Subtilisin from *Bacillus licheniformis*

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

Novozymes A/S

June 2020

[redacted]

Table of Contents

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 subtilisin 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 subtilisin from *Pyrococcus furiosus* as permitted source for subtilisin.

The application is applied for assessment by the general procedure.

Description of enzyme preparation

The enzyme is a subtilisin (EC 3.4.21.62).

Subtilisin hydrolyses peptide bonds in proteins with broad specificity.

The enzyme is produced by submerged fermentation of a *Bacillus licheniformis* microorganism expressing a subtilisin from *Pyrococcus furiosus*.

The subtilisin 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 subtilisin preparation is used as a processing aid in potable alcohol production. During production subtilisin hydrolyses protein peptide bonds. In potable alcohol production the subtilisin is used in order to degrade proteins into peptides and amino acids.

Benefits

The benefits of the action of the subtilisin in potable alcohol production:

- lowering viscosity
- release of peptides and amino acids used as nutrients for yeast for fast and complete fermentation

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 subtilisin does not pose food allergenic or toxic concern.
- A mutagenicity study *in vitro* showed no evidence of genotoxic potential of the enzyme preparation.
- An oral feeding study in rats for 14 days showed that all dose levels were generally well tolerated and no evidence of toxicity.

Conclusion

Based on the Novozymes A/S safety evaluation, we respectfully request the inclusion of this enzyme in Schedule 18—Processing aids.

INTRODUCTION

The present dossier describes a subtilisin enzyme preparation produced by submerged fermentation of a *Bacillus licheniformis* microorganism expressing a subtilisin from *Pyrococcus furiosus*.

The enzyme is subtilisin (EC 3.4.21.62). The enzyme catalyses the hydrolysis of protein peptide bonds with broad specificity.

The subtilisin enzyme preparation is intended to be used as a processing aid in the potable alcohol production industry to hydrolyse proteins into peptides and amino acids.

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 noted that in some reports, the subtilisin enzyme preparation is described as NS250133, or by the internal production batch codes PPA34557 and PPA48131.

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

(a) Applicant's name/s
[redacted]

(b) Company/organisation name
Novozymes Australia Pty Ltd

(c) [redacted]

(d) Telephone number
[redacted]

(e) Email address
[redacted]

(f) Nature of applicant's business
Biotechnology

(g) Details of other individuals, companies or organisations associated with the application.
Dossier prepared by:

[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 subtilisin.

D Justification for the application

The need for the proposed change

Schedule 18—Processing aids contains a list of permitted enzymes of microbial origin. Schedule 18 does contain serine proteinases (EC 3.4.21.14), including a serine proteinase from *Bacillus licheniformis*. Serine proteinases have been transferred to new entries by the International Union of Biochemistry and Molecular Biology (IUBMB)[1] and are now divided into subtilisin (EC 3.4.21.62), oryzin (EC 3.4.21.63), endopeptidase K (EC 3.4.21.64), thermomycolin (EC 3.4.21.65), and endopeptidase So (EC 3.4.21.67). Therefore, the entry on Schedule 18 for a serine proteinase from *Bacillus licheniformis* includes subtilisin. However, Schedule 18 does not contain a subtilisin (EC 3.4.21.62) from *Bacillus licheniformis* containing the gene for subtilisin from *Pyrococcus furiosus.*

Bacillus licheniformis is an approved host and production strain for a number of enzymes in Schedule 18—Processing aids, e.g. other proteases such as chymotrypsin and serine proteases but also alpha-amylase, beta-galactosidase, and pullulanase.

The advantages of the proposed change over the status quo

The subtilisin preparation is used as a processing aid during the manufacture of potable alcohol products. Subtilisin hydrolyses proteins into peptides and amino acids.

The benefits of the action of the subtilisin in potable alcohol production:

- lowering viscosity
- release of peptides and amino acids used as nutrients for yeast for fast and complete fermentation

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 subtilisin enzyme in Schedule 18—Processing aids will provide the beverage industry with the opportunity to improve the yield of potable alcohol production 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.

[1] https://www.qmul.ac.uk/sbcs/iubmb/enzyme/EC3/4/21/14.html

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 subtilisin 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 subtilisin does not pose food allergenic or toxic concern.
- A mutagenicity study in vitro showed no evidence of genotoxic potential of the enzyme preparation.
- An oral feeding study in rats for 14 days 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 enzyme, it is considered appropriate that the assessment procedure is characterised as “General Procedure, Level 1”.

G Confidential commercial information (CCI)

Detailed information on the construction and characteristics of the genetically modified production strain is provided in Appendix 6. A summary of this information is given in section 3.3.2 E. The formal request for treatment of selected parts of Appendix 6 as confidential commercial information (CCI) is included as Appendix 1.1.

