# Pullulanase from *Bacillus subtilis*

An application to amend the Australia New Zealand Food Standards Code with a pullulanase preparation produced by a genetically modified strain of *Bacillus subtilis*

[redacted]

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# Executive summary

The present application seeks to amend Schedule 18—Processing aids of the Australia New Zealand Food Standards Code (the Code) to approve a pullulanase enzyme preparation produced by Novozymes.

**Proposed change to Australia New Zealand Food Standards Code – Schedule 18—Processing aids**

Schedule 18—Processing aids is proposed to be amended to include a genetically modified strain of *Bacillus subtilis* expressing a pullulanase from *Bacillus deramificans* as permitted source for pullulanase.

The application is applied for assessment by the general procedure.

**Description of enzyme preparation**

The enzyme is a pullulanase (EC 3.2.1.41).

Pullulanase catalyses the hydrolysis of (1→6)-α-D-glucosidic linkages in pullulan, amylopectin and glycogen, and in the alpha- and beta-limit dextrins of amylopectin.

The enzyme is produced by submerged fermentation of a *Bacillus subtilis* microorganism expressing a pullulanase from *Bacillus deramificans*.

The pullulanase enzyme preparation is available as a liquid preparation complying with the JECFA recommended purity specifications for food-grade enzymes.

The producing microorganism, *Bacillus subtilis*, is absent from the commercial enzyme product.

**Use of the enzyme**

The pullulanase enzyme preparation is used as a processing aid in starch processing for glucose syrups production and other starch hydrolysates. Generally, pullulanase hydrolyses 1,6-alpha-D-glucosidic linkages in pullulan and partially hydrolysed amylopectin as well as alpha- and beta-amylase limit dextrins of amylopectin[1]. When the substrate is partially hydrolysed amylopectin, linear maltodextrins like maltotriose and maltotetraose are released.

**Benefits**

The benefits of the action of the pullulanase in starch processing for glucose syrups production and other starch hydrolysates are:

- Efficient degradation of starch increasing the substrate availability for other enzymes, thereby enabling higher yield of the substrate (dextrins) used for further processing and production of syrups.

[1] Pullulanase also hydrolyses 1,6-alpha-D-glucosidic linkages in glycogen, partially hydrolysed glycogen and glycogen limit dextrins. This is not known to have industrial food use

## Safety evaluation

The safety of the production organism and the enzyme product has been thoroughly assessed:

- The production organism has a long history of safe use as production strain for food-grade enzyme preparations and is known not to produce any toxic metabolites.
- The genetic modifications in the production organism are well-characterised and safe and the recombinant DNA is stably integrated into the production organism and unlikely to pose a safety concern.
- The enzyme preparation complies with international specifications ensuring absence of contamination by toxic substances or noxious microorganisms
- Sequence homology assessment to known allergens and toxins shows that oral intake of the pullulanase does not pose food allergenic or toxic concern.
- Two mutagenicity studies *in vitro* showed no evidence of genotoxic potential of the enzyme preparation.
- An oral feeding study in rats for 13-weeks showed that all dose levels were generally well tolerated and no evidence of toxicity.

Furthermore, the safety of the pullulanase preparation was confirmed by external expert groups, as follows:

- Denmark: The enzyme preparation was safety assessed resulting in the authorisation of the enzyme product by the Danish Veterinary and Food Administration.

## Conclusion

Based on the Novozymes safety evaluation, confirmed by the above-mentioned bodies, we respectfully request the inclusion of the pullulanase in Schedule 18—Processing aids.

# Introduction

The present application describes a pullulanase enzyme preparation produced by submerged fermentation of a *Bacillus subtilis* microorganism producing a pullulanase from *Bacillus deramificans*.

The enzyme is a pullulanase (EC 3.2.1.41). The enzyme catalyses the hydrolysis of (1→6)-α-D-glucosidic linkages in pullulan, amylopectin and glycogen, and in the alpha- and beta-limit dextrins of amylopectin.

The pullulanase enzyme preparation is intended to be used as a processing aid in the starch-processing industry for the production of syrups and other starch hydrolysates.

The following sections describe in detail the construction of the genetically modified *Bacillus subtilis* used as the production organism, the production process, the product specification, the application of the enzyme preparation and finally the safety evaluation of the product including the toxicology program, which has been carried out confirming the safety of the product for its intended use.

The documentation has been elaborated according to the Application Handbook from Food Standards Australia New Zealand as of 1 July 2019, applied as relevant for an enzyme application, *i.e.* outlining the following section:

- Section 3.1.1 – General requirements
- Section 3.3.2 – Processing aids, subsections A, C, D, E, F

**NB!** When reading this document it should be noticed that in some reports, the pullulanase enzyme preparation is described by its commercial name, Promozyme D2, or by the internal production batch code PPY27880 and PPY6454.

# Chapter 3.1, General requirements for applications

## A Executive summary

An Executive Summary is provided as a separate copy together with this application.

