expressed in enzyme unit, is calculated as 33.19 NPUN/mg TOS x 1,285 mg TOS/kg bw/day = 42,649 NPUN/kg bw/day.

Studies of Pullulanase Preparations Manufactured by Other Combinations of *Bacillus* Species

Pullulanase from *Bacillus subtilis* (GRN 20 stamped pages 128-134; FDA, 1999)

In GRN 20, the acute oral toxicity of pullulanase from *Bacillus subtilis* B1-163/pEB301 was assessed in young adult albino rats (Crl:CD® [SD]BR strain) (FDA, 1999). The rats (10 males and 10 females per group) received either a single dose of 5 g/kg bw of pullulanase or vehicle control (deionized water) via oral gavage and were observed for 14 days (GRN 20). No mortality and treatment-related abnormalities were observed. The LD_{50} value was estimated to be greater than 5 g/kg bw for male and female rats. In this study, no enzyme unit was reported for the test substance, thus, it was not possible to convert LD_{50} value into enzyme unit/kg bw.

In a subacute toxicity study, Crl:CD® (SD)VAF/Plus rats (10 males and 10 females per group) were fed diets containing 0, 0.625% (466 to 659 mg/kg bw/day for males and 540 to 664 mg/kg bw/day for females), or 1.25% pullulanase enzyme product (914 to 1,313 mg/kg bw/day for males and 995 to 1,226 mg/kg bw/day for females) for 4 weeks. No treatment-related adverse effects were noted. The NOAELs were found to be the highest levels tested, i.e., 914 to 1,313 mg/kg bw/day for males and 995 to 1,226 mg/kg bw/day for females.

Pullulanase from *Bacillus licheniformis* via *B. deramificans*

Modderman and Foley (1995) investigated the safety of a pullulanase enzyme preparation (1,300 ASPU/g) produced by a strain of *Bacillus licheniformis* that has been transformed by the introduction of a pullulanase gene from *B. deramificans*. A 4-week dietary toxicity study in rats was conducted in which Crl:CD BR rats (10 males and 10 females per group; 5 weeks of age at the initiation of dosing) received pullulanase in the feed at concentrations of 0, 0.2, 1.0, and 5.0%. Body weight, food consumption, clinical signs were recorded weekly. At the last week, neurobehavioral evaluations were conducted. At the end of the 4-week administration, animals were sacrificed, and the analysis of blood chemistry and hematology, ophthalmoscopic examinations, urinalysis, organ weights, and microscopic examination of organs were conducted. No adverse treatment-related effects were observed in any parameters tested.

A 2-week dietary toxicity study of pullulanase (1,300 ASPU/g) in rats also did not show any treatment-related adverse effects when pullulanase was added to the feed at concentrations of 0, 0.1, 0.5, 1.0, 3.0, and 5.0% (Modderman and Foley, 1995).

This pullulanase preparation was also tested for primary dermal irritation and primary eye irritation in New Zealand white rabbits and acute inhalation toxicity in Sprague-Dawley (SD) rats using standard protocols. No dose levels were specified in this study. The enzyme preparation has been shown to be a nonirritant in the eye and primary dermal irritation tests in rabbits (Modderman and Foley, 1995).

This pullulanase preparation tested the acute inhalation toxicity in SD rats using standard protocols. No deaths occurred as a result of exposure in rats (Modderman and Foley, 1995).

Conclusion

Based on the studies summarized above, for the purpose of the safety evaluation, the NOAEL of 1,285 mg TOS/kg bw/day (or up to 42,649 pullulanase U/kg bw/day) was chosen for pullulanase from *Bacillus deramificans* expressed in *Bacillus subtilis*.

6.B.2.2. Mutagenicity and Genotoxicity Studies of Pullulanase

The subject of the present GRAS notice is pullulanase from *Bacillus deramificans* expressed in *Bacillus subtilis* (or from *B. subtilis* via *B. deramificans*). Table 9 summarizes the mutagenicity, genotoxicity, and cytotoxicity studies of pullulanase preparations produced by *B. subtilis* or other *Bacillus* species.

Table 9. Summary of Mutagenicity, Genotoxicity and Cytotoxicity Studies of Pullulanase

Pullulanase Source	Test system	Concentration of Pullulanase	Reference
The study of GenScript/Bestzyme's Pullulanase (from *B. subtilis* via *B. deramificans*)			
Pullulanase from *Bacillus subtilis* via *Bacillus deramificans*	*S. typhimurium* TA97, TA98, TA100, TA102, and TA1535	253, 126.5, and 63.25 U/plate, ± S9	Gao, 2018a, Appendix C
Studies of Similar Pullulanase Enzyme Preparation from *B. subtilis* via *B. deramificans*			
Pullulanase from *Bacillus subtilis* via *Bacillus deramificans*	*S. typhimurium* TA98, TA100, TA1535, TA1537, and *E. coli* WP2 uvrA	156, 313, 625, 1,250, 2,500, or 5,000 ug/mL or per plate, ± S9; or up to 19.75 pullulanase U/plate	GRN 205*, stamped pages 47, 50, and 51
	In vitro chromosomal aberration test with human lymphocytes	Up to 5,000 µg/mL or up to 19.75 pullulanase U/mL, ± S9	
Studies of Pullulanase Enzyme Preparations Using Other Combinations of Bacillus Species			
From *B. licheniformis* via *Bacillus deramificans* or *B. acidopullulyticus*	Bacterial reverse mutation assay: *S. typhimurium* TA1535, TA100, TA1537, TA98 and *E. coli* WP2uvrA	156, 313, 625, 1,250, 2,500, and 5,000 µg dry matter pullulanase/mL, ± S9	GRN 645, stamped pages 51 to 54
From *B. licheniformis* via *Bacillus deramificans* or *B. acidopullulyticus*	*In vitro* Micronucleus Test in cultured human lymphocytes	5,000 µg TOS/mL of pullulanase, ± S9	GRN 645, stamped pages 51, 52 and 54
From *B. licheniformis* via *Bacillus deramificans* or *B. acidopullulyticus*	*In vitro* cytotoxicity study: Neutral Red Uptake in BALB/c 3T3 cell culture	50% viability at the highest concentrations (2-30 mg/mL); less cytotoxic at 0.1-1 mg/mL	GRN 645, stamped pages 51 to 53

From *B.licheniformis* via *Bacillus deramificans*	*S. typhimurium* TA98, TA100, TA1535, TA1537, and TA1538	5 quarter log dilutions up to 5,000 µg dry matter pullulanase/plate, ± S9	Modderman and Foley, 1995
From *B. licheniformis* via *Bacillus deramificans*	Forward mutation assay using mouse lymphoma L5178Y cells	Pullulanase concentrations of 1,000-5,000 µg/mL	Modderman and Foley, 1995
From *B. licheniformis* via *Bacillus deramificans*	*In vivo* mouse micronucleus assay with Swiss-Webster mice	0, 500, 889, 1,581, 2,811, or 5,000 mg pullulanase/kg bw (i.p.)	Modderman and Foley, 1995

*GRN 205 is related to pullulanase from *Bacillus acidopullulyticus* expressed in *Bacillus subtilis*. However, in this GRAS notice, the safety data on the pullulanase from *Bacillus deramificans* expressed in *Bacillus subtilis* were presented to support the safety of pullulanase expressed in *B. subtilis*.

A Study of GenScript/Bestzyme's Pullulanase (from *B. subtilis* via *B. deramificans*)

The potential mutagenicity of GenScript/Bestzyme's pullulanase (2,530 U/mL) was tested using a bacterial reverse mutation assay (Gao, 2018b). In the reverse mutation assay using five strains of *Salmonella typhimurium* (TA97, TA98, TA100, TA102, and TA1535), pullulanase (253, 126.5, and 63.25 U/plate, respectively) did not increase the number of revertant colonies in any tester strain, regardless of metabolic activation by S9 mix. The data indicated that pullulanase was non-mutagenic under the conditions used in this test. Details are described in Appendix C. The unpublished status of this study will not impact the safety evaluation since the data from mutagenicity and cytogenic studies of a similar preparation have been available through GRN 205 (FDA, 2016).

Studies of Another Pullulanase from *B. subtilis* via *B. deramificans* (Studies Reviewed in GRN 205)

In GRN 205, the data from mutagenicity and *in vitro* cytogenic studies of pullulanase from *Bacillus deramificans* expressed in *Bacillus subtilis* (GRN 205, stamped pages 47, 50, and 51) were presented to support the safety of pullulanase expressed in *Bacillus subtilis*. The subject of this GRAS notice is pullulanase derived from the same species of source and production microorganisms as that described in GRN 205, i.e., from *Bacillus deramificans* expressed in *Bacillus subtilis*. Therefore, this notice incorporates, by reference, the toxicity studies discussed in GRN 205, and will not discuss previously reviewed references in detail.

The potential for gene mutations by pullulanase was evaluated in *S. typhimurium* TA98, TA100, TA1535, and TA1537, as well as in *E. coli* WP2 uvrA. The concentrations used were 156, 313, 625, 1,250, 2,500, or 5,000 ug /mL (*S. typhimurium* strains) or ug/plate (*E. coli*), respectively, with or without S9. Corresponding pullulanase unit were up to 19.7 U/plate (Tested pullulanase had 3,950 U/g and 5000 ug would contain 19.75 U/plate). No dose-related increases in revertants were noted in the absence or presence of S9. This bacterial reverse mutation study

demonstrated that pullulanase did not induced gene mutations in bacteria in the absence or presence of S9.

In an *in vitro* chromosomal aberration test, human lymphocytes were exposed to doses of pullulanase, up to 5,000 µg/mL, with or without S9 (GRN 205). Corresponding pullulanase unit were up to 19.75 U/mL. Pullulanase-treated lymphocytes had similar numbers of aberrations as the solvent controls. This study showed that pullulanase did not induce chromosome aberrations in cultured human blood lymphocytes.

Studies of Pullulanase Enzyme Preparation Using Other Combinations of *Bacillus* Species

Pullulanase from *Bacillus licheniformis* via *B. deramificans* and *Bacillus acidopullulyticus* (GRN 645, stamped pages 51 to 55)

The safety of pullulanase from *B. deramificans* and *Bacillus acidopullulyticus* expressed in *Bacillus licheniformis* was evaluated in three mutagenicity tests: bacterial reverse mutation assay, *in vitro* cytotoxicity test, and *in vitro* micronucleus test in cultured human lymphocytes (GRN 645; FDA, 2016). The pullulanase preparation had 5,530 PUN(G)/g.

