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Cellulase Enzyme Preparation Produced by *Trichoderma reesei*

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PART 2 - IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS AND PHYSICAL OR TECHNICAL EFFECT OF THE NOTIFIED SUBSTANCE

2.1 IDENTITY OF THE NOTIFIED SUBSTANCE

The subject of this notification is a multi-component cellulase enzyme preparation, produced by submerged fermentation of a genetically modified *Trichoderma reesei.* The cellulase enzyme preparation consists of cellobiohydrolase 1, beta-glucosidase and endo-glucanase 1 enzymes. It is well established that the *Trichoderma reesei* complex is multi-enzymatic, there are at least three enzyme components that are physically and enzymatically distinct and that all three components are essential for the conversion of cellulose to glucose (1).

The characterizing activity of the preparation is cellulase (E.C. 3.2.1.4; systematic name: 1,4-(1,3:1,4)-β-D-glucan 4-glucanohydrolase), an enzyme responsible for the endohydrolysis of 1,4-β-D-glucosidic linkages in cellulose, lichenin and cereal β-D-glucans. Key enzyme and protein chemical characteristics of the enzymes are given below.

Table 1. Key enzyme characteristics

CHARACTERISTICS			
Classification	Cellulase	Cellulase	Cellulase
Systematic name	4-beta-D-glucan cellobiohydrolase	beta-D-glucoside glucohydrolase	4-beta-D-glucan-glucanohydrolase
Accepted name	Cellobiohydrolase 1	Beta-glucosidase	Endo-glucanase 1
EC No.	3.2.1.176	3.2.1.21	3.2.1.4
CAS No.	37329-65-0	9001-22-3	9012-54-8
Specificity	Hydrolysis of (1->4)-beta-D-glucosidic linkages in cellulose	Hydrolysis of terminal, non-reducing beta-D-glucosyl	hydrolysis of (1->3)-beta-D-glucosidic linkages in (1->3)-beta-D-glucans
Molecular Weight	54 kDa	91 kDa	48 kDa
Amino Acid Count	506	844	437

2.2 IDENTITY OF THE SOURCE

2.2(a) *Trichoderma reesei* Production Strain

The *Trichoderma reesei* production strain, designated AyGm61-2C-2, was constructed via the intermediate strain, BTR213. Strain BTR213 is derived from a natural isolate of *Trichoderma reesei* parental strain RUT-C30 (ATCC 56765). RUTC30 is derived from the well-known wild type strain QM6a. QM6a is the parent of practically all *Trichoderma reesei* industrial production strains (2). *Trichoderma reesei* is classified as a Biosafety Level 1 microorganism by the American Type Culture Collection (ATCC) based on risk assessment from U.S. department of Public Health guidelines (3).

The expression plasmids used in the strain construction contain well defined chromosomal DNA fragments and synthetic DNA linker sequences. The DNA sequence

for the introduced genes are based on the sequences encoding the cellobiohydrolase 1 and beta-glucosidase variant from *Aspergillus fumigatus* and the endo-glucanase 1 from *Trichoderma reesei*.

This genetically modified *Trichoderma reesei* production strain complies with the OECD (Organization for Economic Co-operation and Development) criteria for GILSP (Good Industrial Large-Scale Practice) microorganisms (4). It also meets the criteria for a safe production microorganism as described by Pariza and Foster (5) and later Pariza and Johnson (6) and several expert groups (7) (8) (9) (4) (10) (11) (12).

2.2(b) Recipient Strain

The recipient strain used in the construction of the *Trichoderma reesei* production strain AyGm61-2C-2, was modified by several rounds of classical mutagenesis and genetic modifications of RUTC30. Furthermore, the paracelsin gene (*parS*) has been deleted in the recipient strain to eliminate the potential production of paracelsin. The recipient strain was prepared for targeted homologous recombination in two loci.

2.2(c) Expression Plasmids

The expression plasmids used to transform the recipient strain are based on the replication origin of the E. coli standard vector *pUC19* (13). No elements of the vectors are left in the production strain. The plasmids contain expression cassettes consisting of; promotors *cbh1AF* (encoding cellobiohydrolase 1 from *Aspergillus fumigatus*), *eg1TR* (encoding endo-glucanase 1 from *Trichoderma reesei*), *bglAF4M* (encoding a modified beta-glucosidase from *Aspergillus fumigatus*) and transcriptional terminators. The expression plasmids also contain a selective marker encoding an acetamidase.

The expression cassettes are flanked by DNA regions used for targeted integration. Only the expression cassettes are present in the final production strain. This has been confirmed by PCR analysis followed by DNA sequencing.

2.2(d) Construction of the Recombinant Microorganism

The production strain, *Trichoderma reesei* AyGm61-2C-2, was constructed from the recipient strain through several modification steps.

The expression plasmids were integrated into two specific loci in the recipient strain by targeted integration. Targeted integration of the expression cassettes at these loci allows the expression of the cellobiohydrolase 1, beta-glucosidase and endo-glucanase 1.

The resulting strain contains one copy of the *bglAF4M* and *eg1* genes and two copies of *cbh1AF* gene. The strain was named AyGm61-2C-2. Only the expression cassettes are

present in the final production strain. This has been confirmed by PCR analysis followed by DNA sequencing.

2.2(e) Stability of the Introduced Genetic Sequences

The transforming DNA is stably integrated into the *Trichoderma reesei* chromosome and, as such, is poorly mobilized for genetic transfer to other organisms and is mitotically stable. This genetic stability is confirmed on the three different fermentation batches using automated gel electrophoresis.

2.2(f) Antibiotic Resistance Gene

As a result of the genetic modifications, no functional antibiotic resistance genes were left in the strain. The absence of these genes was verified by genome sequence analysis.

2.2(g) Absence of Production Organism

The absence of the production organism is an established specification for the commercial product. The production organism does not end up in food and therefore the first step in the safety assessment as described by IFBC (7) is satisfactorily addressed.

2.3 METHOD OF MANUFACTURE

The quality management system used in the manufacturing process for the enzyme preparation complies with the requirements of ISO 9001. It is manufactured in accordance with current Good Manufacturing Practices, using ingredients that are accepted for general use in foods, and under conditions that ensure a controlled fermentation. These methods are based on generally available and accepted methods used for production of microbial enzymes (14) (15) (16)

The enzyme preparation complies with the purity criteria recommended for enzyme preparations as described in the Food Chemicals Codex (17). It also conforms to the General Specifications for Enzyme Preparations Used in Food as proposed by JECFA (18).

2.3(a) Raw Materials

The raw materials used in the fermentation and recovery process for the enzyme concentrate are standard ingredients used in the enzyme industry (14) (15) (16). The raw materials conform to Food Chemicals Codex specifications except those raw materials which do not appear in the FCC. For those not appearing in the FCC, internal specifications have been made in line with FCC requirements. On arrival at Novozymes A/S, the raw materials are sampled by the Quality Control Department and subjected to the appropriate analyses to ensure their conformance to specifications.

Any antifoams or flocculants used in fermentation and recovery are used in accordance with the Enzyme Technical Association submission to FDA on antifoams and flocculants dated April 10, 1998. The maximum use level of the antifoams and or flocculants, if used in the product, is not greater than 1%.

2.3(b) Fermentation Process

The cellulase enzyme preparation is produced by pure culture submerged fed-batch fermentation of a genetically modified strain of *Trichoderma reesei* as described in Part 2. All equipment is carefully designed, constructed, operated, cleaned, and maintained to prevent contamination by foreign microorganisms. During all steps of fermentation, physical and chemical control measures are taken, and microbiological analyses are done to ensure absence of foreign microorganisms and confirm strain identity.