## B Applicant details

# C Purpose of the application

This application is submitted to provide for amendment of the Australia New Zealand Food Standards Code, Schedule 18—Processing aids to include a genetically modified strain of *Bacillus subtilis* as permitted source for a pullulanase.

# D Justification for the application

## The need for the proposed change

Schedule 18—Processing aids contains a list of permitted enzymes of microbial origin, among others pullulanases (EC 3.2.1.41) from different sources, including *Bacillus subtilis*. However, Schedule 18—Processing aids does not contain a pullulanase (EC 3.2.1.41) from *Bacillus subtilis* containing the gene for pullulanase from *Bacillus deramificans*.

*Bacillus subtilis* is an approved host and production strain for a number of enzymes in Schedule 18—Processing aids, e.g. a wide range of enzymes that can be used in starch processing such as alpha-amylase, beta-amylase, endo-1,4-beta-xylanase, maltogenic α-amylase.

## The advantages of the proposed change over the status quo

The pullulanase preparation is used as a processing aid during the manufacture of starch-based products. Pullulanase hydrolyses 1,6-alpha-D-glucosidic linkages in pullulan and partially hydrolysed amylopectin as well as alpha- and beta-amylase limit dextrins of amylopectin. When the substrate is partially hydrolysed amylopectin, linear maltodextrins like maltotriose and maltotetraose are released.

The benefits of the action of the pullulanase in starch processing for glucose syrups production and other starch hydrolysates are:

- Efficient degradation of starch increasing the substrate availability for other enzymes, thereby enabling higher yield of the substrate (dextrins) used for further processing and production of syrups.

The benefits, which are described above, are not exclusively obtainable by means of enzyme treatment but can be achieved without the use of enzymes, or with a reduced use of enzymes, through *e.g.* modified maybe more expensive or less environmentally friendly production processes or recipe changes.

## D.1 Regulatory impact information

### D.1.1 Costs and benefits of the application

The application is not likely to place costs or regulatory restrictions on industry or consumers. Inclusion of the pullulanase enzyme in Schedule 18—Processing aids will provide the food and beverage industry with the opportunity to improve the yield of substate (dextrins) used for further processing and production of syrups under environmentally friendly and cost efficient production conditions. For the government, the burden is limited to necessary activities for a variation of Schedule 18—Processing aids.

#### D.1.2 Impact on international trade

The application is not likely to cause impact on international trade.

# E Information to support the application

## E.1 Data requirements

No public health and safety issues related to the proposed change are foreseen. As outlined in sections 3.3.2 C, D, E, F, the pullulanase is produced by submerged fermentation of a genetically modified *Bacillus subtilis* strain.

The safety of the production organism and the enzyme product has been thoroughly assessed:

- The production organism has a long history of safe use as production strain for food-grade enzyme preparations and is known not to produce any toxic metabolites.
- The genetic modifications in the production organism are well-characterised and safe and the recombinant DNA is stably integrated into the production organism and unlikely to pose a safety concern.
- The enzyme preparation complies with international specifications ensuring absence of contamination by toxic substances or noxious microorganisms
- Sequence homology assessment to known allergens and toxins shows that oral intake of the pullulanase does not pose food allergenic or toxic concern.
- Two mutagenicity studies *in vitro* showed no evidence of genotoxic potential of the enzyme preparation.
- An oral feeding study in rats for 13-weeks showed that all dose levels were generally well tolerated and no evidence of toxicity.

# F Assessment procedure

Because the application is for a new source organism for an existing enzyme in the Code, it is considered appropriate that the assessment procedure is characterised as “General Procedure, Level 1”.

# G Confidential commercial information (CCI)

Detailed information on the raw materials used in production of the enzyme preparation and construction and characteristics of the genetically modified production strain are provided in **Appendix 4** and **6**, respectively. Summaries of the information are given in section A.4 and 3.3.2 E. The formal request for treatment of selected parts of **Appendix 4** and **6** as confidential commercial information (CCI) is included as **Appendix 1.1**.

# H Other confidential information

Apart from the selected parts of **Appendix 4** and **6** identified as confidential commercial information (CCI), no other information is requested to be treated as confidential.

# I Exclusive capturable commercial benefit (ECCB)

This application is not expected to confer an Exclusive Capturable Commercial Benefit.

# J International and other national standards

## J.1 International Standards

Use of enzymes as processing aids for food production is not restricted by any Codex Alimentarius Commission (Codex) Standards.

## J.2 Other national standards or regulations

With few exceptions on national, commodity standards, use of enzymes as processing aids for food production is in general not restricted by standards or regulations in other countries.

# K Statutory declaration

The Statutory Declaration is provided as a separate document together with this submission.

# L Checklist

This application concerns an enzyme product intended to be used as a processing aid. Therefore, the relevant documentation according to the Application Handbook from Food Standards Australia New Zealand as of 1 July 2019, are the following sections:

- Section 3.1.1 – General requirements
- Section 3.3.2 – Processing aids, subsections A, C, D, E, F

Accordingly, the checklist for General requirements as well as the Processing aids part of the checklist for applications for substances added to food was used and is included as **Appendix 1.2** and **1.3**.