The bacterial reverse mutation assay (Ames test) exposed *S. typhimurium* TA1535, TA100, TA1537, TA98 and *E. coli* WP2uvrA to 156, 313, 625, 1,250, 2,500, and 5,000 µg dry matter/mL with or without rat liver metabolic activation (S9) for 3 hours (GRN 645, stamped pages 51 to 54). No dose related increases in revertant numbers were observed. Pullulanase did not induce gene mutations in the absence or presence of S9.

In the *in vitro* cytotoxicity study, BALB/c 3T3 cells were exposed to a range of pullulanase concentrations for 48 hours to evaluate the cytotoxicity of Pullulanase, Batch PPY38874, using a Neutral Red Uptake (NRU) assay in 3T3 cells (GRN 645; FDA, 2016a; stamped pages 51 to 53). Pullulanase was toxic at approximately 50% viability at the highest concentrations (2-30 mg/mL) and less toxic at concentrations of 0.1 to 1 mg/mL. 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 observation for pullulanases.

In the *in vitro* micronucleus test, human lymphocytes were exposed to up to 5,000 µg TOS/mL in the absence or presence of S9 (GRN 645; FDA, 2016a; stamped pages 51, 52 and 54). The pulse 3+21 h treatment in absence or presence of S9 was similar to the results of the vehicle control. The 24+24 h treatment had a small significant increase in micronucleated Bi-nucleate cells (MNBN) cells with the 5,000 µg TOS/mL dose in the absence of S9, inducing a 52% cytotoxicity, which did not exceed the observed historical vehicle control values. This was not considered as biological importance. A single culture at 4,000 µg TOS/mL showed a marginal increase in MNBN cell frequency above the normal values, which induced a 42% cytotoxicity; however, this was not significant. Pullulanase, up to 5,000 µg TOS/mL concentration, did not induce biologically relevant increases in micronuclei in cultured human peripheral blood lymphocytes following treatment in the absence or presence of S9.

Overall, the mutagenicity/genotoxicity studies demonstrated no significant toxicological effects of pullulanase.

Pullulanase from *Bacillus licheniformis* via *B. deramificans*

Modderman and Foley (1995) investigated the safety of a pullulanase enzyme preparation produced by a strain of *Bacillus licheniformis* that has been transformed by introduction of genetic material from *B. deramificans*. Lack of genetic toxicity potential was demonstrated by the results of a bacterial mutation assay in *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537, and TA1538, in an *in vitro* histidine forward mutation study in mouse lymphoma cells, and in *in vivo* mouse bone marrow chromosome aberration and micronucleus assays.

The bacterial reverse mutation assay (Ames test) exposed *S. typhimurium* TA98, TA100, TA1535, TA1537, and TA1538 to 5 quarter log dilutions of up to 5,000 µg dry matter pullulanase/plate with or without the rat liver metabolic activation system (S9) (Modderman and Foley, 1995). No concentration related increases in revertant colonies/plate or no significant increases in mutation frequency were observed for any tested concentrations of pullulanase in any *Salmonella* strains with and without metabolic activation.

In the forward mutation assay, the ability of pullulanase to induce gene and chromosomal mutations at the thymidine kinase locus was tested using mouse lymphoma L5178Y cells (Modderman and Foley, 1995). Pullulanase concentrations of 1,000-5,000 µg/mL in the absence of S9 and 1,000-5000 µg/mL in the presence of S9 resulted in negative results. Non pullulanase concentrations yielded an induced mutation frequency (IMF) of greater than or equal to 70 $x10^{-6}$ in the absence or presence of metabolic activation.

In the *in vivo* micronucleus assay, Swiss-Webster mice were exposed to 500, 889, 1,581, 2,811, or 5,000 mg pullulanase/kg bw through i.p injection to test the ability of pullulanase to induce chromosomal breakage and aberrations (Modderman and Foley, 1995). Groups of 5 mice/sex/dose were sacrificed at intervals of 12, 24, and 48 h after exposure, and bone marrow cells were collected. No statistically significant increases were observed in the incidence of micronucleated polychromatic erythrocytes (MNPCE) in polychromatic erythrocytes (PCE) in any test groups compared with the negative control group. A significant increase in the incidence of MNPCE in PCE was observed in the positive control group compared with the negative control group. There were no statistically significant differences in the ratio of PCE to total erythrocytes in any test groups compared with the negative control. Thus, pullulanase was shown to be negative in the mouse bone marrow micronucleus and chromosome aberration assays.

6.B.3. Safety of the Production Microorganism, *Bacillus subtilis*

B. subtilis is a gram positive, aerobic, endospore-forming, rod-shaped, non-pathogenic, and non-toxigenic bacterium that is well characterized and widely used in the production of food-grade enzymes (Kunst et al., 1997). *B. subtilis* strains are an important source of industrial enzymes (such as amylases and proteases), and much of the commercial interest in these bacteria arises from their capacity to secrete these enzymes at gram per liter concentrations. *Bacillus subtilis* 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 microorganism found in many foods such as natto, a Japanese fermented food.

Bacillus subtilis has been used in the fermentation industry for the production of enzymes. Various enzymes have been produced by *Bacillus subtilis* and are GRAS substances as mentioned in Part 6.A. In addition, *Bacillus subtilis* is classified as a Risk Group 1 organism according to the National Institutes of Health Guidelines for Research Involving Recombinant Molecules (NIH, 2001). Risk Group 1 organisms are those not associated with disease in healthy adult humans. The Centers for Disease Control and Prevention (CDC) defines BSL-1 organisms as those "not known to consistently cause disease in immunocompetent adult humans, and present minimal potential hazard to laboratory personnel and the environment" (CDC, 2009).

The recipient *Bacillus subtilis*, designated as 8001-YF, was genetically modified by deletion of six genes and a gene cluster that are residential in the parental strain BS-YF (CICC 20632). *Bacillus subtilis* is classified as a GILSP (Good Industrial Large Scale Practice) microorganism, as well as meets the criteria for a safe production microorganism as described by Pariza and Foster (1983). According to the European Food Safety Authority (EFSA), the primary concern associated with the *Bacillus* genus is the potential for producing *Bacillus cereus* enterotoxins, haemolysin BL, cytotoxin, and bacteriocin of *B. cereus* G9241 (EFSA, 2014). However, *B. subtilis* has been shown not to contain genes with homology to those that encode for known *B. cereus* enterotoxin and not to produce such toxins (de Boer and Diderichsen, 1991; Pedersen et al*.,* 2002; Olempska-Beer et al*.,* 2006). There are no reports in the public literature that any strains of *B. subtilis* are capable of producing any of these toxins. Additionally, published articles have reported the use of *B. subtilis* in traditionally-fermented food from Asia and Africa (Isu and Ofuya, 2000; Omafuvbe et al., 2000; Kiers et al., 2000). These articles support the safe use of the source, *B. subtilis*, in food applications.

6.B.3.1. Absence of Antibiotic Resistance Gene in the Production Microorganism

The antibiotic resistance genes for selection were removed from the production strain after integration; therefore, the production strain is free of any antibiotic resistance genes (please see the attached genome sequencing results for details).

In order to confirm that there is no acquired antibiotic resistance genes, the comprehensive antibiotic resistance database from CARD database was downloaded (https://card.mcmaster.ca/, published paper: Jia et al., 2017, Nucleic Acids Research). This database is a rigorously curated collection of characterized, peer-reviewed resistance determinants and associated antibiotics. A total of 2238 reference sequences of antibiotic resistance gene (Supplementary file: nucleotide_fasta_protein_homolog_model.fasta and its function annotation file aro.csv) was downloaded and included in https://card.mcmaster.ca/download/0/broadstreet-v3.0.0.tar.gz and then analyzed through NGS read mapping of target reference genes. The count of mapped reads was summarized in Table 10. It was determined that the test strain had originally instinctive genes of *Bacillus subtilis* and that no external antibiotic resistance gene existed.

Table 10. Antibiotic Resistance Gene Detection of *B. subtilis* through NGS Read Mapping to Curated Collection of Annotated Resistance Genes

ID	length (bp)	# mapped read	memo
gb|AL009126|+|1376854-1377172|ARO:3003064|ykkD	318	60	ARO:3003064 ykkD ykkD is an SMR-type protein that is a subunit of the ykkCD efflux pump
gb|AL009126|-|2735681-2736536|ARO:3002627|aadK	855	549	ARO:3002627 aadK aadK is a chromosomal-encoded aminoglycoside nucleotidyltransferase gene in *B. subtilis* and *Bacillus* spp.
gb|JYFL01000006.1|-|112069-113503|ARO:3002813|lmrB	1434	1217	ARO:3002813 lmrB lmrB is a chromosomally-encoded efflux pump that confers resistance to lincosamides in *Bacillus subtilis*
gb|M33768|+|194-1364|ARO:3003007|bmr	1170	577	ARO:3003007 bmr bmr is an MFS antibiotic efflux pump that confers resistance to multiple drugs including acridine dyes, fluoroquinolone antibiotics, chloramphenicol, and puromycin
gb|AL009126.3|-|339155-339749|ARO:3003059|tmrB	594	375	ARO:3003059 tmrB tmrB is an ATP-binding tunicamycin resistance protein found in *Bacillus subtilis*
gb|AL009126|+|1376516-1376855|ARO:3003063|ykkC	339	126	ARO:3003063 ykkC ykkC is an SMR-type protein that is a subunit of the ykkCD efflux pump
gb|L32599|+|1236-2439|ARO:3003006|blt	1203	845	ARO:3003006 blt blt is an MFS efflux pump that confers resistance to multiple drugs such as rhodamine and acridine dyes, and fluoroquinolone antibiotics
gb|AL009126|+|916777-919348|ARO:3003324|Bacillus	2571	2579	ARO:3003324 *Bacillus subtilis* mprF MprF is an integral membrane protein that modifies the negatively-charged phosphatidylglycerol on the membrane surface. This confers resistance to cationic peptides that disrupt the cell membrane, including defensins. Additionally, large-scale mutations causing loss of function of the gene result in increased susceptibility to daptomycin.

NGS=next generation sequencing.

6.B.3.2. Absence of the Allergenic Potential in the Production Microorganism

Following the guidelines developed by FAO/WHO (2001) and modified by Codex Alimentarius Commission (2003), the whole genomic sequence (WGS) of pullulanase was compared to allergens from the FARRP allergen protein database (http://allergenonline.org). There is no significant homology founded between the pullulanase and any of the allergens in the databases mentioned above.