2.3(c) Production Organism

Each batch of the fermentation process is initiated with a stock culture of the production organism, *Trichoderma reesei.* Each new batch of the stock culture is thoroughly controlled for identity, absence of foreign microorganisms, and enzyme-generating ability before use.

2.3(d) Criteria for the Rejection of Fermentation Batches

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

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

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

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

Any contaminated fermentation is rejected.

2.3(e) Recovery Process

The recovery process is a multi-step operation designed to separate the desired enzyme from the microbial biomass and partially purify, concentrate, and stabilize the enzyme.

2.3(f) Purification Process

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

1) Pretreatment - pH adjustment and flocculation (if required)

2) Primary Separation – vacuum drum filtration or centrifugation

3) Concentration - ultrafiltration and/or evaporation

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

5) Final concentration – evaporation and/or ultrafiltration.

6) Preservation and Stabilization of the liquid enzyme concentrate

The enzyme concentrate is stabilized with sucrose. The liquid product is formulated by the addition of water and preserved with potassium sorbate and sodium benzoate. See Table 2 below.

2.4 COMPOSITION AND SPECIFICATIONS

The final products are analyzed according to the specifications given below.

2.4(a) Quantitative Composition

The enzyme preparation is sold in a liquid form. Table 2 below identifies the substances that are considered diluents, stabilizers, preservatives and inert raw materials used in the enzyme preparations. This enzyme preparation does not contain any major food allergens from the fermentation media.

Table 2. Typical compositions of the enzyme preparations

Substance	Approximate Percentage
Enzyme Solids (TOS*)	12.5%
Water	40 - 55%
Sucrose	20 - 30%
Potassium Sorbate	<0.5%
Sodium Benzoate	<0.5%

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

2.4(b) Specifications

The cellulase enzyme preparation complies with the recommended purity specification criteria for "Enzyme Preparations" as described in *Food Chemicals Codex* (17) . 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 (18).

This is demonstrated by analytical test results of three representative enzyme batches in Table 3 below.

Table 3. Analytical data for three food enzyme batches

Parameter	Specifications	PPC50472	WAM1	WAM4
Cellulase	CNU(B)/g	1060	2797	2910
Total viable count	Upper limit 50,000	<100	<100	200
Lead	Not more than 5 mg/kg	<0.5	<0.5	<0.5
Salmonella sp.	Absent in 25 g of sample	ND	ND	ND
Total coliforms	Not more than 30 per gr	< 4	< 4	< 4
Escherichia coli	Absent in 25 g of sample	ND	ND	ND
Antimicrobial activity	Not detected	ND	ND	ND
Production Organism	Not detected	ND	ND	ND

2.5 PHYSICAL OR TECHNICAL EFFECT

2.5(a) Mode of Action

The enzymes (cellobiohydrolase 1, beta-glucosidase and endo-glucanase 1) expressed by the production strain belong to the cellulase class of enzymes (EC 3.2.1.4). Cellulases catalyse the hydrolysis of the 1,4-beta-D-glycosidic linkages in cellulose, hemicellulose, lichenin and cereal beta-D-glucans to break down the cellulose present in plants.

2.5(b) Intended Use

Cellulase enzymes are used as processing aids in a wide range of food products (19) (20) (21) (22) (23). The typical food applications where this cellulase will be used are: Fruit and vegetable processing, starch and grain processing, brewing and other cereal based beverages and potable/fuel alcohol production.

<u>Brewing and Other Cereal Based Beverages:</u>
Brewing processes rely on cereals (malted or not) as the primary raw material. And, are the primary raw material in the production of beer and other cereal based beverages.

Cellulase enzymes are typically added during the mashing step to reduce the viscosity of the wort and improve the separation of the wort from the spent grains. Also, the cellulase enzyme degrades the polymeric beta-glucans present in the endosperm cell wall of grain, into smaller less viscous molecules, thereby lessening the filtration time and

reducing haze problems (24) (25). The enzyme is typically denatured during the lautering, mash filtration or during the pasteurization step after fermentation.

Potable and Fuel Ethanol:
The cellulase enzymes will be used in potable ethanol production in the mashing of malted and un-malted cereal and other plant sources such as rice, barley, corn wheat etc. The addition of enzymes facilitates the degradation of starch and non-starch polysaccharides in to fermentable sugars, improves yield and allows for better processing conditions. In the production of potable and fuel ethanol the ethanol is concentrated and recovered by distillation.
After saccharification and fermentation are completed, the slurry goes through distillation at — 85° C. The water phase goes to evaporation and the solids go to dryers. Denatured enzyme ends up in the distillers grains (used in animal feed), but the primary steps in the distillation process are considered harsh enough to conclude that enzymes added during ethanol processing are removed or inactivated during that processing and there is no carry-over of organic solids (TOS) to the final potable alcohol product (26) (27).

Additionally, the European Food Safety Agency (EFSA) has published their opinion regarding the exposure to food enzymes when used in distillation processes. According to EFSA, there is evidence to conclude that the presence of residual amounts of TOS, after distilling or filtration and purification during the distilled alcohol processing, is negligible (28).

Wine Processing:
Cellulases are added during maceration, vinification (storage, ageing) and/or before filtration. They catalyse the degradation of structural polysaccharides thereby, lowering the viscosity which results in improved juice yield, clarification and filterability. Cellulases also liberate and solubilize the phenolic compounds (tannins) and glycoside precursors from grape cells wall and flesh leading to better colour intensity, stability and improved overall mouth feel and aroma (29).

The enzymes may be inactivated by pasteurization or removed by bentonite addition and/or filtration. Therefore, the enzymes will not be present/active in the final food.

Fruit and Vegetable Processing:
In the juice industry, cellulases are applied in combination with other macerating enzymes. They are used to increase process performance and yield, improve extraction methods and clarify and stabilize juice. They can also reduce viscosity in nectars and purees (23). Here the enzymes are denatured during the pasteurisation steps included in the processing, rendering the enzyme inactive.

Starch/Grain processing:
Complex structures in cereals can cause processing issues when the grain is milled and when fractionated in to starch, gluten and fiber. The use of cellulases during grain milling

can provide a smooth and efficient processing of that grain, enable separation of the grain structures and ensure quality polysaccharide and gluten fractions.

Cellulases are typically added in grain processing during the milling, mixing and steeping processes. Enzymes are inactivated when used in further food processing, such as baking and starch liquefaction, and are considered non-functional.

2.5(c) Use Levels

Food enzyme preparations are used by food manufacturers according to the *Quantum Satis* (QS) principle i.e. at a level not higher than the necessary dosage to achieve the desired enzymatic reaction in accordance with Good Manufacturing Practices (GMP).

The dosage applied in practice by a food manufacturer depends on the process. The initial recommendation by the enzyme manufacturer is only the starting point for the food producer and is optimised by the manufacturer of the food to fit the process conditions. From a technological position, there are no "normal or maximum use levels". But, a food producer who would add much higher doses than what is needed would experience untenable costs as well as negative technological consequences.

Table 4 below shows the maximum recommended use levels for each application where the food enzyme may be used.

Table 4. Use levels for applications

Application	Maximum Recommended Use levels (activity per/kg RM)
Brewing and other cereal based beverage processes	**3000 CNU(B)**
Potable and fuel ethanol	**750 CNU(B)**
Wine processing	**1500 CNU(B)**
Fruit & vegetable processing	**150 CNU(B)**
Starch/grain processing	**1300 CNU(B)**

2.5(d) Enzymes Residues in the Final Food

The cellulase enzyme preparation is used during processing and does not exert any enzymatic activity in the final food. This is due to a combination of various factors and depend on the process conditions used by the individual food producer. These factors include; denaturation of the enzyme during heat processing, depletion of the substrate, physical removal of the enzyme, etc. In most cases, a heat treatment step is part of the manufacturing process for production of food ingredients and this process will be enough to inactivate or denature the enzyme protein.