# Chapter 3.3, Guidelines for applications for substances added to food

## 3.3.2 Processing Aids

The pullulanase enzyme preparation described in this application is representative of the commercial food enzyme product for which approval is sought.

# A Technical information on the processing aid

## A.1 Information on the type of processing aid

The pullulanase enzyme preparation belongs to the category of processing aids described in Schedule 18—Processing aids.

The pullulanase enzyme preparation is to be used in the food industry as a processing aid during the processing of raw materials containing starch.

The pullulanase enzyme preparation is used in the following food manufacturing processes:

- starch processing for glucose syrups production and other starch hydrolysates

The highest dosage of the pullulanase during a food manufacturing process is 5,000 NPUN per kg starch (or starch-derived) dry matter.

## A.2 Information on the identity of the processing aid

### A.2.1 Enzyme

| | |
|---|---|
| Generic name | pullulanase |
| IUBMC nomenclature | pullulanase |
| IUBMC No. | EC 3.2.1.41 |
| Cas No. | 9075-68-7 |

### A.2.2 Enzyme preparation

The enzyme concentrate is formulated into a final enzyme preparation. The enzyme concentrate may be intended for a single enzyme preparation or a blend with other food enzymes and formulated as a liquid product depending on the characteristics of the intended food process in which it will be used.

The typical composition of the enzyme concentrate is:

| | |
|---|---|
| Enzyme solids (TOS)[2] | approx. 1.8 % |
| Sucrose/Glucose, D- | Approx. 40.0% |
| Sodium benzoate | approx. 0.4 % |
| Potassium sorbate | approx. 0.1 % |
| Water | approx. 57.7 % |

The enzyme concentrate is standardised in pullulanase units to an activity of 1350 NPUN/g. The Novozymes method used to determine the NPUN activity is enclosed in **Appendix 3.2**.

### A.2.3 Host organism

The production strain was developed from the *Bacillus subtilis* A164 cell lineage, which was obtained from The American Type Culture Collection (ATCC). The A164 cell lineage has a long history of safe use at Novozymes for production of food enzymes and has given rise to a number of food enzyme production strains, which are used for production of previously evaluated and regulatory approved food enzymes. The taxonomic classification of the strain is:

| | |
|---|---|
| Phylum: | Firmicutes |
| Class: | Bacilli |
| Order: | Bacillales |
| Family: | Bacillaceae |
| Genus: | *Bacillus* |
| Species: | *Bacillus subtilis* |

For a more detailed description of the host organism and the genetic modifications, please see section 3.3.2 E.

[2] TOS = Total Organic Solids, defined as: 100% - water - ash - diluents

### A.2.4 Donor organism

The donor for the pullulanase gene is *Bacillus deramificans*.

| | |
|---|---|
| Phylum: | Firmicutes |
| Class: | Bacilli |
| Order: | Bacillales |
| Family: | Bacillaceae |
| Genus: | *Bacillus* |
| Species: | *Bacillus deramificans* |

For a more detailed description of the donor and the donor gene, please see section 3.3.2 E and **Appendix 6**.

## A.3 Information on the chemical and physical properties of the processing aid

The enzyme is a pullulanase (EC 3.2.1.41). Pullulanase catalyses the hydrolysis of (1→6)-α-D-glucosidic linkages in pullulan, amylopectin and glycogen, and in the alpha- and beta-limit dextrins of amylopectin and glycogen.

The enzyme preparation is available as liquid product. The appearance and storage stability of the enzyme product are given in **Appendix 2.1**.

The pullulanase is active at temperatures up to approximately 60 °C with an optimum around 50 °C at pH 5.5, and within a pH range of 4 to 7 with an optimum of pH 6 at 30 °C. The temperature stability of the pullulanase was determined at pH 5.5 and 30 minutes incubation showing that the enzyme retained full activity up to 30 °C, where after the activity rapidly declines with no activity remaining at 70 °C and above. The food processes, in which the enzyme is applied, include processing steps where it is denatured at high temperatures.

The food enzyme object of the present application is not added to final foodstuffs but used as a processing aid during food manufacturing. The enzyme exerts no function in the final food. In the final food the enzyme protein is either denatured by high temperature, which means that it does not have any action or any function, and is thus, like any other protein, inert, and/or removed in certain processing steps (e.g. filtration, ion exchange).

No reaction products, which could not be considered normal constituents of the diet, are formed during the production or storage of the enzyme-treated food.

## A.4 Manufacturing process

The manufacturing process comprises a fermentation process, a purification process, a formulation process and finally a quality control of the finished product, as outlined by Aunstrup (1979). This section describes the processes used in manufacturing of the pullulanase enzyme product.

The enzyme preparation is manufactured in accordance with current Good Manufacturing Practices (**Appendix 4.1**). The quality management system used in the manufacturing process complies with ISO 9001:2015 (**Appendix 4.2**).

