Beta-Amylase from *Bacillus licheniformis*

An application to amend the *Australia New Zealand Food Standards Code* with a beta-amylase preparation produced by a genetically modified strain of *Bacillus licheniformis*

Novozymes A/S

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[redacted]

Regulatory Affairs

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 beta-amylase enzyme preparation produced by Novozymes A/S.

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 licheniformis* expressing a beta-amylase from *Bacillus flexus* as permitted source for beta-amylase.

The application is applied for assessment by the general procedure.

Description of enzyme preparation

The enzyme is a beta-amylase (EC 3.2.1.2).

Beta-amylases catalyse the hydrolysis of (1→4)-α-D-glucosidic linkages in polysaccharides so as to remove successive maltose units from the non-reducing ends of the chains.

The enzyme is produced by submerged fermentation of an *Bacillus licheniformis* microorganism expressing a beta-amylase from *Bacillus flexus*.

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

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

Use of the enzyme

The beta-amylase preparation is used as a processing aid in starch processes to produce syrups where it degrades starch into maltose.

Benefits

The benefits of the action of the beta-amylase in starch processing are:

- More consistent and efficient production of maltose syrups from starch compared to the use of enzymes present in malt

- Consistent maltose content in the syrup product
- Reduced risk of contamination because the enzyme can be used at high operating temperature
- Stable process allowing for variations in pH and temperature

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 beta-amylase 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 beta-amylase 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.
- France: The enzyme is included in the French positive list for processing aids, including food enzymes (The French order of October 19, 2006 on use of processing aids in the manufacture of certain foodstuff), as amended.
- Brazil: The enzyme was evaluated, approved and included in the Brazilian positive list – RDC 26/2009.

- Mexico: Based on a dossier submitted by Novozymes, the Mexican food authorities, COFEPRIS, have approved the enzyme.

Conclusion

Based on the Novozymes A/S safety evaluation (confirmed by the above-mentioned bodies), we respectfully request the inclusion of the beta-amylase in Schedule 18—Processing aids.

Contents

INTRODUCTION

The present dossier describes a beta-amylase enzyme preparation produced by submerged fermentation of a *Bacillus licheniformis* microorganism producing a beta-amylase from *Bacillus flexus*.

The enzyme is a beta-amylase (EC 3.2.1.2). The enzyme catalyses the hydrolysis of (1→4)-α-D-glucosidic linkages in polysaccharides so as to remove successive maltose units from the non-reducing ends of the chains.

The beta-amylase enzyme preparation is intended to be used as a processing aid in the starch-processing industry to hydrolyse starch for the production of syrups.

The following sections describe in detail the construction of the genetically modified *Bacillus licheniformis* 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 beta-amylase enzyme preparation is described by its commercial name, Secura®, or by the internal production batch code PPY36295.

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

[redacted]

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 licheniformis* as permitted source for a beta-amylase.

D Justification for the application

The need for the proposed change

Schedule 18—Processing aids contains a list of permitted enzymes of microbial origin. There are a number of approved beta-amylases (EC 3.2.1.2) from different sources, including *Bacillus* spp. However, Schedule 18—Processing aids does not contain a beta-amylase (EC 3.2.1.2) from *Bacillus licheniformis* containing the gene for beta-amylase from *Bacillus flexus*.

Bacillus licheniformis 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 carbohydrate processing such as alpha-amylase, endo-1,4-beta-xylanase, beta-galactosidase, maltotetraohydrolase and pullulanase.

The advantages of the proposed change over the status quo

The beta-amylase preparation is used as a processing aid during the manufacture of starch-based products. Beta-amylases convert starch by removing maltose units from the non-reducing end of the substrate molecule for further processing.

The benefits of the action of the beta-amylase in starch processing are:

- More consistent and efficient production of maltose syrups from starch compared to the use of enzymes present in malt
- Consistent maltose content in the syrup product
- Reduced risk of contamination because the enzyme can be used at high operating temperature
- Stable process allowing for variations in pH and temperature

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.