Pullulanase is not added to the final foodstuffs, but is used as a processing aid during food manufacturing to hydrolyze starch. Even if the enzymes are not completely removed from the foods, the enzyme action is expected to be over before the food product is available for consumers. Any residual inactivated pullulanase produced by the *Bacillus subtilis* strain is not anticipated to pose any food allergenic concern. The amino acid sequence of pullulanase has been identified and did not show any homology with those of known allergenic proteins when screened by overall FASTA alignment, 80 amino acid alignments by FASTA, and 8 amino acid exact match (details are described in Appendix D).

Bindslev-Jensen et al. (2006) also reported no cases of IgE-mediated food allergy to commercial enzymes and no indications of cross-reactivity between the tested industrial food enzymes and the main known allergens including inhalation allergens, food allergens, or bee or wasp allergens.

6.B.3.3. Absence of Virulence Genes in the Production Microorganism

The backend database (updated Oct 12, 2018, downloaded from https://bitbucket.org/genomicepidemiology/virulencefinder_db/downloads/), affiliated to VirulenceFinder 2.0 (https://cge.cbs.dtu.dk/services/VirulenceFinder/; Joensen et al., 2014), was used as a database for searching possible virulence gene through detecting homologous sequences of the virulence genes related to four well-known pathogens (*E. coli*, *Enterococcus*, *Listeria*, and *Staphylococcus aureus*) (included in Supplementary file: virulencefinder_db.rar). No virulence factors (such as *E. coli* Shiga toxin gene and *S. aureus* exoenzyme genes, hostimm genes, toxin genes, and so on) were found by Blastn all unmapped.fa against all fsa files compressed in virulencefinder_db.rar with default parameter. Thus, it was confirmed that the genomic sequences do not include toxic or pathogenic genes found in *E. coli*, *Enterococcus*, *Listeria*, and *S. aureus*.

6.B.4. Safety of the Donor Strain, *Bacillus deramificans*

The pullulanase gene is synthesized based on the sequence data from public databases of *Bacillus deramificans*. The introduced DNA does not code for any known harmful or toxic substances.

6.B.5. Absence of the Production Organism in Product

No production strain has ever been found in the commercial product by colony growing, counting, and identification tests on the red-pullulan plates.

6.B.6. Stability of the Introduced Genetic Sequences

The production strain was grown for at least 15 generations to evaluate the stability and mobility of the integrated genetic material, and polymerase chain reaction (PCR) and Southern

hybridization experiments showed no genetic transfer of integrated components on the chromosome in any generations (please see the attached genome sequencing results for details), and the introduced DNA sequence for pullulanase expression was stably resided at the AmyE locus while being tested monthly by PCR.

6.C. Safety Determination

The subject of the present GRAS notice is pullulanase from *Bacillus deramificans* expressed in *Bacillus subtilis* (from *B. subtilis* via *B. deramificans*). This GRAS determination is based on the data and information generally available about the safety of pullulanase, *B. subtilis*, and *B. deramificans.* There is broad-based and widely disseminated knowledge concerning the safety of pullulanase and its source and production microorganisms (*B. deramificans* and *B. subtilis*).

The following safety evaluation fully considers the composition, intake, and microbiological and toxicological properties of GenScript/Bestzyme's pullulanase and its source and production microorganisms as well as appropriate corroborative data.

1. The pullulanase is used for hydrolysis of carbohydrates during processing of starch-containing foods. Pullulanase is not added to final foodstuffs, but is used as a processing aid during food manufacturing to hydrolyze starch. The enzyme preparation is used at the minimum levels necessary to achieve the desired effect and per requirements for normal production following cGMP.

2. GenScript/Bestzyme follows the principles of HACCP-controlled manufacturing process and rigorously tests its final production batches to verify adherence to quality control parameters and specifications.

3. FDA has determined that mixed carbohydrase and protease enzyme preparations derived from *B. subtilis* are GRAS (21 CFR 184.1148 and 21 CFR 184.1150). The U.S. FDA had no questions in response to GRAS notices for pullulanase enzyme preparations obtained from a genetically modified strain of *B. subtilis* or *B. licheniformis* for use as a processing aid (GRN 645- FDA, 2016a; GRN 205- FDA, 2006; and GRN 20- FDA, 1999). In addition, a recent review by Ladics and Sewalt (2018) summarizes that enzymes, in general, don't produce acute toxicity, dermal sensitization, genotoxicity, or repeated dose oral toxicity.

4. Acute toxicity and mutagenicity studies of GenScript/Bestzyme's pullulanase found that the LD_{50} was far above 60 mL/kg bw (or 151,800 U/kg bw), the highest dose tested, and it was not mutagenic. Additional subchronic oral toxicity study of similar pullulanase preparations from *B. subtilis* via *B. deramificans* suggests that the enzyme was well tolerated in rats with no side effects: in a 90-day subchronic toxicity study, the NOAEL was determined to be higher than 1,285 mg TOS/kg bw/day (or up to 42,649 pullulanase U/kg bw/day).

5. GenScript/Bestzyme's maximum suggested use levels are as follows: up to 10,000 U/kg starch dry matter for brewing processes and up to 1,500 U/kg starch dry matter for starch processing. Even under the assumption that all the enzyme product is retained in the final food product to provide a worst-case scenario, the theoretical maximum daily intake of consumers of the food enzyme based on the contribution by both starch and brewing and cereal based beverage would be 0.496 mg TOS/kg bw/day or 27.9 pullulanase U/kg bw/day. After consideration of the safety margin of 100, the theoretical maximum daily intake values are estimated to be less than one fifteenth of the safe intake levels estimated from a 13-week oral toxicity study of pullulanase in rats. However, these EDIs are highly amplified since the presence of the enzyme residues in the final food is not expected to have any effect in or on the final food, and the enzyme action is expected to be over before the food product is available for consumers.

6. GenScript/Bestzyme's production microorganism, *B. subtilis*, is absent of any antibiotic resistance genes, allergenic genes, and virulent genes. *B. subtilis* has been safely used in the production of many carbohydrases and proteases. *Bacillus subtilis* is described as the production organism for different enzymes in many GRAS notifications (GRN 114- FDA, 2003; GRN 274- FDA, 2009; GRN 476- FDA, 2014; GRN 579- FDA, 2015a, GRN 592- FDA, 2015b; GRN 649- FDA, 2016b; GRN 714- FDA 2018a; GRN 746- FDA, 2018b). All strains of *B. subtilis*, the production microorganism, available from the American Type Culture Collection (ATCC), are classified as Biosafety Level I (BSL-1) (ATCC, 2016). The Centers for Disease Control and Prevention (CDC) defines BSL-1 organisms as those "not known to consistently cause disease in immunocompetent adult humans, and present minimal potential hazard to laboratory personnel and the environment" (CDC, 2009).

7. GenScript/Bestzyme's source microorganism, *Bacillus deramificans*, has been recognized as a safe source microorganism for pullulanase (GRN 645, FDA 2016a; GRN 72, FDA, 2001).

Thus, it is concluded that the pullulanase enzyme preparation from *Bacillus deramificans* expressed in *B. subtilis* is safe and Generally Recognized as Safe (GRAS) for its intended use in food processing.

6.D. Conclusions and General Recognition of the Safety of Pullulanase

6.D.1. Common Knowledge Element of the GRAS Determination

Pullulanase is classified as a debranching enzyme that specifically hydrolyzes α-1,6-glucosidic bonds in starch, pullulan, amylopectin, and glycogen, and in the alpha- and beta-limit dextrins of amylopectin and glycogen. Pullulanase is a naturally occurring enzyme in many microbial species. The safety of pullulanase is well acknowledged by authorities. Carbohydrase and proteases from *Bacillus subtilis* are affirmed as GRAS by the U.S. FDA and are covered in the regulations under 21 CFR 184.1148 and 21 CFR 184.1150. The enzyme, pullulanase, has a history of safe use in food production and, even more specifically, in the production of starch and alcohol and cereal based beverages. FDA has issued several "no questions" letters about the GRAS status of the following sources of pullulanase: GRN 645 (FDA, 2016a), GRN 205 (FDA, 2006), GRN 72 (FDA, 2001), and GRN 20 (FDA, 1999). In addition, *Bacillus subtilis*, the production microorganism, has been safely used in production of many carbohydrases and proteases. *Bacillus subtilis* is described as the production organism for different enzymes in many GRAS notifications. *Bacillus deramificans*, the source microorganism, also has been recognized as a safe source microorganism for pullulanase. In all the studies summarized in these GRAS determinations, there were no significant adverse effects/events or tolerance issues attributable to pullulanase and the production and source microorganisms. A recent review by Ladics and Sewalt (2018) also summarizes that food industrial enzymes, in general, don't produce acute toxicity, dermal sensitization, genotoxicity, or repeated dose oral toxicity. Because this safety evaluation was based on generally available and widely accepted data and information, it satisfies the so-called “common knowledge” element of a GRAS determination.

6.D.2. Technical Element of the GRAS Determination (Safety Determination)

The intended uses of pullulanase have been determined to be safe though scientific procedures as set forth in 21 CFR 170.3(b); thus, satisfying the so-called “technical” element of the GRAS determination. The intended use and use levels are the same or similar to those described in GRN 645. The pullulanase is used for hydrolysis of carbohydrates during processing of starch-containing foods and brewing processes. However, the enzyme used during processing is not expected to exert any unintentional enzymatic activity in the final food when using the pullulanase in food and brewing processes. The enzyme preparation is used at the minimum levels necessary to achieve the desired effect and per requirements for normal production following cGMP. No toxicants have been detected from pullulanase enzyme preparations.

Literature or publicly available data did not identify safety or toxicity concerns related to pullulanase or its source and production microorganisms. Toxicity studies of GenScript/Bestzyme’s pullulanase (an acute toxicity in rats and a mutagenicity test) found no adverse effects of pullulanase. After consideration of the safety margin of 100, the theoretical maximum daily intake values are estimated to be less than one fifteenth of the safe intake levels estimated from a 13-week oral rat toxicity study of a pullulanase, whose source and production microorganism species are the same as the subject of this GRAS notice. However, these EDIs are highly amplified since the presence of the enzyme residues in the final food is not expected to have any effect in or on the final food, and the enzyme action is expected to be over before the food product is available for consumers. This evidence is sufficient to support the safety and GRAS status of the proposed use of pullulanase as a processing aid for the manufacture of foods.

GenScript/Bestzyme has concluded that GenScript/Bestzyme's pullulanase is GRAS under the intended conditions of use on the basis of scientific procedures, and other experts qualified to assess the safety of food ingredients would concur with these conclusions. Therefore, it is excluded from the definition of a food additive and may be marketed and sold for its intended purpose in the U.S. without the promulgation of a food additive regulation under Title 21 of the CFR. Therefore, GenScript/Bestzyme has concluded that pullulanase, when used as described in this dossier, is GRAS based on scientific procedures.