H Other confidential information

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

The subtilisin enzyme preparation is to be used in the food industry as a processing aid during the processing of raw materials containing proteins. Subtilisin hydrolyses proteins into peptides and amino acids.

The subtilisin enzyme preparation is used in the production of potable alcohol.

The highest recommended dosage of the subtilisin during potable alcohol production is up to 90 PROT(A) per kg starch dry matter.

Information on the identity of the processing aid

A.2.1 Enzyme

Generic name	subtilisin
IUBMC nomenclature	subtilisin
IUBMC No.	EC 3.4.21.62
Cas No.	9014-01-1

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 or a granulate depending on the characteristics of the intended food process in which it will be used.

The typical composition of the enzyme concentrate is shown below:

Enzyme solids (TOS)	approx. 3.0 %
Glycerol	approx. 47.0 %
Sodium benzoate	approx. 0.3 %
Potassium sorbate	approx. 0.2 %
Water	approx. 49.5 %

The enzyme concentrate is standardised in subtilisin units to an activity of 300 PROT(A)/g. The Novozymes A/S method used to determine the PROT(A) activity is enclosed in Appendix 3.1.

Briefly, subtilisin hydrolyses a peptide substrate that carries a nitroaniline chromophore. The release of chromophore upon hydrolysis can be measured as the increase of absorbance at 405 nm. The increase in signal 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:

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 subtilisin gene is from *Pyrococcus furiosus*.

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 subtilisin (EC 3.4.21.62). Subtilisin hydrolyses peptide bonds of proteins with a broad specificity.

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 subtilisin 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 Food Grade Quality and have been subjected to appropriate analysis to ensure their conformity with the specifications.

A.4.1 Fermentation

The subtilisin 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 plus minerals and vitamins necessary for growth. The choice of raw materials used in the fermentation process (the feed, the seed fermenter, the main fermenter and dosing) is listed below.

- potable water
- carbohydrates (e.g. sucrose, glucose syrup, corn starch)
- vegetable protein (e.g. potato protein)
- salts (e.g. ammonium sulphate, magnesium sulphate, potassium phosphate, potassium sulphate, sodium hydrogen phosphate)
- trace metals (e.g. $MnSO_4$, $NiCl_2$, $FeSO_4$, $CuSO_4$, $ZnSO_4$)

- alkali and acid for pH adjustments (e.g. citric acid, potassium hydroxide, sodium hydroxide)

- antifoaming agents (e.g. modified polyalkoxyether, polyalkylene glycol)

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 raw materials typically used in the recovery process are as follows:

- potable water
- filter aids or pre-coats (e.g. diatomaceous earth, perlite)
- acids and bases for pH adjustment (e.g. acetic acid, potassium hydroxide, sodium hydroxide)
- flocculants (e.g. anionic polyacrylamide, calcium chloride, poly(aluminium hydroxy) chloride)
- stabilisation (e.g. glycerol, potassium sorbate, sodium benzoate)

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 pre-coat used in the filter and the filter aid used in the process is diatomaceous earth (diatomite or perlite).

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 20-65 °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 glycerol is added to the enzyme concentrate. pH is adjusted by acetic acid, potassium hydroxide, or sodium hydroxide.

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

In addition to this, the subtilisin 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, available online at: http://www.fao.org/food/food-safety-quality/scientific-advice/jecfa/jecfa-additives/en/.

Analytical data for an unstandardized, representative batch of the subtilisin 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 (DL, limit of detection; ND, not detected)

Control parameter	**Unit**	**Specification**	**Batch 1**
Subtilisin activity	PROT(A)/g		**240**
Heavy Metals	ppm	Max 30	**5.4**
Pb	ppm	Max 5	**ND (DL < 0.5)**
As	ppm	Max 3	**ND (DL < 0.3)**
Cd	ppm	Max 0.5	**ND (DL < 0.05)**
Hg	ppm	Max 0.5	**ND (DL < 0.05)**
Total viable count	per g	Not more than 50000	**<100**
Total coliforms	per g	Not more than 30	**<4**
Enteropathogenic *Escherichia coli*	per 25 g	Not detected	**ND**
***Salmonella* spp.**	per 25 g	Not detected	**ND**
Antibiotic activity		Not detected	**ND**
Production strain	per g	Not detected	**ND**

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

The subtilisin enzyme preparation is available as a liquid enzyme concentrate. The concentrate is standardised in subtilisin units (PROT(A)/g; Appendix 3.1). The preparation does not contain known food allergens (Appendix 2.1).

A.6 Analytical method for detection

The subtilisin 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 protein-containing raw materials in a range of countries, where there are no restrictions of the use of enzyme processing aids.

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 subtilisin, have a long history of use in food (Pariza and Foster, 1983 and Pariza and Johnson, 2001).