As a response to international customer interests, registration activities have been done globally, e.g. the beta-amylase enzyme preparation has been approved in Denmark, France, Brazil and Mexico for the described applications.

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 beta-amylase enzyme in Schedule 18—Processing aids will provide the food and beverage industry with the opportunity to improve the yield of fermentable sugars for starch processing and distilling 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 beta-amylase is produced by submerged fermentation of a genetically modified *Bacillus licheniformis* 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 beta-amylase 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 4.3 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 4.3 and 6 as confidential commercial information (CCI) is included as Appendix 1.1.

H Other confidential information

Apart from the selected parts of Appendix 4.3 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 beta-amylase 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 beta-amylase enzyme preparation belongs to the category of processing aids described in Schedule 18—Processing aids.

The beta-amylase enzyme preparation is to be used in the food industry as a processing aid during the processing of raw materials containing starch. Beta-amylase converts starch to maltose.

The beta-amylase enzyme preparation is used in, but not limited to, the following food manufacturing processes:

- Starch processing to produce syrups during which the beta-amylase degrades polysaccharides into maltose. The use of the enzyme leads to a higher maltose yield.

The highest dosage of the beta-amylase during a food manufacturing process is in starch processes, where dosages up to 10,000 BAMU per kg starch dry matter are used.

A.2 Information on the identity of the processing aid

A.2.1 Enzyme

Generic name	beta-amylase
IUBMC nomenclature	beta-amylase
IUBMC No.	EC 3.2.1.2
Cas No.	9000-91-3

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 compositions of the enzyme concentrate is shown below:

Enzyme solids (TOS[1])	approx. 4.4 %
Polyols	approx. 43.0 %
Sodium benzoate	approx. 0.4 %
Potassium sorbate	approx. 0.2 %
Water	approx. 52.0 %

The enzyme concentrate is standardised in beta-amylase units to an activity of 5,000 BAMU/g (Appendix 2.1). The Novozymes A/S method used to determine the BAMU activity is enclosed in Appendix 3.1.

Briefly, beta-amylase hydrolyses maltohexaose into maltotetraose and maltose. An oxidase oxidises the maltotetraose and reduces oxygen to hydrogenperoxide. Subsequently, a two compounds form a purple product through oxidation by a peroxidase. This reaction can be quantified following the increase in absorbance at 540 nm. The increase is proportional to the enzyme activity.

A.2.3 Host organism

The *Bacillus licheniformis* host strain (Si3) was developed from the natural isolate *Bacillus licheniformis* Ca63. The Si3 cell lineage has a long history of safe use at Novozymes A/S 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 as follows:

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

Phylum Firmicutes

Class Bacilli

Order Bacillales

Family Bacillaceae

Genus *Bacillus*

Species *Bacillus licheniformis*

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

A.2.4 Donor organism

The donor for the beta-amylase gene is *Bacillus flexus*.

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

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

The enzyme is a beta-amylase (EC 3.2.1.2). Beta-amylases catalyse the hydrolysis of (1→4)-α-D-glucosidic linkages in polysaccharides so as to remove successive maltose units from the non-reducing ends of the chains.

The enzyme preparation is available as liquid product.

The food enzyme object of the present dossier is not added to final foodstuffs but used as a processing aid during food manufacturing.

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 is composed of a fermentation process, a purification process, a formulation process and finally a quality control of the finished product, as outlined by Aunstrup *et al*. (1979). This section describes the processes used in manufacturing of the beta-amylase 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 beta-amylase is produced by submerged fed-batch pure culture fermentation of the genetically modified strain of *Bacillus licheniformis*, 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 beta-amylase enzyme product complies with the purity criteria recommended for Enzyme Preparations in Food, Food Chemicals Codex, 11th edition, 2018.