6.E. Discussion of Information Inconsistent with GRAS Determination

GenScript/Bestzyme has reviewed the available data and information and is not aware of any data and information that are, or may appear to be, inconsistent with its conclusion of the GRAS status.

PART 7. REFERENCES

7.A. References that are Generally Available

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Appendix A. Certificate of Analysis

Appendix A. Certificate of Analysis

Product description					
Product name	HighDEX P1000				
Lot number					
Date manufactured	2018-07-29				
Inspection Report					
Sequence	Item	Unit	Standard	Result	Executive standard
1	Pullulanase	U/ml	≥2300	2560	GB1886.174-2016
2	pH	--	3.5-5.0	4.28	--
3	Specific gravity	g/ml	1.05-1.25	1.20	--
4	Lead	mg/kg	≤5.0	No detected	GB1886.174-2016
5	Total arsenic (calculated as As)	mg/kg	≤3.0	No detected	GB1886.174-2016
6	Total number of colonies	CFU/g	≤50000	<100	GB1886.174-2016
7	Coliforms	CFU/g	≤30	< 3	GB1886.174-2016
8	*Escherichia coli*	CFU/g	≤10	<10	GB1886.174-2016
9	*Salmonella*	——	No detected	No detected	GB1886.174-2016
10	Antibacterial activity	——	No detected	No detected	GB1886.174-2016

***No limitation of pH in GB1886.174-2016.**

Conclusion：Qualified

Inspector： N14 N13 **Auditor：** Liu Peng

Date： 2018-07-31 **Date：** 2018-07-31 **Sealed**

Appendix A. Certificate of Analysis

Product description					
Product name	HighDEX P1000				
Lot number					
Date manufactured	2018-10-07				
Inspection Report					
Sequence	Item	Unit	Standard	Result	Executive standard
1	Pullulanase	U/ml	≥2300	2700	GB1886.174-2016
2	pH	--	3.5-5.0	4.39	--
3	Specific gravity	g/ml	1.05-1.25	1.20	--
4	Lead	mg/kg	≤5.0	undetected	GB1886.174-2016
5	Total arsenic (calculated as As)	mg/kg	≤3.0	undetected	GB1886.174-2016
6	Total number of colonies	CFU/g	≤50000	300	GB1886.174-2016
7	Coliforms	CFU/g	≤30	< 3	GB1886.174-2016
8	*Escherichia coli*	CFU/g	≤10	<10	GB1886.174-2016
9	*Salmonella*	——	No detected	No detected	GB1886.174-2016
10	Antibacterial activity	——	No detected	No detected	GB1886.174-2016

***No limitation of pH in GB1886.174-2016.**

Conclusion：Qualified

Inspector： N12 N13 **Auditor：** Li Xinmei

Date： 2018-10-09 **Date：** 2018-10-09

Sealed

Appendix A. Certificate of Analysis

Product description					
Product name	HighDEX P1000				
Lot number					
Date manufactured	2018-11-15				
Inspection Report					
Sequence	Item	Unit	Standard	Result	Executive standard
1	Pullulanase	U/ml	≥2300	2750	GB1886.174-2016
2	pH	--	3.5-5.0	4.26	--
3	Specific gravity	g/ml	1.05-1.25	1.20	--
4	Lead	mg/kg	≤5.0	No Detected	GB1886.174-2016
5	Total arsenic (calculated as As)	mg/kg	≤3.0	No Detected	GB1886.174-2016
6	Total number of colonies	CFU/g	≤50000	100	GB1886.174-2016
7	Coliforms	CFU/g	≤30	< 3	GB1886.174-2016
8	*Escherichia coli*	CFU/g	≤10	<10	GB1886.174-2016
9	*Salmonella*	——	No Detected	No Detected	GB1886.174-2016
10	Antibacterial activity	——	No Detected	No Detected	GB1886.174-2016

***No limitation of pH in GB1886.174-2016.**

Conclusion：Qualified

Inspector： N11 N3 **Auditor：** Li Xinmei **Sealed**

Date： 2018-11-17 **Date：** 2018-11-17

Appendix B.

Title of Study	Oral Acute Toxicity Study of Pulullanase in Rats
Study Number	A2018-T006
Entrustment Company	NutraSource, Inc.
Address of Entrustment Company	NutraSource, Inc. 6309 Morning Dew Ct Clarksville, MD 21029
Contact Person	Susan Cho, Ph.D. and Albert W. Lee
Contact Tel. and E-mail	+1-410-531-3336 (O) +1-301-875-6454 (C)
Primary Test Facility	School of Life Sciences, Yantai University
Address of Research Institute	30, Qingquan RD, Laishan District, Yantai, China
Contact Person	Yonglin Gao [redacted]
Contact Tel. and E-mail	86-15854569558; gylbill@163.com;gaoyonglin@ytu.edu.cn.
Study Director	Yonglin Gao
Study Participants	Yonglin Gao *Operator* Shuqing Qu *Test products management* Yiran Wang *Animal management*
Study Start and End Dates	July 2018

Oral Acute Toxicity Study of Pulullanase in Rats

ABSTRACT
The aim of this study was to evaluate the acute toxicity of Pulullanase after oral administration in rats. Test substances were administered to young rats by oral gavage at doses of 0 (control), 10 ml/kg body weight (BW) 30 ml/kg BW, and 60 ml/kg BW (5 males and 5 females per group). Animals were observed for 14 days to monitor changes in clinical signs (i.e., changes in eyes, mucous membranes, or behavior patterns; loss of fur or scabbing), body weight and clinical signs, as well as food consumption. At the end of the study, animals were sacrificed and major organs (such as liver, kidneys, spleen, heart, and lungs) were examined macroscopically and microscopically if needed. No animal died during the 14-day observation period and no clinical signs of abnormality were observed at any dose level. Furthermore, no significant differences in mean body weight, food consumption and organ weights were found among the four test and control group. No treatment-related abnormalities were observed in macroscopic examinations. In summary, the present study found that the lethal dose (LD_{50}) of Pulullanase was far above 60 ml/kg BW, the highest dose tested. These results will provide guidance for selecting a safe dose in its use in the future.

Key words: Pulullanase; Acute Toxicity Study; Rat

METHODS

1. Study design

The study was performed in accordance with the Food and Drug Administration (FDA) Redbook 2000: chapter IV.C.3.a Short-Term Toxicity Studies with Rodents. Pulullanase was administered by oral gavage to rats (0, 10 ml/kg BW 30 ml/kg BW, and 60 ml/kg BW; 5 males and 5 females for each group) and observed for 14 days. Clinical signs, body weight, food consumption and death rates were observed. On day 15, all surviving animals were sacrificed and organs were weighed, including lungs, heart, kidneys, liver, and spleens. The study was performed in accordance with Good Laboratory Practices (GLP) regulations.

2. Animals

Sprague-Dawley rats, 6 weeks of age, were housed in cages under hygienic conditions and placed in a controlled environment with a 12-h light/dark cycle at 23±3 °C and 40-60% humidity. Animals were allowed a commercial standard rat cube diet and water *ad libitum*. All procedures involving the use of laboratory animals were in accordance with the Guidelines of the Animal Care.

3. Treatment

Based on stratified randomization by body weights taken before treatment, rats were divided into five groups (each group of 10 rats consisted of 5 male and 5 female rats): control (purified water), 10, 30, and 60 ml/kg BW Pulullanase (orally administered dose by gavage). Group assignments are outlined in Table 1.

Table 1. Experimental design of a 14-day rat acute toxicity study.

Groups	Test substance	Number of animals
1	0 (Control)	10 (♀:5+♂:5)
2	10 ml/kg BW Pulullanase	10 (♀:5+♂:5)
3	30 ml/kg BW Pulullanase	10 (♀:5+♂:5)
4	60 ml/kg BW Pulullanase	10 (♀:5+♂:5)

Abbreviations: BW =Body weight.

4. Observations and clinical tests

All animals were observed twice daily for clinical signs of toxicity, mortality, and morbidity. The body weight of each rat was measured pre-test, weekly thereafter, and at sacrifice. Food consumption also was noted.

5. Organ weights, gross necropsy, and histopathological examinations

At the end of treatment, all surviving animals were fasted overnight. The body weight and the main organ weights including liver, kidneys, spleen, heart, and lungs, were measured. Moreover, the coefficient was reported as the organ/body weight ratio. These tissues were examined, and gross lesions were examined microscopically. If treatment-related effects were noted in certain tissues, they were examined microscopically.

6. Statistical analysis

We used SPSS 11.5 software for Windows to perform all analyses. One-way ANOVA with Dunnet's post-hoc test was used to compare the test and control group data. A P-value less than 0.05 was considered statistically significant.

RESULTS

7.1 General clinical signs and mortality

All rats survived to the end of the experiment and appeared healthy throughout the study period. No obvious abnormal clinical signs (i.e., changes in eyes, mucous membranes, or behavior patterns; loss of fur or scabbing) were observed in all groups. As shown in Table 2,3 and Figure 1,2, there were no significant differences in body weight between Pulullanase treated groups and control group.

7.2 Food consumption

In the experiment, food consumption was studied in rats during the 14-day study. The results showed that all data were within historic controls obtained in our facility. There were also no

significant differences in food consumption (Table 4,5; Figure 3,4) between Pululllanase treated groups and control group.

7.3 The organ/body weight ratio (the organ coefficient)

The organ/body weight ratios (the organ coefficient) are shown in Table 6,7 and Figure 5,6. No consistent, statistically significant, or dose-dependent, adverse effects were observed in all groups. On macroscopic examination, there are no treatment-related effects noted in these tissues.

CONCLUSION

Under our test conditions, the present study found that the lethal dose (LD_{50}) of Pululllanase was far above 60 ml/kg BW, the highest dose tested.

Table 2. Body weight change of female rats during a 14-day study (g)

Groups	Test substance	Before	1st week	2nd week
1	0 (Control)	117.60±4.16	145.80±5.40	174.20±9.12
2	10 ml/kg BW Pululllanase	118.20±4.82	144.00±6.44	176.60±7.83
3	30 ml/kg BW Pululllanase	115.80±5.36	140.80±3.03	174.20±4.97
4	60 ml/kg BW Pululllanase	119.20±4.21	142.80±5.17	175.20±6.61

Abbreviations: BW =Body weight.