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 has taken place during the food

manufacturing process and is complete before the food product is available for delivery to consumers.

PART 3 - DIETARY EXPOSURE

A "worst case" scenario is provided for the calculation of the possible daily human exposure. The assumption was made that all the enzyme product is retained in the final food product. The general population is the target population for consumption. There is no specific subpopulation.

3(a) Assumptions in Dietary Exposure

Overall, the human exposure to the cellulases will be negligible because the enzyme preparation is used as a processing aid and generally at lower dosages.

The food enzyme is used in the manufacture of a wide variety of foods, food ingredients and beverages. Due to this wide variety of applications, the most appropriate way to estimate the human consumption in the case of food enzymes is using both the Budget Method (30) (31) and specific human consumption.

An exaggerated human intake is estimated using the Budget method was used for the intake associated with starch/grain processing. Specific consumption data is used to estimate the intake associated with fruit/vegetable processing, brewing and other cereal based beverages, wine processing and potable alcohol.

Data summarizing the intake of: juice from fruit/vegetable food products, brewing and other cereal based beverages and wine and potable alcohol beverage consumption was taken from the EFSA Comprehensive European Food Consumption Database using the consumption data from 17 countries and at the 95th percentile (32) (33) (34) (35).

The total TMDI represents a highly exaggerated value because of (among others) the following reasons:

- ➢ It is assumed that ALL producers of the intended uses mentioned above for both solid foodstuffs and beverages, use the food enzyme atthe highest recommended level.
- ➢ For the calculation of the TMDI in food as well as in beverage, the TOS for each application was combined and the total sum was used as the factor for the TMDI in the MOS (margin of safety) calculation.
- ➢ It is assumed that the final food containing the calculated theoretical amount of TOS is consumed daily over the course of a lifetime.

Using these assumptions, the enzyme preparation will be consumed by humans at the maximum recommended dose for all applications. and will provide a highly conservative margin of safety.

Also, the consumption is further exaggerated since the enzyme protein and the other substances resulting from the fermentation are diluted or removed in certain processing steps.

The cellulase enzyme preparation has an average activity of 2256 CNU(B) per gram and approximately 12.5% TOS (Total Organic Solids) content.

This corresponds to an activity/TOS ratio of 18 CNU(B) per mg TOS.

3(b) Food Consumption Data

Assumptions in the Budget Method

Solid food	The maximum energy intake over the course of a lifetime is 50 kcal/kg body weight/day. 50 kcal corresponds to 25 g foods. Therefore, adults ingest 25 g foods per kg body weight per day. Assuming that 50% of the food is processed food, the daily consumption will be 12.5 g processed foods per kg body weight. It is further assumed that, in average, all processed food contains 25% starch (or starch-derived) dry matter = 3.12 g starch derived dry matter per kg body weight per day.
Liquids	The maximum intake of liquids (other than milk) is 100 ml/kg body weight day. Assuming that 25% of the non-milk beverages is processed, the daily consumption will be 25 ml processed beverages per kg body weight. It is further assumed that all processed beverages contain 10% starch hydrolysates = 2.50 g starch derived dry matter per kg body weight per day. It is assumed that the densities of the beverages are ~ 1.

TMDI calculation – Starch/Grain Processing

Solid Food:
The dosage given in Table 4 for starch/grain processing is 1300 CNU(B) per kg starch based raw material.

1300 CNU(B) corresponds to:

1300 CNU(B) ÷ 18 CNU(B) per mg TOS = 72 mg TOS per kg starch based raw material

Based on this, 3.12-gram starch-derived dry matter in solid food will maximally contain:

72 mg TOS per kg ÷ 1000 g per kg x 3.12 g = 0.22 mg TOS per kg bw/day

Liquid Food:

The dosage given in Table 4 for starch/grain processing is 1300 CNU(B) per kg starch based raw material.

1300 CNU(B) corresponds to:

1300 CNU(B) ÷ 18 CNU(B) per mg TOS = 72 mg TOS

Based on this, 2.50-gram starch-derived dry matter in liquids will maximally contain:

72 mg TOS ÷ 1000 g per kg x 2.50 g = 0.18 mg TOS per kg bw/day

The theoretical maximum daily intake (TMDI) of consumers of the food enzyme is:
0.22 + 0.18 = 0.4 mg TOS/kg body weight/day

TMDI Calculation - Fruit and vegetable processing

To demonstrate a worst-case calculation, an exaggerated human intake for fruit and vegetable juice was used. This intake value was taken from the EFSA Comprehensive European Food Consumption Database using the consumption data for fruit and vegetable juices from 17 countries and at the 95th percentile (32). Based on this, the intake value of 33 g of juice is consumed per kg of body weight per day.

The dosage given in Table 4 for fruit and vegetable processing is 1300 CNU(B) per kg fruit/vegetable raw material. This corresponds to 72 mg TOS per kg of fruit/vegetable product.

The theoretical maximum daily intake (TMDI) of consumers of the food enzyme is:

72 mg TOS x 33 g juice per kg bw/day ÷ 1000 = 2.4 mg TOS per kg bw/day

Intake associated with beer and other cereal based beverage processes:

To demonstrate a worst-case calculation, an exaggerated human intake for beer and beer like beverages was used. This intake value was taken from the EFSA Comprehensive European Food Consumption Database using the consumption data for beer and beer-like beverages from 17 countries and at the 95th percentile (33). Based on this, the intake value of 11.4 g of beer and beer-like beverage is consumed per kg of body weight per day.

Typical values for the starch content of malt and barley is 65% (24). As a rule of thumb 1 kg of grits will be used to produce 6 kg of beer. Therefore, an intake per kg bw perday of 11.4 g “Beer and beer-like beverage” corresponds to:

11.4 g beer/kg bw/day ÷ 6 g beer/g grits = 1.86 g grits/kg bw/day x 0.65 g starch/per g grits = 1.21g starch/kg bw/day.

TMDI calculation - Brewing and Cereal Based Beverage

The dosage given in Table 4 for beer and other cereal based beverages is 3000 CNU(B) per kg starch dry matter. This corresponds to 166 mg TOS per kg of starch dry matter.

The theoretical maximum daily intake (TMDI) of consumers of the food enzyme is:

166 mg TOS x 1.21 g starch per kg bw/day ÷ 1000 = 0.20 mg TOS per kg bw/day

TMDI Calculation – Wine processing

To demonstrate a worst-case calculation, an exaggerated human intake for wine and wine-like beverages was used. This intake value was taken from the EFSA Comprehensive European Food Consumption Database using the consumption data for wine and wine-like beverages, from 17 countries and at the 95th percentile (35). Based on this, the intake value of 9.2 g of wine and wine-like beverages are consumed per kg of body weight per day.

The dosage given in Table 4 for wine processing is 1500 CNU(B) per kg wine material. This corresponds to 83 mg TOS per kg of wine material.