In addition to this, the beta-amylase 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 an unstandardized, representative batch of the beta-amylase enzyme preparation is shown in (Table 1). These data show compliance with the purity criteria of the specification.

Table 1 Analytical data of an unstandardized enzyme product batch

Control parameter	**Unit**	**Specification**	**Batch PPY36295**
beta-amylase activity	BAMU/g		9544
Pb	ppm	< 5	ND (LOD < 0.5)
As	ppm	< 1	ND (LOD < 0.3)
Cd	ppm	< 1	ND (LOD < 0.05)
Hg	ppm	< 1	ND (LOD < 0.05)
Total viable count	CFU per g	< 50,000	100
Total coliforms	CFU per g	< 30	<4
Enteropathogenic *Escherichia coli*	CFU per 25 g	ND	ND
***Salmonella* spp.**	CFU per 25 g	ND	ND

Antimicrobial activity		ND	ND
Production strain	CFU per g	ND	ND

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

The methods of analysis used to determine compliance with the specifications are enclosed (Appendix 3).

The beta-amylase enzyme preparation is available as a liquid enzyme concentrate. The concentrate is standardised in beta-amylase units (BAMU/g; Appendix 3.1). The preparation does not contain known food allergens (Appendix 2.1).

A.6 Analytical method for detection

The beta-amylase 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 country positive list or specific approval.

The safety of the beta-amylase 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.
- France: The enzyme is included in the French positive list for processing aids, including food enzymes (The French order of October 19, 2006 on use of processing aids in the manufacture of certain foodstuff), as amended.

- Brazil: The enzyme was evaluated, approved and included in the Brazilian positive list – RDC 26/2009.

- Mexico: Based on a dossier submitted by Novozymes, the Mexican food authorities, COFEPRIS, have approved the enzyme.

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 beta-amylase, have a long history of use in food (Pariza and Johnson, 2001).

Beta-amylases are widespread in nature and are universally distributed in plants and micro-organisms. The primary sources of beta-amylase today are barley, wheat and soy. The first industrial-scale microbial beta-amylase was found in 1974 (Kojima, 2010). Beta-amylase enzyme preparations from various sources are widely authorised in, e.g. Australia and New Zealand, Brazil, China, Denmark, France, Japan, Mexico.

(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 beta-amylase enzyme to known toxins was conducted. The amino acid sequence of the beta-amylase 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 a representative batch (PPY36295) that was 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 5.3.

The following studies were performed:

- Ames Test. Test for mutagenic activity (Appendix 5.4)

- *In vitro* micronuclei test (Appendix 5.5)

- Subchronic (13 week) oral toxicity study in rats (Appendix 5.6)

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

- Beta-amylase PPY36295 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.

- Beta-amylase PPY36295 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 batch PPY36295 to Sprague-Dawley rats at doses up to 100 % of the tox test batch (1199 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 1199 mg TOS/kg bw/day).

Based on the present toxicity data it can be concluded that the beta-amylase enzyme preparation, represented by batch PPY36295, 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 beta-amylase enzyme is produced by an *Bacillus licheniformis* microorganism expressing the beta-amylase from *Bacillus flexus*. *Bacillus licheniformis* 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 enzyme 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 beta-amylase enzyme to known allergens was conducted (Appendix 5.1). The amino acid sequence of the beta-amylase provided in Appendix 6.4 was used as input for the search. The beta-amylase was compared to allergens from the FARRP allergen protein database (http://www.allergenonline.org).

The analyses of the beta-amylase sequence identified homology to one allergen, Tri a 17, a known food allergen, above the threshold of 35 % across an 80 amino acid window (Appendix 5.1)

Tri a 17 of *Triticum aestivum* (wheat) had a 44.7 % identity with the beta-amylase produced by *Bacillus licheniformis* across an 76 amino acids window. However, it had only a 25.7 % identity over the full-length sequence. It has to be emphasised that full-length comparison produces fewer false positives compared to the 80 amino acid window comparison. Thus, full-length comparison has been recommended to be used to compare identities of proteins to allergens (Ladics et al., 2007; Cressman and Ladics, 2009).