Table 3. Body weight change of male rats during a 14-day study (g)

Groups	Test substance	Before	1st week	2nd week
1	0 (Control)	120.80±4.32	178.20±6.42	232.20±7.95
2	10 ml/kg BW Pululllanase	123.00±7.78	177.60±6.54	238.40±9.34
3	30 ml/kg BW Pululllanase	122.40±8.26	175.60±8.62	238.00±10.20
4	60 ml/kg BW Pululllanase	123.20±5.93	174.80±7.19	235.00±9.75

Abbreviations: BW =Body weight.

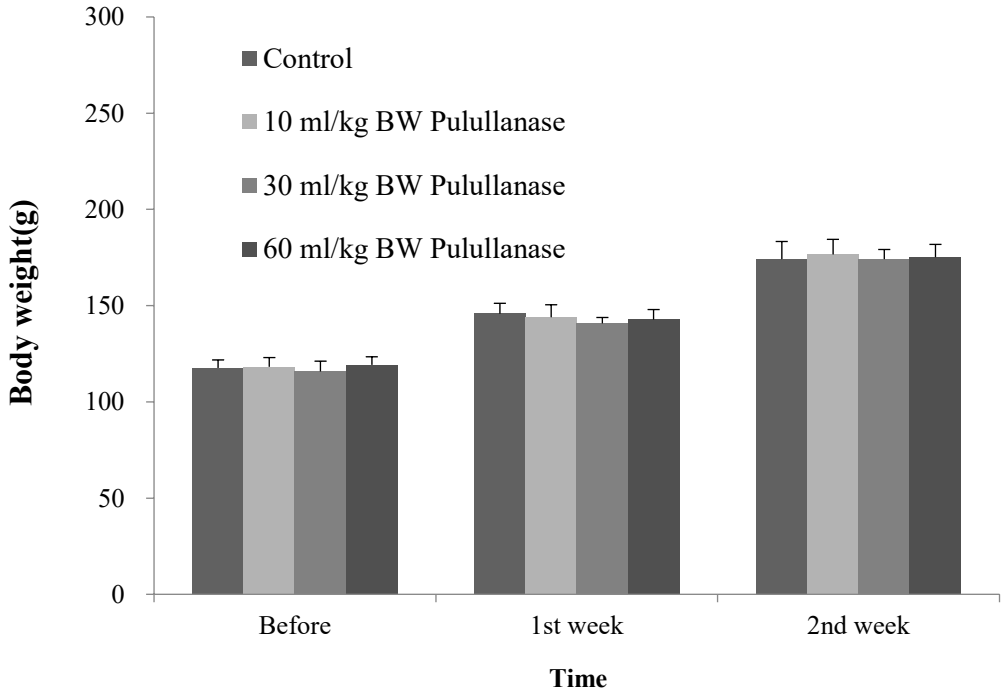

Figure 1. Body weight change of female rats during a 14-day study
Abbreviations: BW =Body weight.

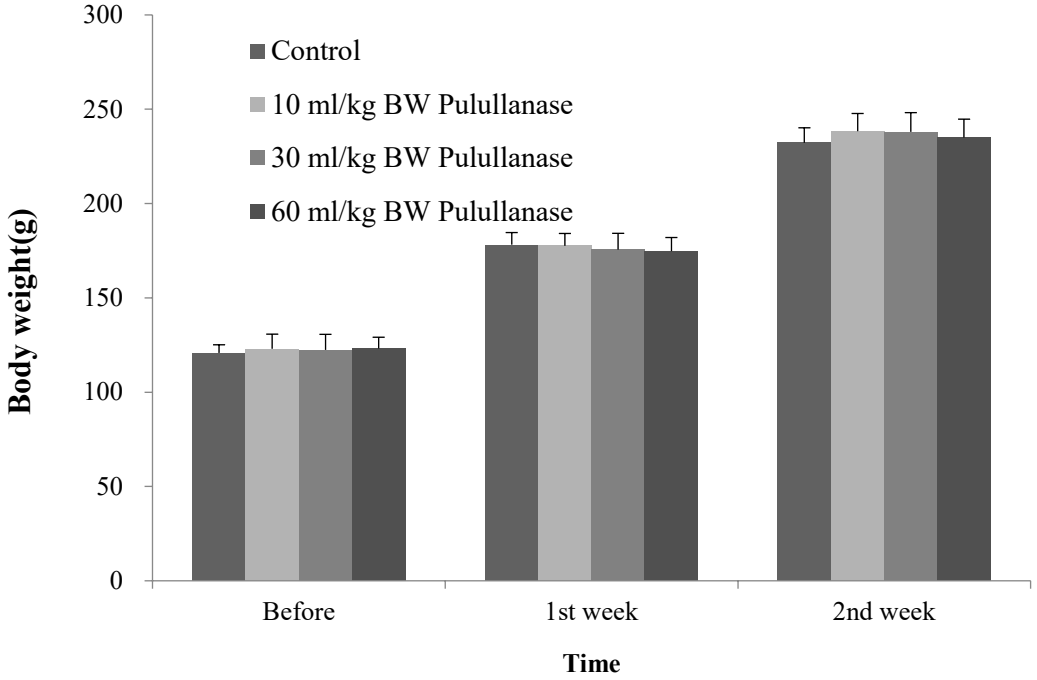

Figure 2. Body weight change of male rats during a 14-day study
Abbreviations: BW =Body weight.

Table 4. Food consumption of female rats during a 14-day study (g/100g BW/day)

Groups	Test substance	1st week	2nd week
1	0 (Control)	10.58±0.63	10.24±1.16
2	10 ml/kg BW Pulullanase	10.76±0.62	10.10±0.73
3	30 ml/kg BW Pulullanase	10.73±1.23	10.89±1.61
4	60 ml/kg BW Pulullanase	10.83±2.03	10.58±2.07

Abbreviations: BW =Body weight.

Table 5. Food consumption of male rats during a 14-day study (g/100g BW/day)

Groups	Test substance	1st week	2nd week
1	0 (Control)	11.02±0.81	10.15±1.66
2	10 ml/kg BW Pulullanase	10.60±0.69	10.56±1.40
3	30 ml/kg BW Pulullanase	11.31±1.10	10.63±1.49
4	60 ml/kg BW Pulullanase	11.12±1.28	10.32±0.87

Abbreviations: BW =Body weight.

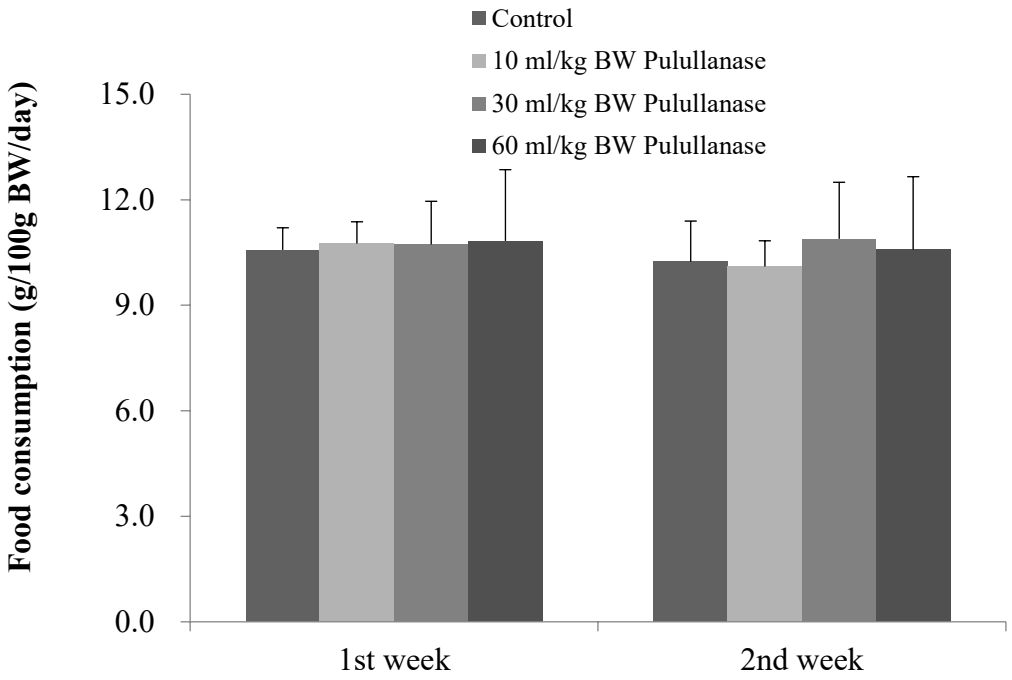

Figure 3. Food consumption of female rats during a 14-day study
Abbreviations: BW =Body weight.

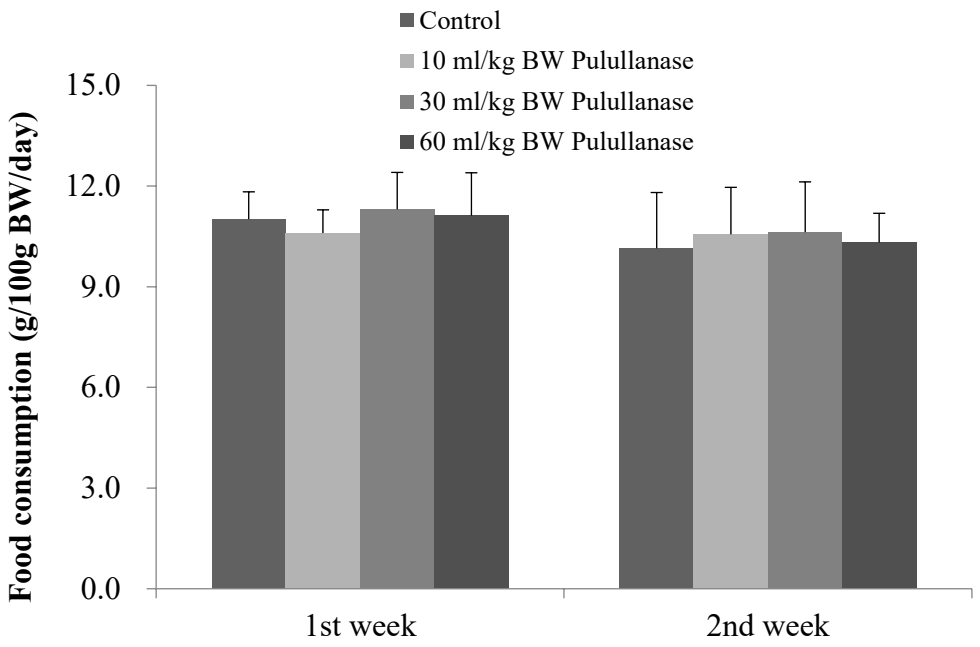

Figure 4. Food consumption of male rats during a 14-day study
Abbreviations: BW =Body weight.