The theoretical maximum daily intake (TMDI) of consumers of the food enzyme is:

83 mg TOS x 9.2 g wine per kg bw/day ÷ 1000 = 0.76 mg TOS per kg bw/day

TMDI Calculation – Potable ethanol

The maximum intake of any alcoholic drink will be limited largely to the maximum intake of alcohol that the body can tolerate. Therefore, the potential exposure to the cellulase enzymes from the consumption of potable alcohol is negligible. But, to demonstrate a worst-case calculation, an exaggerated human intake for alcoholic beverages was used. This intake value was taken from the EFSA Comprehensive European Food Consumption Database using the consumption data for alcoholic beverages from 17 countries and at the 95th percentile (34). Based on this, the intake value of 0.28 g of alcoholic beverages is consumed per kg of body weight per day.

The dosage given in Table 4 for beverage alcohol is 750 CNU(B) per kg fruit/vegetable raw material. This corresponds to 42 mg TOS per kg of fruit/vegetable product.

The theoretical maximum daily intake (TMDI) of consumers of the food enzyme is:

42 mg TOS x 0.28 g alcohol per kg bw/day ÷ 1000 = 0.01 mg TOS per kg bw/day

Total TMDI:

To represent a worst-case scenario for the maximum human exposure value, it is assumed that foods represented for each application are consumed daily. The final TMDI is calculated taking the TOS value for all applications.

Starch/grain processing:	0.4 mg TOS/kg body weight/day
Fruit/vegetable processing:	2.4 mg TOS per kg bw/day
Beer and other cereal based beverage processing:	0.20 mg TOS per kg bw/day
Wine processing:	0.76 mg TOS per kg bw/day
Potable alcohol:	0.01 mg TOS per kg bw/day
Total:	**3.83 mg TOS per kg bw/day**

Theoretical Maximum Daily Intake (TMDI)

The safety margin is calculated as the dose level with no adverse effect (NOAEL) divided by the estimated human consumption. The NOAEL dose level in the 14-day oral toxicity study in rats conducted on cellulase, PPC50472 was the highest dosage possible, 1314 mg TOS/kg bw/day. See Appendix 1 and Table 5 below.

Table 5. NOAEL Calculation

NOAEL (mg TOS/kg bw/day)	1314
*TMDI (mg TOS/kg bw/day)	3.83
Safety margin	343

*based on the worst-case scenario

PART 4 - SELF-LIMITING LEVELS OF USE

This part does not apply

PART 5 - COMMON USE IN FOOD BEFORE 1958

This part does not apply

PART 6 - NARRATIVE ON THE CONCLUSION OF GRAS STATUS

The information provided in the following parts is the basis for our determination of the general recognition of safety (GRAS) of the cellulase enzyme preparation. The evaluation follows the generally recognized methodology and the decision tree by Pariza and Johnson (2001) (Appendix 2) and includes published information that provides the common knowledge element of the GRAS conclusion. Our safety evaluation in Part 6 follows the approach described in the Enzyme Technical Association publication (Appendix 3) which includes an evaluation of the production organism, the donor strain, the introduced DNA, the enzyme and the manufacturing process (36). Data and information cited in this notification is generally available and Part 6 does not contain any data or information that is exempt from disclosure under the FOIA.

The production organism for the cellulase, *Trichoderma reesei,* is discussed in Part 2 and also in this Part. The names *Trichoderma reesei, Trichoderma longibrachiatum,* and *Hypocrea jecorina* may appear in different cited reference documents, but they refer to essentially the same fungal species.

6(a) Safety of the Production Organism

The safety of the *Trichoderma reesei* production organism must be the prime consideration in assessing the degree of safety of an enzyme preparation intended for use in food (5) (6). If the organism is non-toxigenic and non-pathogenic, then it is assumed that food or food ingredients produced from the organism, using current Good Manufacturing Practices, is safe to consume (37). Pariza and Foster (5) define a non-toxigenic organism as "one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure" and a non-pathogenic organism as "one that is very unlikely to produce disease under ordinary circumstances".

Trichoderma reesei has a long history (more than 35 years) of safe use in industrial scale enzyme production and can be considered as a safe production organism for enzymes for food as well as feed processing and numerous other industrial applications. The original isolate, QM6a, and its subsequent derivatives have been the subject of intense research due to their usefulness in the production of cellulases.
Trichoderma reesei is not present on the list of pathogens used by the EU (Directive Council Directive 90/679/EEC) and major culture collections worldwide (38). It is classified as a Biosafety Level 1 (BSL 1) microorganism by the American Type Culture Collection (ATCC) based on assessment of the potential risk using U.S. Department of Public Health guidelines. BSL 1 microorganisms are not known to cause diseases in healthy adult humans.

Cellulases, hemicellulases, beta-glucanases, pectinases and xylanases produced by *Trichoderma reesei* are used in food, animal feed, pharmaceutical, textile, detergent,

bioethanol and pulp and paper industries (2) (39) (40). *Trichoderma reesei* strains are non-pathogenic for healthy humans and animals (2). The safety of *Trichoderma reesei* has been discussed in several review papers (2) (39) (41) (42). *Trichoderma reesei* has been described not to produce mycotoxins or antibiotics under conditions used for enzyme production.

All fungal species produce secondary metabolites to allow them to survive in nature. It is recognized that *Trichoderma reesei* is capable of producing a peptaibol compound (paracelsin) (41). However, the bulk of the literature investigating the capability of *Trichoderma reesei* to produce peptaibol is based on fermentation conditions designed either to mimic natural (and poor) growth conditions or attempt to optimize the conditions for secondary metabolite production. These methods are not representative of the conditions used in controlled industrial fermentation practices (43) (44) (41).

In 2012, the U.S. EPA published a risk assessment (45) to support tiered exemption status for *Trichoderma reesei* QM6a and it's derivate. The EPA acknowledged in this assessment that under normal submerged fermentation conditions paracelsin is not produced. Novozymes has removed the paracelsin gene (*parS*) in the recipient strain to eliminate the potential production of paracelsin.

Enzyme preparations from *Trichoderma reesei* have been approved for use in food in; Canada (Food and Drugs Act Division 16, Table V), France (Arrêté du 19 Octobre 2006), Denmark, Australia/New Zealand (Standard 1.3.3 processing aids), China and Japan. To this date, there are more than ten enzymes produced in *Trichoderma reesei* that have been notified to FDA/CFSAN as GRAS for their intended uses (46). In addition, a cellulase enzyme preparation from *Trichoderma reesei* is the subject of the regulation in 21 CFR §184.1250.

An essential aspect of the safety evaluation of food components, derived from genetically modified organisms, is the identification and characterization of the inserted genetic material (37) (9) (4) (10) (11) (12). An evaluation of the genetically modified *Trichoderma reesei* production organism embodying the concepts initially outlined by Pariza and Foster, 1983 (6) and further developed by IFBC in 1990 (37), the EU SCF in 1991 (9), the OECD in 1992 (4), ILSI Europe Novel Food Task Force in 1996 (47), FAO/WHO in 1996 (11), JECFA in 1998 (18) and Pariza and Johnson in 2001 (6), demonstrates the safety of this genetically modified production microorganism strain. The components of this evaluation: the identity of the recipient strain, a description of the incorporated DNA, the sources and functions of the introduced genetic material, an outline of the genetic construction and characteristics of the production strain and the a description of the enzyme derived from it are given in Part 2.

Novozymes' used the decision tree (Appendix 2) in Pariza and Johnson 2001 (6) as a basis for our safety assessment. The production strain is genetically modified as discussed in Part 2. The expressed enzymes have a history of safe use in food. The

enzyme preparation is free of DNA encoding transferable antibiotic resistance DNA genes. The introduced DNA is well characterized and safe for the construction of microorganisms to be used in the production of food grade products. The DNA is stably integrated into the chromosome and the incorporated DNA is known not to encode or express any harmful or toxic substances.