The intended use of beta-amylase produced by *Bacillus licheniformis* is to hydrolyse starch in order to produce maltose syrups. During the production of the syrup the majority of the enzyme protein (> 99%) is removed. Removal occurs in purification steps, *i.e.* filtration, ion exchange chromatography, carbon treatment and crystallisation. As a result, the presence of residual amounts of enzyme TOS is negligible. Consumers will thus not experience any significant exposure. The European Food Safety Authority's (EFSA) Panel on Food Contact Materials, Enzymes, Processing Aids (CEP) agrees that residual amounts of enzyme TOS are removed by the purification steps applied during the production of syrups (by > 99%) (EFSA, 2016)

On the basis of the available evidence it is concluded that oral intake of the beta-amylase is not anticipated to pose any food allergenic concern. Further details regarding the risk assessment can be found in Appendix 5.2

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

Documentation of approval of the beta-amylase in Denmark, France, Brazil and Mexico 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 beta-amylase enzyme is produced by an *Bacillus licheniformis* microorganism expressing the beta-amylase from *Bacillus flexus*. The *Bacillus licheniformis* host strain (Si3) was developed from the natural isolate *Bacillus licheniformis* Ca63. The Si3 cell lineage has a long history of safe use at Novozymes A/S 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 beta-amylase production strain is a non-pathogenic, non-toxigenic, genetically modified *Bacillus licheniformis* 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

Industrial strains belonging to the *Bacillus licheniformis* species have a long history of safe use in food enzyme manufacturing (OECD, 1986). They have been used for decades in the production of enzymes, and in 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).

The industrial production of alpha-amylase from *Bacillus licheniformis* was introduced in 1973 (Madsen et al, 1973). Since then, the bacterium has been safely used as a source of food enzymes such as carbohydrase (alpha-amylase) and protease for the production of various types of foods and food ingredients.

The Scientific Committee of EFSA has proposed to include a number of Bacillus species, including *Bacillus licheniformis*, on the list of QPS (Qualified Presumption of Safety) microorganisms due to the substantial body of knowledge available about these bacteria. Since all bacteria within the listed species potentially possess toxigenic traits, absence of toxigenic activity (emetic food poisoning toxins with surfactant activity and enterotoxic activity) must be verified (EFSA, 2007).

The Food and Drug Administration has affirmed that mixed carbohydrase and protease enzyme products derived from *Bacillus licheniformis* are generally recognized as safe (GRAS) in the production of certain foods including nutritive sweeteners, see 21CFR §184.1027 (FDA, 1983). In the supplementary information to the final rule in the Federal Register, FDA emphasized that “Published scientific literature as well as standard books on food microbiology demonstrate that *B. licheniformis* is widely recognized as a common

contaminant found in many foods. None of these references report any toxicity or pathogenicity associated with the presence of this organism in food."

In addition, the FDA did not question the conclusion that various other food enzymes obtained from genetically modified *Bacillus licheniformis* strains are GRAS under the intended conditions of use (GRN no. 22, 24, 72, 79, 265, 277, 472, 564, 572, 587, 594, 645, 689, 728, and 774).

JECFA has evaluated food enzymes derived from *Bacillus licheniformis*, including a genetically modified strain, and concluded that these food enzymes do not constitute a toxicological hazard (JECFA 1987, 2004).

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

The genetically modified *Bacillus licheniformis* host strain, used for production of the enzyme product, has been developed in a line of strains which have been used for production at Novozymes A/S for many years and has an extensive history of commercial safe use for production of recombinant enzymes for food. It has been constructed using only well-characterized genetic material from class 1 microorganisms. No genetic material that can give rise to resistance towards antibiotics was left in the recipient strain as a result of the genetic modifications.

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.