Table 6. The organ coefficient of female rats after a 14-day study (% BW).

Test substance	0 (Control)	10 ml/kg BW Pulullanase	30 ml/kg BW Pulullanase	60 ml/kg BW Pulullanase
Heart	0.41±0.02	0.41±0.04	0.38±0.05	0.42±0.03
Liver	3.49±0.43	3.45±0.25	3.51±0.21	3.51±0.15
Spleen	0.23±0.02	0.25±0.03	0.25±0.05	0.26±0.03
Lung	0.56±0.05	0.55±0.04	0.53±0.05	0.55±0.05
Kidney	0.93±0.10	0.89±0.10	0.91±0.09	0.90±0.10

Abbreviations: BW =Body weight.

Table 7. The organ coefficient of male rats after a 14-day study (% BW).

Test substance	0 (Control)	10 ml/kg BW Pulullanase	30 ml/kg BW Pulullanase	60 ml/kg BW Pulullanase
Heart	0.38±0.04	0.37±0.03	0.36±0.02	0.37±0.02
Liver	3.40±0.25	3.46±0.30	3.42±0.23	3.41±0.11
Spleen	0.25±0.03	0.23±0.04	0.24±0.04	0.24±0.04
Lung	0.53±0.03	0.55±0.06	0.51±0.10	0.55±0.06
Kidney	0.88±0.06	0.90±0.06	0.90±0.08	0.91±0.09

Abbreviations: BW =Body weight.

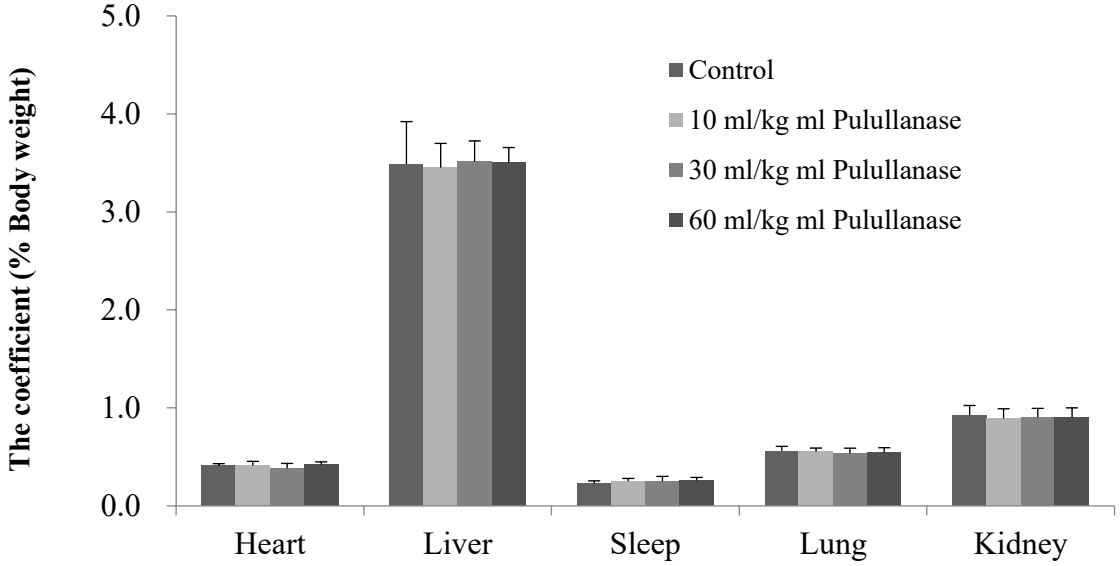

Figure 5. The organ coefficient of female rats after a 14-day study
Abbreviations: BW =Body weight.

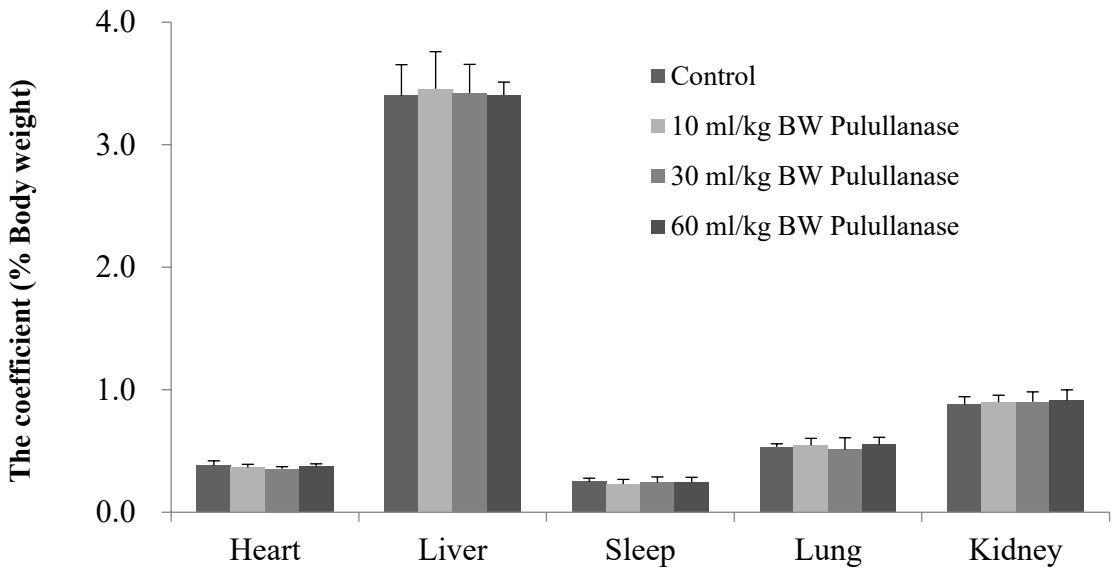

Figure 6. The organ coefficient of male rats after a 14-day study
Abbreviations: BW =Body weight.

Appendix C.

TOXICOLOGY STUDY REPORT

Title of Study	Mutagenicity Study of Pululanase
Study Number	A2018-T012
Entrustment Company	NutraSource, Inc.
Address of Entrustment Company	NutraSource, Inc. 6309 Morning Dew Ct Clarksville, MD 21029
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Primary Test Facility	School of Life Sciences, Yantai University
Address of Research Institute	30, Qingquan RD, Laishan District, Yantai, China
Contact Person	Yonglin Gao
Contact Tel. and E-mail	86-15854569558; gylbill@163.com;gaoyonglin@ytu.edu.cn.
Study Director	Yonglin Gao
Study Participants	YonglinGao *Operator* Meina Wang, Bing Han *Test products management*
Study Start and End Dates	Jul. 2018 – Aug. 2018

ABSTRACT

As part of a safety evaluation, we evaluated the potential mutagenicity of Pulullanase (2530 U/ml) using a bacterial reverse mutation assay. In the reverse mutation assay using five strains of *Salmonella typhimurium* (TA97, TA98, TA100, TA102, and TA1535), Pulullanase (253, 126.5, and 63.25 U/plate, respectively) did not increase the number of revertant colonies in any tester strain regardless of metabolic activation by S9 mix. The data indicated that Pulullanase was non-mutagenic under the conditions used in this test.

Keywords: Pulullanase; Bacterial reverse mutation assay

Study design

As part of a safety evaluation, we evaluated the potential mutagenicity of Pulullanase (2530 U/ml) using a bacterial reverse mutation assay. The study was performed in accordance with Good Laboratory Practices (GLP) regulations.

Materials and methods

Five strains of *Salmonella typhimurium* (TA97, TA98, TA100, TA102, and TA1535) were treated using the plate incorporation method. We selected concentrations for the test based on a preliminary study, and the results indicated that Pulullanase did not show any antibacterial activity up to, the maximum concentration, 253 U/plate (Enz unit is 2530 U/ml and the maximum volume is 0.1 ml/ plate). TA97, TA98, TA100, TA102, and TA1535 were treated with Pulullanase at concentrations of 0 (solvent control), 253, 126.5, and 63.25 U/plate in the presence and absence of an exogenous metabolic activation system (S9) by the plate incorporation method. We prepared triplicate plates for each concentration.
4-Nitro-o-phenylenediamine (NPD), daunomycin (DAM), sodium azide (NaN_3), and methyl methanesulfonate (MMS) were used as positive controls in conditions without S9 mix (Table 1), and 2-aminofluorene (2-AF), 1,8-dihydroxyanthraquinone (1,8-DT), and 2-aminoanthracene (2-AA) were used as positive controls in conditions with S9 mix (Table 1). All plates were incubated at 37 °C for 72 h, and the number of revertant colonies was counted.

Table 1 The positive control for study

Salmonella typhimurium	S9	Dose (μg/plate)
TA97	-S9	NPD (20)
	+S9	2-AF (20)
TA98	-S9	DAM (10)
	+S9	2-AF (20)
TA100	-S9	NaN3 (1.5)
	+S9	2-AF (20)
TA102	-S9	MMS (2)
	+S9	1,8-DT (50)
TA1535	-S9	NaN3 (1.5)

	+S9	2-AA (5)

We declared the test substance mutagenic if the number of revertant colonies in the test dose was more than twofold than that in the control, or if the number of revertant colonies increased in a dose-dependent manner compared to control in at least one strain with or without the metabolic activation system. The validity of the study was confirmed by more than twofold increase in the number of revertant colonies in positive control plates compared to the control.

3. Statistical analysis

We used SPSS 11.5 software for Windows to perform all analyses. One-way ANOVA with Dunnet's post-hoc test was used to compare treatment and control group data. A P-value less than 0.05 was considered statistically significant.

4. Results

The mutagenicity of Pulullanase in bacteria was evaluated up to a maximum dose of 253 U/plate using the plate incorporation method (Table 2, 3). We found no increase in revertant frequencies at any test doses in any of the tester strains with or without S9 compared to those in the vehicle control cultures. The positive control chemicals for each tester strain induced obvious increases in the number of revertant colonies compared to the vehicle control. The data indicated that Pulullanase was non-mutagenic under the conditions used in this test.

5. Conclusion

Under our test conditions, a reverse mutation assay using five strains of *Salmonella typhimurium* (TA97, TA98, TA100, TA102, and TA1535), Pulullanase (253, 126.5, and 63.25 U/plate, respectively) did not increase the number of revertant colonies in any tester strains regardless of metabolic activation by S9 mix. The data indicated that Pulullanase was non-mutagenic under the conditions used in this test.