The raw materials are of food-grade quality and have been subjected to appropriate analysis to ensure their conformity with the specifications.

### A.4.1 Fermentation

The pullulanase is produced by submerged fed-batch pure culture fermentation of the genetically modified strain of *Bacillus subtilis*, described in section 3.3.2 E.

#### A.4.1.1 Raw materials for fermentation

The production strain is grown in a medium consisting of compounds providing an adequate supply of carbon and nitrogen as well as minerals and vitamins necessary for growth. Furthermore, acids and bases for the adjustment of the pH and processing aids (*e.g.* antifoaming agents) are used during fermentation. The choice of raw materials used in the fermentation process (the feed, the seed fermenter, the main fermenter and dosing) is given in the confidential parts of **Appendix 4.3**.

#### A.4.1.2 Hygienic precautions

All equipment is designed and constructed to prevent contamination by foreign microorganisms.

All valves and connections not in use for the fermentation are sealed by steam at more than 120 °C.

After sterilization a positive pressure of more than 0.2 atmosphere is maintained in the fermentation tank.

The air used for aeration is sterilised by passing through a sterile filter. The inside of each fermentation tank is cleaned between fermentations by means of a high-pressure water jet and inspected after the cleaning procedures have been completed.

#### A.4.1.3 Preparation of the inoculum

The inoculum flask containing the prepared medium is autoclaved and checked. Only approved flasks are used for inoculation.

The stock culture suspension is injected aseptically into the inoculum flask and spread onto the medium in the flask. Once growth has taken place in the inoculum flask (typically after a few days at 30°C), the following operations are performed:

- Strain identity and traceability: ampoule number is registered
- Microbial purity: a sample from the inoculum flask is controlled microscopically for absence of microbial contaminants.

When sufficient amount of biomass is obtained and when the microbiological analyses are approved, the inoculum flask can be used for inoculating the seed fermenter.

#### A.4.1.4 The seed fermentation

The raw materials for the fermentation medium are mixed with water in a mixing tank. The medium is transferred to the seed fermenter and heat sterilised (*e.g.* 120 °C/60 min).

The seed fermentation tank is inoculated by transferring aseptically a suspension of cells from the inoculum flask.

The seed fermentation is run aerobically (sterile airflow), under agitation. The overpressure is kept above 0.2 atmosphere at all times, to prevent contamination.

Once a sufficient amount of biomass has developed, microbiological analyses are performed to ensure absence of contamination. The seed fermentation can then be transferred to the main fermentation tank.

#### A.4.1.5 The main fermentation

The raw materials for the medium are mixed with water in a mixing tank. The medium is transferred to the main fermenter and heat sterilised (*e.g.* 120 °C/60 min). If necessary, the pH is adjusted after sterilization, with sterile pH adjustment solutions.

The fermentation in the main tank is run as normal submerged fed-batch fermentation.

The main fermentation is run aerobically (sterile airflow), under vigorous agitation. The overpressure is kept above 0.2 atmosphere at all times, to prevent contamination. The fermentation is run at a well-defined temperature.

Fresh medium is added aseptically when the pH increases above its set point, and the dissolved oxygen concentration rises. The feed rate is adjusted so that there is no accumulation of carbohydrates.

Other parameters are measured at regular intervals

- refractive index
- enzyme productivity
- residual glucose
- residual ammonia

Samples are also taken at regular intervals to check absence of microbial contamination.

### A.4.2 Recovery

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

The steps of this process involve a series of typical unit operations:

- pre-treatment
- primary separation
- filtration
- concentration
- evaporation

- preservation and stabilization

#### A.4.2.1 Raw materials for recovery

The choice of raw materials used during recovery is given in the confidential parts of **Appendix 4.3**.

#### A.4.2.2 Pre-treatment

To facilitate the separation, flocculants are used in a pH-controlled process.

#### A.4.2.3 Primary separation

The cell mass and other solids are separated from the broth by well-established techniques such as pre-coat vacuum drum filtration or centrifugation.

The primary separation is performed at well-defined pH and temperature range.

#### A.4.2.4 Filtration

For removal of residual cells of the production strain and as a general precaution against microbial degradation, filtration on dedicated germ filtration media is applied. Pre-filtration is included when needed.

The filtrations are performed at well-defined pH and temperature intervals, and result in an enzyme concentrate solution free of the production strain and insoluble substrate components from the fermentation.

#### A.4.2.5 Concentration

Ultrafiltration and/or evaporation are applied for concentration and further purification. The ultrafiltration is applied to fractionate high molecular weight components (enzymes) from low molecular weight components and is used to increase the activity/dry matter ratio. Evaporation is used to increase the activity while maintaining the activity/dry matter ratio.

The pH and temperature are controlled during the concentration step, which is performed until the desired activity and activity/dry matter ratio has been obtained.

#### A.4.2.6 Evaporation

Evaporation is performed to remove water and increase the refractive index. The concentration is run at 0-45 °C and the refractive index is controlled during the concentration step to ensure that the dry matter content is within a given range.