Proteases, including subtilisin, have been used extensively for food processing for many centuries (Sumantha et al., 2006). Proteases are essential to produce cheese, but they are also used in brewing, baking, and flavour enhancement processes (Pariza and Foster, 1983; Sumantha et al., 2006; Singh et al., 2016). Protease enzyme preparations from various sources are widely authorised in, e.g. Australia and New Zealand, Brazil, Canada, China, Denmark, France, 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 subtilisin enzyme to known toxins and allergens was conducted. The amino acid sequence of the subtilisin provided in Appendix 6.4 was used as input for the search. No homologies to toxins above threshold were found. A range of homologies to allergens were found. However, given the process conditions in which the enzyme product is used as a processing aid and the negligible exposure in the final product, it was concluded that they do not pose a risk. The complete search report is enclosed in Appendix 5.1.

Furthermore, *Bacillus licheniformis* is part of the list of Qualified Presumption of Safety (QPS) organisms (EFSA 2007). In essence, any strain of microorganism considered QPS is freed from the need for further safety assessment other than meeting specific qualifications, *i. e.* establishing identity, body of knowledge, absence of toxigenic activity, and end use. The EFSA BIOHAZ Panel confirmed that QPS can also be extended to genetically modified production strains if the recipient strain qualifies as QPS (EFSA BIOHAZ Panel, 2018). The identity of the parental strain was established using molecular taxonomical methods (Appendix 6.2). In order to verify absence of toxigenic activity, the parental strain and a predecessor strain of the production strain were tested in Chinese hamster ovary cells (Pedersen et al., 2002) and VERO cells (Appendix 6.3, EFSA FEEDAP, 2014) in accordance with recommendations from the Scientific Committee on Animal Nutrition (SCAN, 2000). No cytotoxicity was observed for either of the tested strains in both analyses, demonstrating the lack of cytotoxicity of the strain lineage. A detailed description of the analyses and the results are presented in Appendix 6.3.

Based on the information presented above it is concluded that the genetically modified *Bacillus licheniformis* production strain is considered QPS and a safe production strain for the subtilisin enzyme preparation.

Given that the production strain is considered QPS, has a long history of safe use (cf. section D.2; Appendix 6.1) and the subtilisin enzyme preparation is used in the production of potable alcohol, where the enzyme TOS is negligible in the final product, a reduced safety programme was conducted. The studies were carried out as described below on representative batches (PPA34557, PPA48131) that were produced according to the description given in section 3.3.2 A.4, omitting stabilization and standardization.

Batches PPA34557 and PPA48131 are produced by closely related *Bacillus licheniformis* production strains from the Si3 lineage. The production strain for batch PPA48131 is an optimised strain of the PPA34557 production strain. A description of the differences can be found in Appendix 6.1.

The following studies were performed:

- Test for mutagenic activity on batch PPA48131 (Ames test; Appendix 5.3)
- Repeated dose 14 days toxicity study in rats on batch PPA34557 (Appendix 5.4)

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

- Subtilisin, batch PPA48131, 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.
- Oral administration of batch PPA34557 to Wistar rats at doses up to 100 % of the tox test batch (1032 mg TOS/kg bw/day) for 14 days was well-tolerated and did not cause any adverse effect. The NOAEL was considered to be 100 % of the tox test batch (equivalent to 1032 mg TOS/kg bw/day).

Based on the present toxicity data it can be concluded that the subtilisin enzyme preparation, represented by batches PPA34557 and PPA48131 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 subtilisin enzyme is produced by a *Bacillus licheniformis* microorganism expressing the subtilisin from *Pyrococcus furiosus*. *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 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 subtilisin enzyme to known toxins and allergens was conducted (Appendix 5.1). The amino acid sequence of the subtilisin provided in Appendix 6.4 was used as input for the search. The subtilisin was compared to allergens from the FARRP allergen protein database (http://www.allergenonline.org) as well as the World Health Organisation and International Union or Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee (http://www.allergen.org) using the recommended allergen method by EFSA.

Allergen risk assessment analysis of the subtilisin was performed according to the EFSA scientific opinion using allergen online and allergen.org databases. The analyses of the subtilisin sequence identified homology to a number of allergens above the threshold of 35 % across an 80 amino acid window (Appendix 5.1). The majority of hits are occupational allergens (by inhalation) and therefore should not be of concern to oral intake. Additionally, Tri r 2, a contact allergen, and Cuc m 1, a known food allergen, were identified to have an identity above the 35 % threshold.

Tri r 2 of *Trichophyton rubrum* had a 47.5 % identity with the subtilisin produced by *Bacillus licheniformis* across an 80 amino acids window using the allergen.org database. However, it had only a 19.5 % identity over the full-length sequence. Cuc m 1 (cucumisin) of *Cucumis melo (*muskmelon) was revealed to have up to 39 % identity with the subtilisin produced by *Bacillus licheniformis* across an 80 amino acids window using the allergen.org database. In contrast, the full-length sequence comparison showed only up to 19.1 % identity between Cuc m 1 and subtilisin produced by *Bacillus licheniformis*. 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).