Table 2. Bacterial mutation assay results (- S9) [a]

Group	Dose	Mean revertant colony counts per plate				
		TA97	TA98	TA100	TA102	TA1535
Vehicle control	—	113.7±17.8	48.0±6.6	213.0±11.8	274.7±26.2	70.3±17.0
Pulullanase	253 U/Plate	98.3±9.0	44.7±11.0	210.0±33.5	2650±49.1	89.3±10.5
	126.5 U/Plate	115.0±13.9	40.7±8.7	199.3±12.0	293.7±8.1	84.0±10.4
	63.25 U/Plate	96.7±9.1	38.0±8.7	232.7±34.5	294.3±16.2	99.0±10.0
NPD	20 μg /Plate	888.3±106.4**	—	—	—	—
DAM	10 μg /Plate	—	829.7±61.2**	—	—	—
NaN_3	1.5 μg /Plate	—	—	1104.3±108.0**	—	792.0±91.4 **
MMS	2 μg /Plate	—	—	—	851.7±73.8 **	—

Abbreviations: NPD = 4-Nitro-o-phenylenediamine; DAM = daunomycin; NaN_3 = sodium azide; MMS = methyl methanesulfonate.
[a] Values are the mean of triplicate plates. ** $P<0.01$, compared with vehicle control.

Table 3. Bacterial mutation assay results (+ S9) [a]

Group	Dose	Mean revertant colony counts per plate				
		TA97	TA98	TA100	TA102	TA1535
Vehicle control	—	123.0±22.5	60.7±6.5	256.0±13.1	313.3±22.4	107.3±23.2
Pulullanase	253 U/Plate	106.7±13.3	51.3±11.8	222.7±24.0	294.3±46.2	94.0±13.2
	126.5 U/Plate	138.3±13.8	55.3±10.0	258.3±74.1	315.7±20.8	117.7±16.9
	63.25 U/Plate	117.0±15.1	69.7±15.8	242.3±48.8	334.3±6.1	134.7±11.4
2-AF	20 μg /Plate	783.7±116.0**	940.7±103.7**	1014.0±178.8**	—	—
1,8-DT	50 μg /Plate	—	—	—	530.3±101.7**	—
2-AA	5 μg /Plate	—	—	—	—	480.0±82.5**

Abbreviations: 2-AF = 2-aminofluorene; 1,8-DT = 1,8-dihydroxyanthraquinone; 2-AA = 2-aminoanthracene.
[a] Values are the mean of triplicate plates.
** P<0.01, compared with vehicle control.

APPENDIX D. Comparison of Amino Acid Sequence of Pullulanase with Those of known Allergenic Proteins

Prepared for GenScript/Bestzyme

GenScript - 860 Centennial Ave, Piscataway, NJ 08854

Bestzyme Biotech Inc. (Bestzyme) - 860 Centennial Ave, Piscataway, NJ 08854

OBJECTIVE

The aim of this study was to determine if Pullulanase has any allergenic potential. Thus, Bestzyme Biotech Inc. determined the amino acid sequence of Pullulanase and compared Pullulanase amino acid sequence with those of known allergenic proteins.

1. Brief overview of methods

Following the guidelines developed by FAO/WHO (2001) and modified by Codex Alimentarius Commission (2003), the whole amino acid sequence of Pullulanase (in Fasta format) pullulanase was compared to allergens from the FARRP allergen protein database (http://allergenonline.org). There is no significant homology founded between the pullulanase and any of the allergens in the databases mentioned above.

2. Amino acid sequence of Pullulanase (in Fasta format)

> Pullulanase

EDAAKPAVSNAYLDASNQVLVKLSQPLTLGEGASGFTVHDDTANKDIPVTSVKD
ASLGQDVTAVLAGTFQHIFGGSDWAPDNHSTLLKKVTNNLYQFSGDLPEGNYQYKVAL
NDSWNNPSYPSDNINLTVPAGGAHVTFSYIPSTHAVYDTINNPNADLQVESGVKTDLVT
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LMALLGKDTMSKAASELHAINPGIALYGEPWTGGTSALPDDQLLTKGAQKGMGVAVF
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GDAVNEFDWSRKAQYPDVFNYYSGLIHLRLDHPAFRMTTANEINSHLQFLNSPENTVA
YELTDHVNKDKWGNIIVVYNPNKTVATINLPSGKWAINATSGKVGESTLGQAEGSVQV
PGISMMILHQEVSPDHGKK

3. Homologies search

The allergenicity between pullulanase and allergenic proteins included in the publicly available databases was analyzed through online tool allergenonline with three methods (http://www.allergenonline.org/)

3.1 Known allergens

The Food Allergy Research and Resource Program (FARRP) AllergenOnline.org database (http://www.allergenonline.org/) has been updated to version 19 on February 10, 2019. Version 19 contains a comprehensive list (2129 protein [amino acid]) sequence entries that are categorized into 852 taxonomic-protein groups of unique proven or putative allergens (food, airway, venom/salivary and contact). Its annual update process includes collecting new sequences designated as "allerg*" in reference files from NCBI protein database (compiled from GenBank, RefSeq, and TPA databases as well as protein sequences from SwissProt, PIR, PRF, and PDB databases).

3.2 Methodology (http://www.allergenonline.org/) including the criteria to identify it as allergenic potential (the following methodology overview has been adopted from Ladics, 2008).

Typically, sequence homology searches comparing the structure of novel proteins to known allergens in a database are conducted using various algorithms such as FASTA to predict overall structural similarities. As recommended by FAO/WHO (2001), IgE cross-reactivity between a novel protein and a known allergen is considered a possibility when there is more than 35% identity over a segment of 80 or greater amino acids. It should also be pointed out, however, that for cross-reactivity to occur, Aalberse (2000) has reported that a high degree of homology is needed, likely in excess of 50–60%, over significant spans of the target protein and allergen. In addition, step-wise contiguous identical amino acid segment searches are also performed to identify amino acid sequences that may represent linear IgE binding epitopes. IgE binding epitopes, however, have only been identified for a few allergens. Therefore, in the absence of an IgE binding epitope database, potential epitopes can be evaluated by producing all overlapping peptides of the allergens contained in a particular database and comparing them in a pair-wise manner to all same-size potential peptides of a novel protein using bioinformatic tools. Eight contiguous amino acid matches between a novel protein and a known allergen(s) are routinely used to identify sequences that may represent linear epitopes. The 2001 FAO/WHO consultation has suggested using a six amino acid match for this type of analysis. The use of a contiguous amino acid match of <8 occurs too commonly between unrelated proteins and therefore, is not a reliable criterion for predicting allergenic potential. Many random matches that are very unlikely to indicate potential IgE epitopes are observed using such a short sequence.

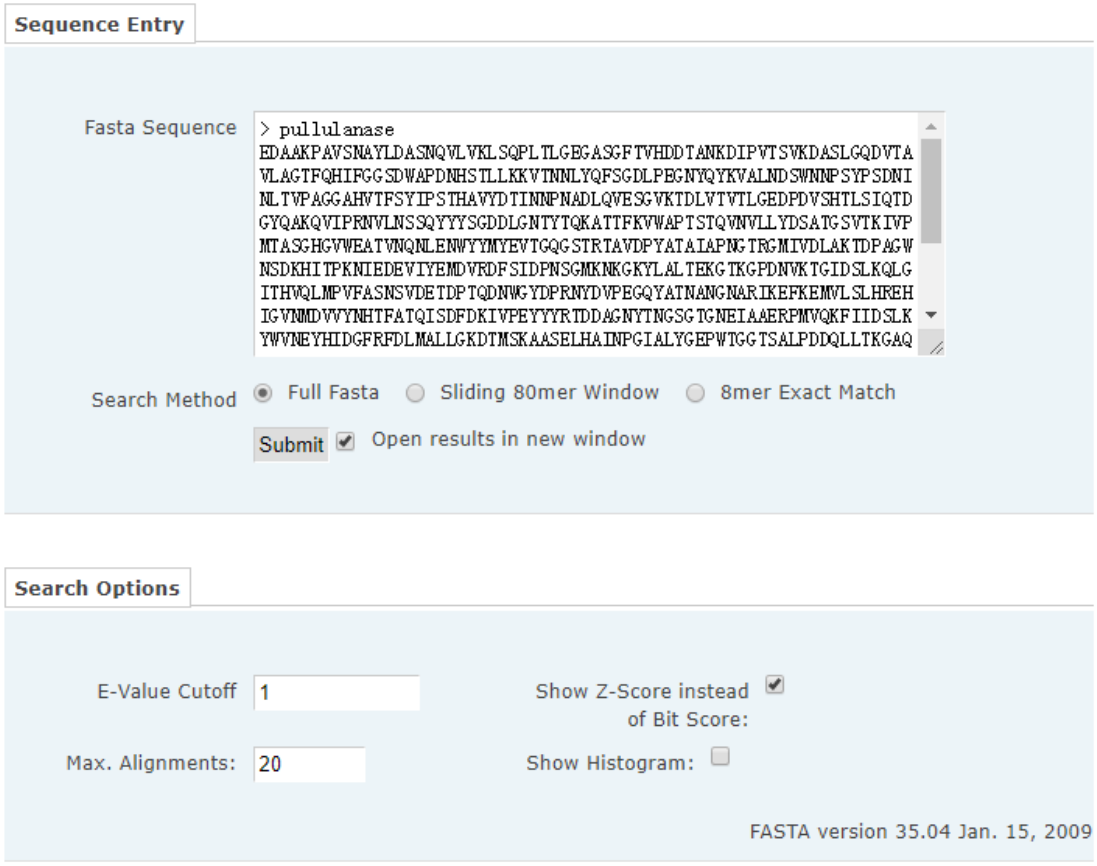
In our analysis, three methodologies with default parameters were used and described below:
Sequence search routines
Search for full-length alignments by FASTA:
The most predictive search is the overall FASTA alignment (see FASTA Help Page), with identity matches greater than 50% indicating possible cross-reactivity (Aalberse, 2000).

Search for 80 amino acid alignments by FASTA:
A precautionary search using a sliding window of 80 amino acid segments of each protein to find identities greater than 35% (according to CODEX Alimentarius guidelines, 2003).