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 *Bacillus licheniformis* host strain (Si3) was developed from the natural isolate *Bacillus licheniformis* Ca63. The Si3 cell lineage has a long history of safe use at Novozymes A/S 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 as follows:

Phylum Firmicutes

Class Bacilli

Order Bacillales

Family Bacillaceae

Genus *Bacillus*

Species *Bacillus licheniformis*

The classification of *Bacillus licheniformis* Ca63 was confirmed by Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH.

The recipient strain used in the construction of the *Bacillus licheniformis* 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 licheniformis* recipient strain are based on the *Staphulococcus aureaus* standard vectors pUB110 (Gryczan *et al.*, 1978) and pE194 (Horinouchi and Weisblum, 1982). No elements of the vectors are left in the production strain. The vectors contain the beta-amylase expression cassette consisting of a hybrid *Bacillus* promoter, the coding sequence for beta-amylase from *Bacillus flexus* and a hybrid *Bacillus* terminator. The inserted beta-amylase gene was provided as a synthetic gene.

E.1.3 Construction of the Recombinant Microorganism

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

1. The beta-amylase expression cassette was integrated at specific integration sites present in the recipient strain.

2. The beta-amylase expression cassette and the gene encoding a chaperone was integrated at specific integration sites present in the recipient strain. The chaperone was integrated in order to improve production yield.

3. A transformant was screened for rapid growth and high beta-amylase 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 licheniformis* 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 beta-amylase preparation is used as a processing aid during the manufacture of starch-based products. Beta-amylases convert starch by removing maltose units from the non-reducing end of the substrate molecule for further processing.

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

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

The beta-amylase preparation is used as a processing aid in starch processes to produce syrups where it degrades starch into maltose.

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 beta-amylase 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 food is 10,000 BAMU per kg starch dry matter. This corresponds to 2 g of beta-amylase enzyme preparation per kg starch dry matter equivalent to 88 mg TOS per kg starch dry matter.

Enzyme residues in the Final Food

The beta-amylase preparation is used in processing of raw materials containing starch for the hydrolysis of starch to maltose. The enzyme is denatured by heat during processing and the enzyme TOS is removed to negligible amounts by down-stream processes during the manufacturing of syrups.

F.2.1 Estimates of human consumption

Method used for the dietary exposure assessment

An exposure assessment according to the Budget Method (Hansen, 1966; Douglass et al., 1997; 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 beta-amylase 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.

As an example the beta-amylase will be used to produce maltose syrups. During the production of the syrup the majority of the enzyme protein (> 99%) is removed. Removal occurs in purification steps, i.e. filtration, ion exchange chromatography, carbon treatment and crystallisation. As a result, the presence of residual amounts of enzyme TOS is negligible. EFSA's Panel on Food Contact Materials, Enzymes, Processing Aids (CEP) agrees that residual amounts of enzyme TOS are removed by the purification steps applied during the production of syrups (by > 99%) (EFSA, 2016)

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

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 12% starch hydrolysates = 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 10,000 BAMU per kg starch dry matter, corresponding to 88 mg TOS per kg starch dry matter (cf. Section 3.3.2 A.2.2).

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

88 mg TOS per kg / 1000 g per kg x 3.12 g = 0.27 mg TOS

Liquids

The highest dosage given for liquids is 10,000 BAMU per kg starch dry matter, corresponding to 88 mg TOS per kg starch dry matter (cf. Section 3.3.2 A.2.2).

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

88 mg TOS per kg / 1000 g per kg x 3.0 g = 0.26 mg TOS

Total TMDI of starch-derived solid foods and liquids

0.27 mg TOS + 0.26 mg TOS = 0.53 mg TOS

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 1199 mg TOS/kg bw/day (cf. Section 3.3.2 C 2).

The estimated human consumption is 0.53 mg TOS/kg/day

The safety margin can thus be calculated to be 1199/0.53 = 2,262.

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 beta-amylase object of this dossier 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 oils and fats are 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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