Based on the information presented here it is concluded that the *Trichoderma reesei* production strain is considered a safe strain for the production of the cellulase enzyme (39) (2).

6(b) Safe Strain Lineage

The safety of the *Trichoderma reesei* production strain was established following published criteria for the assessment of the safe use of microorganisms when used in the manufacture of food ingredients (6) (37). The *Trichoderma reesei* recipient strain is derived from a safe strain lineage that is comprised of production strains for enzyme preparations which have full toxicological safety studies (i.e. 13-week oral toxicity study in rats, Ames test and chromosomal aberration test or micronucleus assay).

Table 6: Safe Strain Lineage

Enzyme	EC No.	Predecessor strain[1]	Donor strain	Safety studies[2]
Cellulase (21 CFR, §184.1148)	3.2.1.4	*Trichoderma reesei RUTC30*	*Non-GM*	Yes
Xylanase (GRN 675)	3.2.1.8	*Trichoderma reesei BTR213*	*Talaromyces leycettanus*	Yes
Arabinofuranosidase (GRN 680)	3.2.1.55	*Trichoderma reesei BTR213*	*Talaromyces pinophilus*	Yes
Lysozyme (GRN 853)	3.2.1.17	*Trichoderma reesei BTR213*	*Acremonium acalophilum*	Yes

Table 6. Novozymes products derived from *T. reesei* strains. The predecessor strains show strains in the GM construction pathway that are in common with the recipient strain lineage. At least the following: *in vitro* test for gene mutations in bacteria (Ames); *in vitro* test for chromosomal aberration or *in vitro* micronucleus assay; 13-week sub chronic oral toxicity study in rats.

All studies concluded that the test preparations did not exhibit any toxic or mutagenic effect under the conditions of the test. These studies support the view, that strains derived from *Trichoderma reesei* can be used safely to produce food enzymes. Additionally, no safety issues are observed when different enzyme preparations are produced in the same strain (e.g., cellulase, arabinofuranosidase and lysozyme), demonstrating that the safety of the strains in the lineage is not preparation-dependent.

Novozymes' has used the procedures outlined by Pariza and Johnson (6) along with the decision tree (Appendix 2) as a basis for our safety assessment for the *Trichoderma reesei* production strain which is the production organism for the article of commerce (subject of this notification). This same procedure has been repeatedly used for the *Trichoderma reesei* strains listed in Table 6.

An evaluation of the genetically modified *Trichoderma reesei* production organism embodying the concepts initially outlined by Pariza and Foster, 1983 (5) and further developed by IFBC in 1990 (37), the EU SCF in 1991 (9), the OECD in 1992 (4), ILSI

Europe Novel Food Task Force in 1996 (47), FAO/WHO in 1996 (11), JECFA in 1998 (18) and Pariza and Johnson in 2001 (6), demonstrates the safety of this genetically modified production microorganism strain. The components of this evaluation: the identity of the recipient strain, a description of the incorporated DNA, the sources and functions of the introduced genetic material, an outline of the genetic construction of the production strain, and some characteristics of the production strain and the enzyme derived from it are given in Part 2.

Based on the information presented here it is concluded that the *Trichoderma reesei* production strain is part of the safe strain lineage and is considered safe to produce cellulase enzymes.

6(c) Safety of the Donor Organism

Besides *Trichoderma reesei*, other fungi, like *Aspergillus*, have the ability to yield high levels of cellulases (48). For the multi-component cellulase preparation, that is the subject of this notification, the cellulase genes were isolated from *Aspergillus fumigatus* and *Trichoderma reesei*.

Trichoderma reesei
Trichoderma reesei is not present on the list of pathogens used by the EU (Directive Council Directive 90/679/EEC) or major culture collections worldwide (38). It is classified as a Biosafety Level 1 (BSL 1) microorganism by the American Type Culture Collection (ATCC) based on assessment of the potential risk using U.S. Department of Public Health guidelines. BSL 1 microorganisms are not known to cause diseases in healthy adult humans.

Aspergillus fumigatus
Aspergillus is one of the oldest named genera of fungi. It is a filamentous fungus which plays an important role in natural environments in the aerobic decomposition of organic materials and is found virtually everywhere on earth. *Aspergillus fumigatus* is one of the most ubiquitous of the airborne saprophytic fungi and is considered a fungal pathogen. There are a number of activities, both indoors and outdoors, that subject people to exposure; lawn mowing, gardening, home landscaping, potting of household plants in soils and raking leaves (49).

The inhalation of spores is the most common route of human exposure and humans are exposed to hundreds of *Aspergillus fumigatus* conidia per day. But, adverse effects in immunocompetent individuals are rare since the conidia are eliminated by innate immune mechanisms in the body and does not normally cause disease (50).

The safety of the production organism is of primary importance. If the introduced DNA is well defined and characterized, the donor organism is not of concern. As indicated in Part 2, only well characterized DNA fragments, limited solely to the enzymes' s coding

sequence from the donor strain, are used in the construction of the genetically modified production strain. Also, the introduced DNA does not code for any known harmful or toxic substances. Therefore, the donor organisms are considered safe.

6(d) Safety of the Cellulase Enzymes Within the Complex

A wide variety of enzymes are used in food processing (5) (6). Cellulases account for a significant share of the world enzyme market with major uses in the food and feed processing.

There are three main types of cellulase activities (51); cellobiohydrolase, beta-glucosidase and endo-glucanase. Cellulase enzymes, have a long history of use in food and have been commercially available for more than 30 years. Furthermore, Novozymes Celluclast® enzyme preparation was the subject of the GRASP petition (9G0260) which was filed by the FDA in 1979. The Federal Register notice (FR Vol. 64, No. 101) amended the regulations to affirm that cellulase derived by *Trichoderma longibrachiatum* (formerly *T. reesei*) is recognized as GRAS (21 CFR §184.1250). Celluclast® is characterized as a cellulase with cellobiohydrolase, beta-glucosidase and beta-glucanase activity.

These enzymes have represented a target for both academic as well as industrial research (51) (6) (5) (52) (53). The cellulase enzyme complex, subject of this notification, is comprised of the following cellulase components.

Endo-glucanase 1:
Endo-glucanases (beta-glucanases) catalyze the endo-hydrolysis of the 1,3- or 1,4-linkages in beta-D-glucans. They are widely distributed in nature and have been isolated from a variety of sources, such as fungi, yeasts, bacteria and plants (54) (55) (25). The FDA has reviewed beta-glucanase preparations in the past; GRN 149,195, 479 and 482, all of which received “No Questions” letters from the Agency. Several enzyme preparations of beta-glucanase have been evaluated by JECFA and assigned an ADI ‘not specified’ for their use in applications such as the preparation of fruit juices, beer and baking products (56).

Cellobiohydrolase 1:
Cellobiohydrolase 1 (CBH1) is a cellulase which degrades cellulose by hydrolyzing the 1,4-β-D-glycosidic bonds. CBH1 is an exocellulase which cleaves two to four units from the reducing ends of cellulose (57). Exocellulases are among the most abundant components in natural and commercial cellulase mixtures (58). Authorization, in which cellobiohydrolase was listed as one of three cellulase components, was granted by the French authorities for a cellulase enzyme produced by *Trichoderma reesei*, for use as a processing aid in food (59).

Beta-glucosidase:

Beta-glucosidases are enzymes that hydrolyze the glucosidic bond of a carbohydrate moiety to release nonreducing terminal glycosyl residues, glycosides and oligosaccharides. These enzymes are present in all kinds of organisms including bacteria, archaea and eukaryotes. Beta-glucosidases play a role in many important processes; biomass conversion in microorganisms, breakdown of glycolipids and the process of lignification, phytohormones activation, catabolism of cell wall in plants and both plant–microbes and plant–insect interaction (60).