#### A.4.2.7 Preservation and stabilization

For enzymatic, physical and microbial stabilization polyols as well as potassium sorbate and sodium benzoate are added to the enzyme concentrate.

#### A.4.2.8 Process control

Apart from the process controls performed during the various fermentation steps and described above, the following microbial controls are also performed.

Samples are withdrawn from both the seed fermenter and the main fermenter:

- before inoculation
- at regular interval during cultivation
- before transfer/harvest

The samples during all steps are examined by:

- microscopy
- plating culture broth on a nutrient agar and incubating for 24-48 hours

Growth characteristics are observed macroscopically and microscopically.

During the microbiological control steps, the number of foreign microorganisms should be insignificant. The fermentation parameters, *i.e.* enzyme activity, temperature and oxygen as well as pH are also monitored closely. A deviation from the normal course of the fermentation may signal a contamination.

If a significant contamination develops, the fermentation is terminated. The fermentation is regarded as "significantly contaminated" if two independent samples show presence of contaminating organisms after growth on nutrient agar.

Any contaminated fermentation is rejected for enzyme preparations to be used in a food-grade application.

## A.5 Specification for identity and purity

The pullulanase enzyme product complies with the purity criteria recommended for Enzyme Preparations in Food, Food Chemicals Codex, 11th edition, 2018.

In addition to this, the pullulanase enzyme product 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.

Analytical data for three representative batches of the pullulanase enzyme preparation are shown in (**Table 1**). These data show compliance with the purity criteria of the specification.

**Table 1.** Analytical data for three representative enzyme product batches

| Control parameter | Unit | Specification | Batch 1 | Batch 2 | Batch 3 |
|---|---|---|---|---|---|
| pullulanase activity | NPUN/g | | 9140 | 8050 | 5240 |
| Lead | mg/kg | ≤ 2 | ND (LOD < 0.5) | ND (LOD < 0.5) | ND (LOD < 0.5) |
| Arsenic | mg/kg | ≤ 1 | 0.13 (LOD < 0.1) | ND (LOD < 0.1) | ND (LOD < 0.1) |
| Cadmium | mg/kg | ≤ 1 | ND (LOD < 0.05) | ND (LOD < 0.05) | ND (LOD < 0.05) |
| Mercury | mg/kg | ≤ 1 | ND (LOD < 0.03) | ND (LOD < 0.03) | ND (LOD < 0.03) |
| Total viable count | CFU/g | | 100 | <100 | <100 |
| Total coliforms | CFU/g | ≤ 30 | <10 | <10 | <10 |
| Enteropathogenic *Escherichia coli* | CFU/25 g | ND | ND | ND | ND |

| Control parameter | Unit | Specification | Batch 1 | Batch 2 | Batch 3 |
|---|---|---|---|---|---|
| *Salmonella* spp. | CFU/25 g | ND | ND | ND | ND |
| Antimicrobial activity | — | ND | ND | ND | ND |
| Production strain | CFU/g | ND | ND | ND | ND |

ND: not detected; LOD: limit of detection ; CFU: colony forming unit

A certificate of analysis of the presented data is attached as **Appendix 3.1**. The methods of analysis used to determine compliance with the specifications are enclosed (**Appendix 3**).

The pullulanase enzyme preparation is available as a liquid enzyme concentrate. The concentrate is standardised in pullulanase units (NPUN/g; **Appendix 3.2**). The preparation does not contain known food allergens (**Appendix 2.1** and **Appendix 4.3**).

### A.6 Analytical method for detection

The pullulanase enzyme preparation is to be used in the food industry as a processing aid. This information is not required in the case of an enzymatic processing aid.

## B Information related to the safety of a chemical processing aid

Not applicable – this application does not concern a chemical processing aid.

## C Information related to the safety of an enzyme processing aid

### C.1 General information on the use of the enzyme as a food processing aid in other countries

The enzyme is used as processing aid during processing of starch-containing raw materials in a range of countries, where there are no restrictions of the use of enzyme processing aids or where the enzyme is covered by a country positive list or specific approval.

The safety of the pullulanase preparation has been evaluated and confirmed by external expert groups, as follows:

- Denmark: The enzyme preparation was safety assessed resulting in the authorisation of the enzyme product by the Danish Veterinary and Food Administration.

### C.2 Information on the potential toxicity of the enzyme processing aid

#### (a) Information on the enzyme's prior history of human consumption and/or its similarity to proteins with a history of safe human consumption

A wide variety of enzymes are used in food processing. Enzymes, including pullulanase, have a long history of use in food (Pariza and Johnson, 2001).

In principle, pullulanases can be used in the processing of all food raw materials which naturally contain the substrate. Pullulanases have been used extensively in various industrial food

applications such as starch processing and distilling for more than 20 years, and have been used in brewing for at least five years (Lalor et al, 2010).