Search for 8 amino acid exact match:
An 8-amino acid short-sequence identity search is provided since some regulatory authorities demand results of this extremely precautionary search. Our scientific opinion is that there is no evidence that an 8 amino acid match will identify a protein that is likely to be cross-reactive and could be missed by the conservative 80 amino acid match (35%). In our experience, isolated identity matches of 8 contiguous amino acids occur by chance alone at some modest rate, matches of 7 and 6 occur more commonly. Experience (published and unpublished) demonstrates that two proteins sharing only a single short identity match of from 6 to 8 contiguous amino acids do not share IgE binding in the absence of more extensive identity alignments (at least >35% identity over 80 or more amino acids). And that sequences sharing less than 50% identity over their full-lengths are rarely cross-reactive. Thus, we recommend not using these short identity matches as there is no scientific evidence that they predict IgE cross-reactivity and they do not predict shared clinical activities.

4. Results

4.1 Input and output of search for full-length alignments by FASTA



AllergenOnline Search Results

Note: As of August 2015 we have included gid: groupid in the fasta results that provides detailed information on the allergenicity references for the group, type of allergen, other sequences belonging to the same group and more.

%_id 1 = 100% identity, alen=alignment length

AllergenOnline Database v19 (February 10, 2019)

NOTE Addition of Allergenicity* column on the Browse Database page with classification based on Group references was added on 10 May 2018. Please review the "allergenicity" of any matches you find here with the Browse page and look at Group References (gid) if you want to further evaluate relevance of alignments.

fasta35.exe -q -H -B -m 9i -w 80 -E 1 -d 20 C:\Windows\Temp\all215E.tmp fasta/version19.fasta
User Query #1 > pullulanase

User Query #1

```
    > pullulanase
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    LPEGNYQYKV ALNDSWNNPS YPSDNINLTV PAGGAHVTFS YIPSTHAVYD TINNPNADLQ VESGVKTDLV TVTLGEDPDV SHTLSIQTDG YQAKQVIPRN
    VLNSSQYYYS GDDLGNTYTQ KATTFKVWAP TSTQVNVLLY DSATGSVTKI VPMTASGHGV WEATVNQNLE NWYYMYEVTG QGSTRTAVDP YATAIAPNGT
    RGMIVDLAKT DPAGWNSDKH ITPKNIEDEV IYEMDVRDFS IDPNSGMKNK GKYLALTEKG TKGPDNVKTG IDSLKQLGIT HVQLMPVFAS NSVDETDPTQ
    DNWGYDPRNY DVPEGQYATN ANGNARIKEF KEMVLSLHRE HIGVNMDVVY NHTFATQISD FDKIVPEYYY RTDDAGNYTN GSGTGNEIAA ERPMVQKFII
    DSLKYWVNEY HIDGFRFDLM ALLGKDTMSK AASELHAINP GIALYGEPWT GGTSALPDDQ LLTKGAQKGM GVAVFNDNLR NALDGNVFDS SAQGFATGAT
    GLTDAIKNGV EGSINDFTSS PGETINYVTS HDNYTLWDKI ALSNPNDSEA DRIKMDELAQ AVVMTSQGVP FMQGGEEMLR TKGGNDNSYN AGDAVNEFDW
    SRKAQYPDVF NYYSGLIHLR LDHPAFRMTT ANEINSHLQF LNSPENTVAY ELTDHVNKDK WGNIIVVYNP NKTVATINLP SGKWAINATS GKVGESTLGQ
    AEGSVQVPGI SMMILHQEVS PDHGKK

# fasta35.exe -q -H -B -m 9i -w 80 -E 1 -d 20 C:\Windows\Temp\all215E.tmp fasta/version19.fasta
FASTA searches a protein or DNA sequence data bank
 version 35.04 Jan. 15, 2009
Please cite:
 W.R. Pearson & D.J. Lipman PNAS (1988) 85:2444-2448

Query: C:\Windows\Temp\all215E.tmp
  1>>> pullulanase - 826 aa
Library: fasta/version19.fasta  507441 residues in  2129 sequences

 507441 residues in  2129 sequences
Statistics:  Expectation_n fit: rho(ln(x))= 4.1935+/-0.00369; mu= 21.3438+/- 0.193
 mean_var=92.5067+/-25.252, 0's: 1 Z-trim: 1  B-trim: 84 in 1/42
 Lambda= 0.133348
Algorithm: FASTA (3.5 Sept 2006) [optimized]
Parameters: BL50 matrix (15:-5) ktup: 2
 join: 38, opt: 26, open/ext: -10/-2, width:  16
 Scan time:  0.000
!! No sequences with E() < 1.000000


826 residues in 1 query   sequences
507441 residues in 2129 library sequences
 Scomplib [35.04]
 start: Sun Apr 14 23:31:28 2019 done: Sun Apr 14 23:31:28 2019
 Total Scan time:  0.000 Total Display time:  0.000

Function used was FASTA [version 35.04 Jan. 15, 2009]
```
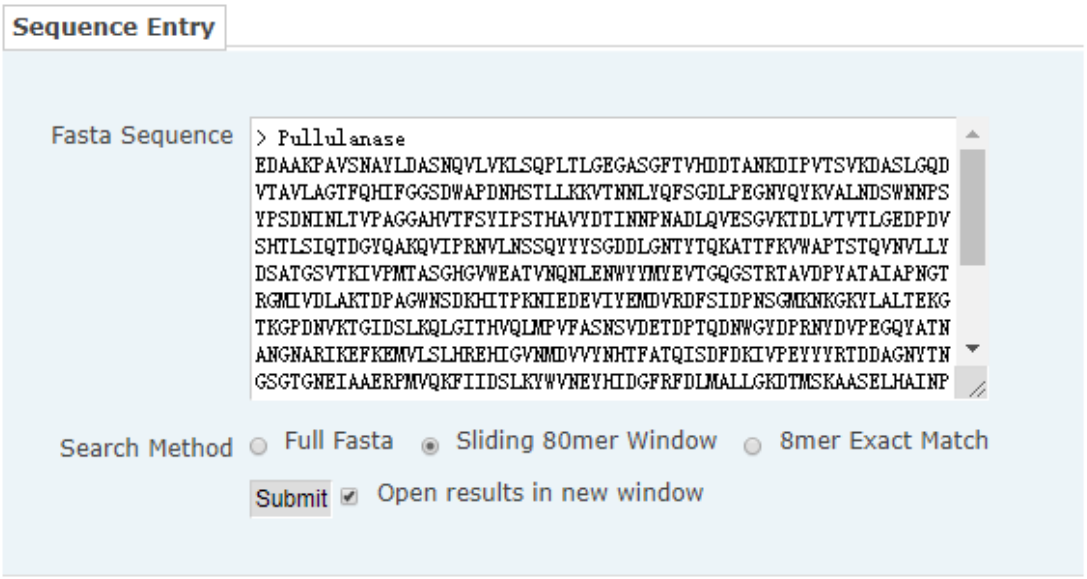

4.2 Input and output of search for 80 amino acid alignments by FASTA



- **Note:** Sliding 80mer Searches Prior to Septermber 12, 2007 may have identified matches of exactly 35% identity. However to be consistant with Codex 2005 the calculation of the cutoff value for a match has been changed to **Greater than 35%**.
- **Note 2:** The sequences in the FASTA searchable database might vary from the sequences described in the public literature, as this database is not updated on a daily basis.
- **Note 3:** We do not observe or log any protein sequences submitted through this website.
- **Note 4:** The E score cutoff for the sliding 80mer search was changed from 100 to 10 on 15 January, 2015 as explained on the "About AllergenOnline" page.

80mer Sliding Window Search Results

Database	AllergenOnline Database v19 (February 10, 2019)
Input Query	> pullulanase EDAAKPAVSNAYLDASNQVLVKLSQPLTLGEGASGFTVHDDTANKDIPVTSVKDASLGQD VTAVLAGTFQHIFGGSDWAPDNHSTLLKKVTNNLYQFSGDLPEGNYQYKVALNDSWNNPS YPSDNINLTVPAGGAHVTFSYIPSTHAVYDTINNPNADLQVESGVKTDLVTVTLGEDPDV SHTLSIQTDGYQAKQVIPRNVLNSSQYYYSGDDLGNTYTQKATTFKVWAPTSTQVNVLLY DSATGSVTKIVPMTASGHGVWEATVNQNLENWYYMYEVTGQGSTRTAVDPYATAIAPNGT RGMIVDLAKTDPAGWNSDKHITPKNIEDEVIYEMDVRDFSIDPNSGMKNKGKYLALTEKG TKGPDNVKTGIDSLKQLGITHVQLMPVFASNSVDETDPTQDNWGYDPRNYDVPEGQYATN ANGNARIKEFKEMVLSLHREHIGVNMDVVYNHTFATQISDFDKIVPEYYYRTDDAGNYTN GSGTGNEIAAERPMVQKFIIDSLKYWVNEYHIDGFRFDLMALLGKDTMSKAASELHAINP GIALYGEPWTGGTSALPDDQLLTKGAQKGMGVAVFNDNLRNALDGNVFDSSAQGFATGAT GLTDAIKNGVEGSINDFTSSPGETINYVTSHDNYTLWDKIALSNPNDSEADRIKMDELAQ AVVMTSQGVPFMQGGEEMLRTKGGNDNSYNAGDAVNEFDWSRKAQYPDVFNYYSGLIHLR LDHPAFRMTTANEINSHLQFLNSPENTVAYELTDHVNKDKWGNIIVVYNPNKTVATINLP SGKWAINATSGKVGESTLGQAEGSVQVPGISMMILHQEVSPDHGKK
Length	826
Number of 80 mers	747
Number of Sequences with hits	0

No Matches of Greater than 35% Identity Found

AllergenOnline Database v19 (February 10, 2019)

4.3 Input and output of search for 8 amino acid exact match

- **Note:** Sliding 80mer Searches Prior to Septermber 12, 2007 may have identified matches of exactly 35% identity. However to be consistant with Codex 2005 the calculation of the cutoff value for a match has been changed to **Greater than 35%**.
- **Note 2:** The sequences in the FASTA searchable database might vary from the sequences described in the public literature, as this database is not updated on a daily basis.
- **Note 3:** We do not observe or log any protein sequences submitted through this website.
- **Note 4:** The E score cutoff for the sliding 80mer search was changed from 100 to 10 on 15 January, 2015 as explained on the "About AllergenOnline" page.

5. Conclusion

Therefore, Bestzyme Biotech Inc. concluded that there is no allergenicity between pullulanase and allergenic proteins included in the publicly available databases through the usage of all of three validation methods provided by AllergenOnline.org.

6. References

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FAO/WHO, 2001. Evaluation of allergenicity of genetically modified foods. Report of a Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology, January 22–25, 2001, Rome, Italy

Ladics GS. Current codex guidelines for assessment of potential protein allergenicity. Food Chem Toxicol. 2008;46 Suppl 10:S20-3.