Although the beta-glucosidase was modified, it is well known that there are natural variations within enzyme families. Modifications to enzymes that improve upon the enzyme function, do not result in the creation of a toxic protein. This is supported by extensive studies on modified enzymes showing that these enzymes retain the same characteristic structure and catalytic activities as found in nature (6). Additionally, toxicological studies (Appendix 1) and the sequence homology to known toxins, Part 6(e), confirm the safety of the cellulase enzyme preparation.

Beta-glucosidase has been evaluated by the FDA as part of the pectinase complex GRAS submission, GRN 89, with "No Questions" affirming the GRAS conclusion (46). Also, beta-glucosidase is on the inventory of substances used as processing aids as outlined by the FAO/WHO Codex committee (61) and is also mentioned as an enzyme used in food processing by Pariza and Johnson (6).

Cellulase, together with other related enzymes, i.e., hemicellulases and pectinases, are among the most important group of enzymes that are employed in the processing of lignocellulosic materials to produce food, feed, fuel, and chemical feedstocks (62). Complete enzymatic hydrolysis of cellulose requires synergistic action of all three cellulase enzymes: endoglucanase, exoglucanase and beta-glucosidases (63). Microbial glucanases are commonly added at the mashing or fermentation stage during the production of beer and play an important role in improving color extraction, clarification and filtration. In fruit and vegetable processing, the use of cellulases, pectinases and hemicellulases increased juice yield. And, β-glucosidases and pectinases are also used to reduce bitterness and improve flavor and texture of fruit and vegetable purees and nectars (51).

GRAS notifications have been submitted and accepted by FDA with "No questions" for the use of cellulases (GRASP petition 9G0260, GRN 584, 479, 292 and 195) from a variety of production organisms (46). All the GRAS notifications mentioned above included sufficient toxicological testing data which showed no evidence of toxicological concern regarding the safety and consumption of cellulase enzymes.

Also, several enzyme preparations of glucanase, cellulase (including cellobiohydrolase) or hemicellulases from *Penicillium funiculosum, Trichoderma longibrachiatum*, *Aspergillus niger* and *Trichoderma reesei*, have been evaluated by JECFA. All were

assigned an ADI of "not specified" for their use in several applications including baking, juice processing and beer (64).

A literature search was performed in September 2019 for the periods 2000 to 2019 on cellulase, utilizing the database *Web of Science* and the keywords "cellulase enzyme", "food safety" and "toxicity". A total of 31 relevant hits were found. Novozymes reviewed the available abstracts and found no indication that that cellulase is associated with toxicity or other adverse effects in humans or animals and the findings did not contradict our determination of the general recognition of safety of the cellulase enzyme. Novozymes also conducted a 14-day oral toxicity study (Appendix 1) and sequence homology to known allergens and toxins. The results showed no indication of allergenic or toxigenic potential of the cellulase.

From the information provided above, it is apparent that cellulase enzymes have a long history of use in food processing and are safe for human consumption.

6(e) Allergenic/Toxigenic Potential of the Cellulase Enzymes

The ingestion of a food enzyme protein is not considered a concern for food allergy. This is based on the following considerations:

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

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

3) Enzymes in foods are added in concentrations in the low range of parts per million. The enzyme is typically removed or denatured during food processing and denatured protein has been shown to be very susceptible to digestion in the gastro-intestinal system. Moreover, a wide range of naturally occurring food enzymes have been shown to be very labile in the gastro-intestinal system even in the native unprocessed form.

The above statements are further supported by the publication: "Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry" (Bindslev-Jensen *et al*, 2006) (65).

To further evaluate the possibility that the cellulases could cross-react with known allergens and induce a reaction in an already sensitized individual, a sequence homology to known food allergens was assessed. Following the guidelines developed by FAO/WHO, 2001 (66) and modified by Codex Alimentarius Commission, 2009 (67) the beta-glucosidase, cellobiohydrolase 1 and endo-glucanase 1 enzymes (cellulase

enzymes) were compared to allergens from the FARRP allergen protein database (http://allergenonline.org) as well as the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee (**http://www.allergen.org**).

A search for more than 35% identity in the amino acid sequence of the expressed protein using a window of 80 amino acids and a gap penalty was done and showed no matches. Alignment of each cellulase enzyme to each of the allergens and identity of hits with more than 35% identity over the full length of the alignment was analyzed. No homology was found between the cellulase enzymes and any of the allergens from the databases mentioned above. Also, a search for 100% identity over 8 contiguous amino acids was completed. Again, no homology was found

In addition, the Association of Manufacturers and Formulators of Enzyme Products (AMFEP) Working Group on Consumer Allergy Risk from Enzyme Residues in Food, performed an in-depth analysis of the allergenicity of enzyme products. In this paper, Dauvrin and colleagues conclude that enzyme exposure by ingestion, in opposition to exposure by inhalation, is extremely unlikely to lead to sensitization. There is compelling evidence that persons affected by occupational asthma can ingest the respiratory allergen without acquiring clinical symptoms of food allergy, suggesting that inhalation is not likely to result in food allergy. Only one single case has been reported in the literature and this case was not verified as a bona fide oral sensitization to enzymes in food (68).

This is backed up by the study conducted by Bindslev-Jensen et al (65) using the generally recognized guidelines for food allergy diagnosis (skin prick test, specific serum IgE and DBPCFC). This study included 400 patients with a diagnosed allergy to one or more of inhalation allergens, food allergens, bee or wasp allergens. The study concluded that no cases of IgE-mediated food allergy to commercial enzymes (including alpha-amylases) could be found. Further, there were no indications of cross-reactivity between the tested enzymes used in food and the main known allergens causing clinical symptoms in the patients included in the study.

A sequence homology of the cellulase enzymes to known toxins was assessed based on the information present in the UNIPROT database. This database contains entries from SWISSPROT and TREMBL. The homology among the emerging entries was below 17% indicating that the homology to any toxin sequence in this database is low and random.

Based on the available evidence and supporting scientific literature, it is concluded that oral intake of cellulase enzymes produced by *Trichoderma reesei* are not anticipated to pose food allergy concerns.

6(f) Safety of the Manufacturing Process

The enzyme preparation follows standard industry manufacturing practices (16) (15) (14). The quality management system used in the manufacturing process complies with the requirements of ISO 9001. The enzyme preparation is produced in accordance with current Good Manufacturing Practices, using ingredients that are accepted for general use in foods and under conditions that ensure a controlled fermentation. The enzyme preparation complies with the purity criteria recommended for enzyme preparations as described in the Food Chemicals Codex (17). It also conforms to the General Specifications for Enzyme Preparations Used in Food as proposed by JECFA (18).

6(g) Safety Studies Conducted

This part describes the studies and analysis performed to evaluate the safety of the cellulase enzyme.

The following studies were performed on test batch PPC50472 all producing favourable results:

- Reverse Mutation Assay (Ames test)
- *In vitro* Cytotoxicity (Neutral Red Uptake – NRU) test
- 14-day oral toxicity study in rats

These tests are summarized in Appendix 1. Based on the presented toxicity data and the history of safe use for the strain it can be concluded that the test preparation, represented by batch PPC50472 exhibits no toxicological effects under the experimental conditions described.

6(h) Description of the Test Article

The toxicological testing of the cellulase was conducted on a batch of cellulase enzyme concentrate (batch PPC50472) which was produced according to the description given in Part 2.3. The cellulase enzyme concentrate test batch does not contain additives or other standardization or stabilization ingredients.