*Bacillus subtilis* species have been used for centuries for the production of natto by solid-state fermentation of soybeans (OECD, 1986; de Boer *et al*, 1991). Industrial strains belonging to the *Bacillus subtilis* species have been used for decades in the production of enzymes, and for more than a decade as recombinant organisms for the production of a variety of bio-industrial products like food grade enzymes, vitamins, antibiotics, and additives (Schallmey *et al*, 2004).

*Bacillus subtilis* is a class 1 organism and is generally considered to be non-pathogenic and non-toxigenic.

**(b) Information on any significant similarity between the amino acid sequence of the enzyme and that of known protein toxins**

A sequence homology assessment of the pullulanase enzyme to known toxins was conducted. The amino acid sequence of the pullulanase provided in **Appendix 6.4** was used as input for the search. No homologies to known toxins were found. The complete search report is enclosed in **Appendix 5.1**.

Furthermore, safety studies as described below were performed on two representative batches (PPY27880 and PPY6454) that were produced according to the description given in section 3.3.2 A.4, omitting stabilization and standardization. A summary of the safety studies is enclosed in **Appendix 5.2**.

The following studies were performed:

- Ames Test. Test for mutagenic activity (**Appendix 5.3**)
- *In vitro* micronuclei test (**Appendix 5.4**)
- Subchronic (13 week) oral toxicity study in rats (**Appendix 5.5**)

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

- Pullulanase PPY27880 did not induce gene mutations in bacteria either in the presence or absence of metabolic activation (S-9) when tested under the conditions employed in this study.
- Pullulanase PPY27880 did not induce micronuclei in cultured human peripheral blood lymphocytes following treatment in the presence or absence of an aroclor induced rat liver metabolic activation system (S-9).
- Oral administration of Pullulanase PPY6454 to Sprague-Dawley rats at doses up to 100 % of the tox test batch (1285 mg TOS/kg bw/day for 13 weeks was well-tolerated and did not cause any adverse change. The NOAEL was considered to be 100 % of the tox test batch (equivalent to 1285 mg TOS/kg bw/day).

Based on the presented toxicity data it can be concluded that the pullulanase enzyme preparation, represented by batches PPY27880 and PPY6454, exhibits no toxicological effects under the experimental conditions described.

## C.3 Information on the potential allergenicity of the enzyme processing aid

### (a) Information of the source of the enzyme processing aid

The pullulanase enzyme is produced by a *Bacillus subtilis* microorganism expressing the pullulanase from *Bacillus deramificans*. *Bacillus subtilis* is ubiquitous in the environment and in general considered as a non-pathogenic fungus (see Section 3.3.2 D).

### (b) Analysis of similarity between the amino acid sequence of the enzyme and that of known allergens

Enzymes have a long history of safe use in food, with no indication of adverse effects or reactions. Moreover a wide variety of enzyme classes (and structures) are naturally present in food.

The allergenicity potential of enzymes was studied by Bindslev-Jensen et al. (2006) and reported in the publication: “Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry”. The investigation comprised enzymes produced by wild-type and genetically modified strains as well as wild-type enzymes and protein engineered variants and comprised 400 patients with a diagnosed allergy to inhalation allergens, food allergens, bee or wasp. It was concluded from this study that ingestion of food enzymes in general is not likely to be a concern with regard to food allergy.

Additionally, food enzymes are used in small amounts during food processing resulting in very small amounts of the enzyme protein in the final food. A high concentration generally equals a higher risk of sensitization, whereas a low level in the final food equals a lower risk (Goodman et al., 2008).

A sequence homology assessment of the pullulanase enzyme to known allergens was conducted (**Appendix 5.1**). The amino acid sequence of the pullulanase provided in **Appendix 6.4** was used as input for the search. The pullulanase was compared to allergens from the FARRP allergen protein database (http://www.allergenonline.org).

No matches with more than 35% identity in the amino acid sequence of the expressed protein were found in the database mentioned above. Thus no significant homology was found between the pullulanase and known allergens listed in the databases and consequently, oral intake of the food enzyme is not anticipated to pose any food allergenic concern.

## C.4 Safety assessment reports prepared by international agencies or other national government agencies, if available

Documentation of approval of the pullulanase in Denmark is enclosed in **Appendix 2**.

# D Additional information related to the safety of an enzyme processing aid derived from a microorganism

## D.1 Information on the source microorganism

The pullulanase enzyme is produced by an *Bacillus subtilis* microorganism expressing the pullulanase from *Bacillus deramificans*. The *Bacillus subtilis* A164 host strain was obtained from The American Type Culture Collection (ATCC). The A164 cell lineage has a long history of safe use at Novozymes for production of food enzymes and has given rise to a number of food enzyme production strains, which are used for production of previously evaluated and regulatory approved food enzymes.

The pullulanase production strain is a non-pathogenic, non-toxigenic, genetically modified *Bacillus subtilis* strain. The production strain is marker-free, and it does not produce secondary metabolites of toxicological concern to humans as explained in Section E 1.3, Section A.5 and **Appendix 6.1**.

## D.2 Information on the pathogenicity and toxicity of the source microorganism

*Bacillus subtilis* species have been used for centuries for the production of natto by solid-state fermentation of soybeans (OECD, 1986; de Boer et al, 1991). Industrial strains belonging to the *Bacillus subtilis* species have been used for decades in the production of enzymes, and for more than a decade as recombinant organisms for the production of a variety of bio-industrial products like food grade enzymes, vitamins, antibiotics, and additives (Schallmey et al, 2004).

*Bacillus subtilis* is a class 1 organism and is generally considered to be non-pathogenic and non-toxigenic.

JECFA has evaluated alpha-amylase, mixed carbohydrase and protease from *Bacillus subtilis*, as well as a range of food enzymes derived from genetically modified strains of *Bacillus subtilis* and concluded that these food enzymes do not constitute a toxicological hazard[3].

Carbohydrases and proteases from *Bacillus subtilis* are affirmed as GRAS by the US FDA and are covered in the regulations under 21 CFR 184.1148 and 21 CFR 184.1150. *Bacillus subtilis* is described as the production organism for different enzymes in GRAS notifications 20, 114, 205, 274, 406, 476 and 751.

Because *Bacillus subtilis* meets the US Environmental Protection Agency (EPA) criteria for non-toxigenicity and non-pathogenicity it is one of ten host organisms eligible for Tier I exemption under the EPA regulations (EPA, 1996).

The non-pathogenicity and non-toxigenicity of *Bacillus subtilis* is thus strongly supported by the historic record of this organism.

[3] JECFA Online Edition: "Combined Compendium of Food Additive Specifications", https://www.fao.org/3/a0691e/a0691e.pdf

## D.3 Information on the genetic stability of the source organism

The inserted recombinant DNA is genetically stable during fermentation, as the inserted DNA is integrated into the chromosome.

Stability of the introduced DNA sequences was analysed using phenotypic characteristics of the production strain, *i.e.* enzyme activity and protein synthesis (**Appendix 6.5**).

For a more detailed description of the strain construction and characteristics, please see section 3.3.2 E.

# E Additional information related to the safety of an enzyme processing aid derived from a genetically-modified microorganism

## E.1 Information on the methods used in the genetic modification of the source organism

This section contains summarised information on the modifications of the host strain, on the content and nature of the introduced DNA and on the construction of the final production strain, as well as the stability of the inserted gene. The detailed information is provided in the confidential **Appendix 6**.

### E.1.1 Host organism

The production strain was developed from the *Bacillus subtilis* A164 cell lineage, which was obtained from The American Type Culture Collection (ATCC). The A164 cell lineage has a long history of safe use at Novozymes for production of food enzymes and has given rise to a number of food enzyme production strains, which are used for production of previously evaluated and regulatory approved food enzymes. The taxonomic classification of the strain is:

| | |
|---|---|
| Phylum: | Firmicutes |
| Class: | Bacilli |
| Order: | Bacillales |
| Family: | Bacillaceae |
| Genus: | *Bacillus* |
| Species: | *Bacillus subtilis* |

The recipient strain used in the construction of the *Bacillus subtilis* production strain, was derived from the parental strain through a combination of classical mutagenesis/selection and GM-steps. These steps were carried out in order to simplify purification, enhance product stability and increase the safety of the strain.

### E.1.2 Introduced DNA

The vectors used to transform the *Bacillus subtilis* recipient strain are based on *Escherichia coli* standard vectors (Heusterspreute and Davison, 1984; Vieira and Messing, 1987). No elements of the vectors are left in the production strain. One vector contains the pullulanase expression cassette consisting of a hybrid *Bacillus* promoter, the coding sequence for pullulanase from *Bacillus deramificans* including its ribosome-binding site and terminator. Furthermore, the other vector was used to remove a marker gene.

### E.1.3 Construction of the recombinant microorganism

The *Bacillus subtilis* production strain was constructed from the recipient strain through the following steps:

1. The pullulanase expression cassette was integrated at a specific integration site present in the recipient strain.
2. A marker gene, used during integration of the pullulanase expression cassette, was removed by double homologous recombination.
3. A transformant was screened for rapid growth and high pullulanase activity leading to the final production strain.

### E.1.4 Antibiotic resistance gene

No functional antibiotic resistance genes were left in the strain as a result of the genetic modifications as shown by genome sequence analysis.

### E.1.5 Stability of the introduced genetic sequences

The transforming DNA is stably integrated into the *Bacillus subtilis* chromosome and, as such, is poorly mobilised for genetic transfer to other organisms and is mitotically stable. Stability of the introduced DNA sequence was analysed using phenotypic characteristics of the production strain, *i.e.* enzyme activity and protein synthesis. Further details can be found in **Appendix 6.5**.

# F Information related to the dietary exposure to the processing aid

## F.1 A list of foods or food groups likely to contain the processing aid or its metabolites

The pullulanase preparation is used as a processing aid during the manufacture of starch-based products. Pullulanases hydrolyse 1,6-alpha-D-glucosidic linkages in pullulan and partially hydrolysed amylopectin as well as alpha- and beta-amylase limit dextrins of amylopectin. When pullulanases hydrolyse amylopectin, which has been prehydrolysed by an alpha-amylase, linear maltodextrins like maltotriose and maltotetraose are released.