6(i) Results and Conclusion

Novozymes has reviewed the publicly available data and information regarding the safety of microbially derived cellulase enzyme preparations used in food processing and found no evidence nor are we aware of any data and/or information that is, or appears to be, inconsistent with our conclusion of GRAS.

Based on this critical review and evaluation, a history of safe use of *Trichoderma reesei* and the limited and the well-defined nature of the genetic modifications, Novozymes

concludes through scientific procedures that the subject of this notification, cellulase enzyme preparation, produced by a genetically modified strain of *Trichoderma reesei* carrying the gene encoding for cellulase from *Trichoderma reesei* and *Aspergillus fumigatus,* meets the appropriate food grade specifications, is produced in accordance with current good manufacturing practices and is safe for human consumption.

Thus, it is generally recognized, among qualified experts, to be safe under the conditions of its intended use.

Part 7 – SUPPORTING DATA AND INFORMATION

All information indicated in the List of Appendices and References is generally available

APPENDICES

1. Summary of Toxicity Data. Cellulase from *Trichoderma reesei* PPC50472. 17, September 2019, File No. 2019-15898-01.

2. Pariza and Johnson Decision Tree Analysis

3. Sewalt Vincent, Shanahan Diane, Gregg Lori, La Marta James and Carrillo Roberts; The Generally Recognized as Safe (GRAS) Process for Industrial Microbial Enzymes. Industrial Biotechnology, Vol. 12, No. 5. October 2016.

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Received 11/05/2019
Via Gateway

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#891

PART 1: Signed statement of the conclusion of GRAS (Generally Recognized as Safe) and certification of conformity to 21 CFR §170.205-170.260.

§170.225(c)(1) – Submission of GRAS notice:

Novozymes North America Inc. is hereby submitting a GRAS (Generally Recognized as Safe) notice in accordance with subpart E of part 170.

§170.225(c)(2) - The name and address of the notifier:

Novozymes North America Inc.
77 Perry Chapel Church Rd., Box 576
Franklinton, NC 27525

§170.225(c)(3) – Appropriately descriptive term:

The appropriately descriptive term for this notified substance is: Cellulase enzyme produced by *Trichoderma reesei.*

§170.225(b) – Trade secret or confidential:

This notification does not contain any trade secret or confidential information.

§170.225(c)(4) – Intended conditions of use:

The cellulase enzyme will be used as a processing aid during the hydrolysis of the 1,4-beta-D-glycosidic linkages in cellulose, hemicellulose, lichenin and cereal beta-D-glucans to break down the cellulose present in plants. The enzyme is used in a wide range of food products; fruit and vegetable processing, wine processing, starch and grain processing, brewing and other cereal based beverages and potable/fuel alcohol production. The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements for normal production following Good Manufacturing Practices. The "general" population is the target population for consumption.

§170.225(c)(5) - Statutory basis for GRAS conclusion:

This GRAS conclusion is based on scientific procedures.

§170.225(c)(6) – Premarket approval:

The notified substance is not subject to the premarket approval requirements of the FD&C Act based on our conclusion that the substance is GRAS under the conditions of the intended use.

§170.225(c)(7) – Availability of information:

This notification package provides a summary of the information which supports our GRAS conclusion of the notified substance. Complete data and information that are the basis for this GRAS conclusion is available to the Food and Drug Administration for review and copying during customary business hours at Novozymes North America, Inc. or will be sent to FDA upon request.

§170.225(c)(8) - FOIA (Freedom of Information Act):

Parts 2 through 7 of this notification do not contain data or information that is exempt from disclosure under the FOIA (Freedom of Information Act).

§170.225(c)(9) – Information included in the GRAS notification:

To the best of our knowledge, the information contained in this GRAS notification is complete, representative and balanced. It contains both favorable and unfavorable information, known to Novozymes and pertinent to the evaluation of the safety and GRAS status of the use of this substance.

11/04/2019

Janet Oesterling — Date

Regulatory Affairs Specialist III

Decision Tree

Appendix 2- This cellulase enzyme preparation produced by *Trichoderma reesei* was evaluated according to the decision tree published in Pariza and Johnson, 2001[1]. The result of the evaluation is presented below in the Decision Tree.

1. Is the production strain genetically modified?
   YES
   If yes, go to 2.
2. Is the production strain modified using rDNA techniques?
   YES
   If yes, go to 3.
3. Issues relating to the introduced DNA are addressed in 3a-3e.

   a. Does the expressed enzyme product which is encoded by the introduced DNA have a history of safe use in food?
   YES
   If yes, go to 3c.
   c. Is the test article free of transferable antibiotic resistance gene DNA?
   YES
   If yes, go to 3e.
   e. Is all other introduced DNA well characterized and free of attributes that would render it unsafe for constructing microorganisms to be used to produce food products?
   YES
   If yes, go to 4.

4. Is the introduced DNA randomly integrated into the chromosome?
   NO
   If no, go to 6.

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

LIST OF REFERENCES

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

Novozymes North America
Regulatory Affairs
77 Perry Chapel Church Rd
Franklinton, NC 27525
Tel. 919-494-3000
Fax 919-494-3420

Product Safety

Date: September 17, 2019
File: 2019-15898-01
Ref.: SGE/JAO

SUMMARY OF TOXICITY DATA

Cellulase, batch PPC50472, from *Trichoderma reesei*

Author:
Signe Gry Elvig-Jørgensen

Issued by:
Novozymes A/S
Krogshoejvej 36
DK-2880 Bagsvaerd
Denmark

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1. ABSTRACT

The below series of toxicological studies were undertaken to evaluate the safety of cellulase, batch PPC50472.

All studies were carried out in accordance with current OECD guidelines and in compliance with the OECD principles of Good Laboratory Practice (GLP). The studies were performed at Envigo (UK) and Covance (UK) during the period December 2017 to May 2018.

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

- Cellulase, batch PPC50472, was tested in a Neutral Red Uptake assay applying the BALB/c 3T3 cell line as test system and observations were in line with previous observations for cellulases.
- Cellulase, batch PPC50472, did not induce gene mutations in the Ames test, in the absence or presence of a rat liver metabolic activation system (S-9).
- In a 14-day oral toxicity study in rats Cellulase, batch PPC50472 was well tolerated and did not cause any toxicologically significant changes at any dose level.

Based on the present toxicity data it can be concluded that Cellulase, represented by batch PPC50472, exhibits no significant toxicological effects under the experimental conditions described.

2. TEST SUBSTANCE

Cellulase hydrolyzes (1,4)-beta-D-glucosidic linkages in cellulose and other beta-D-glucans and belongs to the enzyme group with Enzyme Class (E.C.) 3.2.1.4.

2.1 Characterization

The toxbatch Cellulase, batch PPC50472, was used for the conduct of all the toxicological studies. The characterization data of the toxbatch is presented in Table 1.

Table 1. Characterization data of Cellulase, batch PPC50472

Batch number	PPC50472
Activity	1060 CNU(B)/g
N-Total (% w/w)	1.29
Water (KF) (% w/w)	86.8
Dry matter (% w/w)	13.2
Ash (% w/w)	0.7
Total Organic Solids (TOS[1]) (% w/w)	12.5
Specific gravity (g/mL)	1.051

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

3. MUTAGENICITY

3.1 Bacterial Reverse Mutation assay (Ames test)

Cellulase, batch PPC50472 was assayed for mutation in four histidine-requiring strains (TA98, TA100, TA1535 and TA1537) of *Salmonella typhimurium*, and one tryptophan-requiring strain (WP2 uvrA pKM101) of *Escherichia coli*. The study was carried out according to the OECD test guideline 471 (adopted in 1997) and in compliance with GLP.