## F.2 The levels of residues of the processing aid or its metabolites for each food or food group

The pullulanase enzyme preparation is used in the following food manufacturing processes:

- starch processing for glucose syrups production and other starch hydrolysates

**Use level**

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

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

The highest dosage given for solid food is 5000 NPUN per kg starch (or starch-derived) dry matter. This corresponds to 3.7 g of pullulanase enzyme preparation per kg starch (or starch-derived) dry matter equivalent to 67 mg TOS per kg starch (or starch-derived) dry matter.

The highest dosage given for liquids is 5000 NPUN per kg starch (or starch-derived) dry matter. This corresponds to 3.7 g of pullulanase enzyme preparation per kg starch (or starch-derived) dry matter equivalent to 67 mg TOS per kg starch (or starch-derived) dry matter.

**Enzyme residues in the final food**

The pullulanase enzyme preparation is used in the hydrolysis of starch polysaccharides during processing of starch-containing food. The enzyme will be denatured and removed by purification steps (e.g. by heating, during filtration, carbon treatment and ion exchange), which removes all protein, including enzyme protein.

**F.2.1 Estimates of human consumption**

Method used for the dietary exposure assessment

An exposure assessment according to the Budget Method (Douglass et al., 1997; Hansen, 1966; ILSI, 1997) has been performed, as the processed starch is used as an ingredient in a variety of food products and beverages.

*Budget Method*

Overall, the human exposure to the pullulanase will be negligible because the enzyme preparation is used as a processing aid and in low dosages.

The Budget Method assumptions represent a “maximum worst case” situation of human consumption, in which the food enzyme object of the present application would be used at its maximum recommended dosages in all processed food and all processed beverages and not only in those food and drink processes described in Section F.2.

It is also supposed that the totality of the food enzyme will end up in the final food. This assumption is exaggerated since the enzyme protein and the other substances resulting from the fermentation are diluted or removed in certain processing steps.

Therefore the safety margin calculation derived from this method is highly conservative.

*Assumptions in the Budget Method*

Solids 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 %[4] starch (or starch-derived) dry matter = 3.12 g starch (or 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 12 %[4] starch (or starch-derived) dry matter = 3.0 g starch derived dry matter per kg body weight per day.

It is assumed that the densities of the beverages are ~ 1.

TMDI (Total amount of dietary intake) calculation

*Solid food*

The highest dosage given for solid food is 5000 NPUN per kg starch (or starch-derived) dry matter, corresponding to 5000 / 1350 x 1.8 x 1000 / 100 = 67 mg TOS per kg starch (or starch-derived) dry matter (cf. Section 3.3.2 A.2.2).

Based on this, 3.12 g starch (or starch-derived) dry matter in solid food will maximally contain:

67 mg TOS per kg / 1000 g per kg x 3.12 g = 0.21 mg TOS

*Liquids*

The highest dosage given for liquids is 5000 NPUN per kg starch (or starch-derived) dry matter, corresponding to 5000 / 1350 x 1.8 x 1000 / 100 = 67 mg TOS per kg starch (or starch-derived) dry matter (cf. Section 3.3.2 A.2.2).

Based on this, 3.0 g starch (or starch-derived) dry matter in liquids will maximally contain:

67 mg TOS per kg / 1000 g per kg x 3.0 g = 0.20 mg TOS

*Total TMDI of starch (or starch-derived) solid foods and liquids*

0.21 mg TOS + 0.20 mg TOS = 0.41 mg TOS

[4] This assumption was explained in an answer to application A1248.

### F.2.2. Safety Margin Calculation

The safety margin is calculated as dose level with no adverse effect (NOAEL) divided by the estimated human consumption (TMDI). The NOAEL dose level in the 13 weeks oral toxicity study in rats was concluded to be 1285 mg TOS/kg bw/day (cf. Section 3.3.2 C 2).

The estimated human consumption is 0.41 mg TOS/kg/day

The safety margin can thus be calculated to be 1285/0.41 ≈ 3134.

## F.3 For foods or food groups not currently listed in the most recent Australian or New Zealand National Nutrition Surveys (NNSs), information on the likely level of consumption

Not relevant.

## F.4 The percentage of the food group in which the processing aid is likely to be found or the percentage of the market likely to use the processing aid

It is assumed that all raw materials containing starch are processed using the pullulanase object of this submission as a processing aid at the highest recommended dosage.

## F.5 Information relating to the levels of residues in foods in other countries

As described in F.2.1 above, a “worst case” calculation is made assuming that all organic matter originating from the enzyme is retained in the processed food product. The dietary exposure is estimated using the Budget Method, as the processed starch is used as an ingredient in a variety of food products.

## F.6 For foods where consumption has changed in recent years, information on likely current food consumption

No significant changes in recent years are observed.

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# List of appendices

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