A 'treat and plate' procedure was used for all treatments in this study as Cellulase, batch PPC50472 is a high molecular weight protein, which may cause artefacts through growth stimulation in a standard plate-incorporation test.

Two independent experiments were performed, with and without the inclusion of metabolic activation (S-9 mix). All Cellulase, batch PPC50472 treatments in this study were performed using formulations prepared in water for irrigation (purified water), and all concentrations are expressed in terms of Total Organic Solids (TOS) content. A stock solution of 100 mg TOS/mL was made and from this in each experiment cultures of bacteria were exposed to six doses of the test substance (16, 50, 160, 500, 1600, and 5000 µg dry matter/mL) in a buffered nutrient broth for 1 hour at 37^{o}C. After incubation the tester strains bacteria were collected by centrifugation to remove the treatment mixture. The bacteria pellets were resuspended, prior to mixing with molten top agar and poured onto the surface of agar plate.

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

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

The results showed that Cellulase, batch PPC50472 did not induce mutation in four histidine-requiring strains (TA98, TA100, TA1535 and A1537) of *Salmonella typhimurium*, and one tryptophan-requiring strain (WP2 uvrA pKM101) of *Escherichia coli* when tested under the conditions of this study. These conditions included treatments at concentrations up to 5000 µg TOS/mL (equivalent to the maximum recommended concentration according to current regulatory guidelines) in the absence and in the presence of a rat liver metabolic activation system (S-9) using a modified Treat and Plate methodology.

It was concluded that cellulase, batch PPC50472, did not induce gene mutations in bacteria either in the presence or absence of metabolic activation when tested under the conditions employed in this study.

4. GENERAL TOXICITY

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

The aim of this study was to evaluate the cytotoxicity of cellulase, batch PPC50472, using a Neutral Red Uptake (NRU) assay in 3T3 cells.

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

The relative toxicity for Cellulase, batch PPC50472 did not fall below 79% of the vehicle control at any concentration and was 79% at the highest concentration, 30 mg/mL, indicating that Cellulase, batch PPC50472 had shown no evidence of causing cytotoxicity. Visual assessment of the cell monolayers indicated that the cells were approximately 70% confluent for all concentrations of Cellulase, batch PPC50472 and the vehicle control.

The IC50 value of the positive control, sodium lauryl sulphate, was calculated to be 82.28 µg/mL which lay within the historical control range of this laboratory.

It was concluded that Cellulase, batch PPC50472 demonstrated no evidence of causing cytotoxicity in this assay.

4.2 Toxicity Study by Oral Gavage Administration to Han Wistar Rats for 2 Weeks

Three groups, each comprising five males and five females received doses of 10, 33 or 100 % of Cellulase, batch PPC50472 (equivalent to 131, 434 and 1314 mg TOS/kg bw/day, corresponding to 1114, 3676 or 11141 CNU(B)/kg bw/day, respectively) at a constant dose volume of 10 mL/kg bw/day. A similarly constituted control group received the vehicle (reverse osmosis water) at the same volume-dose (10 mL/kg body weight) as the treated groups.

During the study, clinical condition, body weight, food consumption, water consumption (by daily visual observation), hematology (peripheral blood), blood chemistry, organ weight, macropathology and histopathology investigations were undertaken.

General appearance and behaviour were not affected by treatment and there were no deaths during the treatment period. There was no effect of treatment on bodyweight gain or food and water consumption. There were no treatment-related hematological findings or biochemical changes in the blood plasma. Organ weights were unaffected and there were no treatment-related macroscopic or histopathological findings.

It is concluded that oral administration of Cellulase, batch PPC50472 to Han Wistar rats at doses up to 100% of Cellulase, batch PPC50472 (equivalent to 1314 mgTOS/kg bw/day or 11141 CNU(B)/kg bw/day) for two weeks was well-tolerated and did not cause any adverse response.

The no-observed-adverse-effect level (NOAEL) was therefore considered to be 100% of Cellulase, batch PPC50472 (equivalent to 1314 mgTOS/kg bw/day or 11141 CNU(B)/kg b w/day).

5. REFERENCES

5.1 Study reports

Envigo Study No.: QX13KV; Novozymes Reference No.: 20176061: Cellulase, PPC50472: 3T3 Neutral Red Uptake test. (April 2018). LUNA file: 2018-05413.

Covance Study No.: 8378783; Novozymes Reference No.: 20176059. Cellulase, batch PPC50472: Bacterial Reverse Mutation Assay using a Treat an Plate modification (AMES). (April 2018). LUNA file: 2018-05185.

Envigo Study No.: JF23WJ; Novozymes Reference No.: 20176060: Cellulase, batch PPC50472: Toxicity Study by Oral Gavage Administration to Han Wistar Rats for 2 Weeks. (May 2018). LUNA file: 2018-06237.

Viebrock, Lauren

From: Viebrock, Lauren
Sent: Friday, September 4, 2020 10:14 AM
To: JAO (Janet Oesterling)
Subject: RE: Questions regarding GRN 000891

Hi Janet,

Thank you for the information.

Best,
Lauren

From: JAO (Janet Oesterling) <JAO@novozymes.com>
Sent: Friday, August 21, 2020 3:05 PM
To: Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
Subject: Questions regarding GRN 000891

Hi Lauren,

Attached are our responses to your questions, the page 9 replacement page and the SDS page, as requested. Please let me know if you need additional information.

Have a great weekend,
Janet

Janet Oesterling
Regulatory Affairs Specialist III

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From: Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
Sent: Monday, August 10, 2020 1:35 PM
To: JAO (Janet Oesterling) <JAO@novozymes.com>
Subject: RE: Questions regarding GRN 000891

Hi Janet,

Thank you for your responses to our questions regarding GRN 891. We have a few additional questions, which are provided below:

1. Please confirm that the intended use in “fuel alcohol” production on page 9 is intended to read “alcohol” production and exclude “fuel.”
2. Please clarify that whether the samples analyzed by microfluidic chip electrophoresis are nucleic acids or proteins.
3. Please clarify whether the three enzymes are present individually or in a complex and provide a gel demonstrating this.
4. Please confirm the enzyme assay conditions used reflect the conditions of use of the enzyme preparation.

We respectfully request a response within **10 business days**. If you are unable to complete the response within that time frame, please contact me to discuss further options.

If you have questions or need further clarification, please feel free to contact me. Thank you in advance for your attention to our comments.

Regards,
Lauren

Lauren VieBrock
Regulatory Review Scientist/Microbiology Reviewer

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From: JAO (Janet Oesterling) <JAO@novozymes.com>
Sent: Friday, July 10, 2020 10:45 AM
To: Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
Subject: RE: Questions regarding GRN 000891

Hi Lauren,

Attached are Novozymes response to your questions concerning GRN 891 and two replacement pages.

Have a great weekend!

Best regards,

Janet Oesterling
Regulatory Affairs Specialist III

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From: Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
Sent: Monday, June 29, 2020 3:40 PM
To: JAO (Janet Oesterling) <JAO@novozymes.com>
Subject: Questions regarding GRN 000891

Dear Ms. Oesterling,

During our review of GRAS Notice No. 000891, we noted questions that need to be addressed and are attached to this email.

We respectfully request a response within **10 business days**. If you are unable to complete the response within that time frame, please contact me to discuss further options.

If you have questions or need further clarification, please feel free to contact me. Thank you in advance for your attention to our comments.

Regards,
Lauren