Furthermore, subtilisin from *Bacillus licheniformis* was used as a test material in a study using the generally recognised guidelines for food allergy diagnosis (Bindslev-Jensen et al., 2006; as also described above). The study included 400 patients with diagnosed allergy to one or more of: inhalation allergens, food allergens, bee or wasp allergens. It should be noted that one of the tested enzymes was a subtilisin from *Bacillus licheniformis.* The subtilisin used in the study by Bindslev-Jensen et al. has an identity of 46 % with the subtilisin enzyme object of the present dossier over the full length. It is also of importance to notice that all patients were orally administered with fully active, i.e. not heat-degraded, subtilisin in doses that were orders of magnitude higher than the maximal estimated human consumption (cf. F.2.1). None of the tested patients showed an allergic reaction against the tested subtilisin when applied orally. There were no indications of cross-reactivity between the tested enzymes used in food and the main known allergens represented by the patients included in the study.

As described in section D the enzyme is intended to be used in potable alcohol production. The harsh conditions during potable alcohol processing will completely denature the subtilisin. Additionally, the distillation process will remove any remaining enzyme from the final product.

On the basis of the available evidence it is concluded that oral intake of the subtilisin 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

The subtilisin enzyme preparation has not been assessed for its safety by other international agencies or other national government agencies.

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

D.1 Information on the source microorganism

The subtilisin enzyme is produced by a *Bacillus licheniformis* microorganism expressing the subtilisin from *Pyrococcus furiosus*. 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 subtilisin 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. Further details can be found in 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 *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 identification 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 Si3 parental strain through a combination of classical mutagenesis/selection and GM-steps. This led to a recipient strain with higher product purity, stability and safety features. Furthermore, the changes introduced specific integration sites for the introduction of the gene of interest.

E.1.2 Introduced DNA

The vectors pMDT351 and pMDT251A, 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 these vectors are left in the production strain. Both vectors contain an expression cassette, *aprPFU2*, consisting of a hybrid *Bacillus* promoter with promoter elements from *Bacillus licheniformis* and *Bacillus amyloliquefaciens*, the coding sequence for subtilisin from *Pyrococcus furiosus* and a hybrid terminator with terminator elements from *Bacillus clausii* and *Bacillus licheniformis*. The inserted subtilisin gene was provided as a synthetic gene. Furthermore, vector pPP3708 was used in order to remove a marker gene present in the recipient strain.

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 subtilisin expression cassette, *aprPFU2*, was integrated at three specific integration sites using plasmid pMDT351.

2. Further copies of the subtilisin expression cassette, *aprPFU2*, were introduced into a single specific integration site using plasmid pMDT251A.

3. Plasmid pPP3708 was used to replace the marker gene in the recipient strain with a transcriptional terminator sequence.

E.1.4 Antibiotic Resistance Gene

No functional antibiotic resistance genes were left in the strain as a result of the genetic modifications.

E.1.5 Stability of the Introduced Genetic Sequences

Stability of the introduced DNA sequences 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 subtilisin preparation is used as a processing aid during potable alcohol production. Subtilisin hydrolyses peptide amide bonds in proteins, present as a natural part of the raw materials, with broad specificity, thereby converting them into peptides and amino acids.

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

The subtilisin preparation is used as a processing aid during potable alcohol production.

Use level

The subtilisin 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 subtilisin 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 liquids is 90 PROT(A) per kg starch dry matter. This corresponds to 0.3 g of subtilisin enzyme preparation per kg starch dry matter equivalent to 9 mg TOS per kg starch dry matter (cf. Section 3.3.2 A.2.2).

Enzyme residues in the Final Food

The subtilisin preparation is used as a processing aid during the manufacture potable alcohol, using raw materials containing proteins as a natural part of the material. The enzyme is denatured by heat during processing and removed by distillation.

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 raw material is used as an ingredient in a variety of beverages.

Budget Method

Overall, the human exposure to the subtilisin 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 beverages and not only in those 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 largely exaggerated since the enzyme protein and the other substances resulting from the fermentation are removed in the processing steps of potable alcohol production.

As an example, distilled beverage spirits will neither contain any TOS (Total Organic Solids) originating from the food enzyme preparation nor from the fermentation mash due to the distillation step(s).

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

Assumptions in the Budget Method

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

Starch-derived liquids

The highest dosage given for liquids is 90 PROT(A) per kg starch dry matter, corresponding to 9 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:

9 mg TOS per kg / 1000 g per kg x 3.0 g = 0.03 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 14 days oral toxicity study in rats was concluded to be 1032 mg TOS/kg bw/day (cf. Section 3.3.2 C 2).

The estimated human consumption is 0.03 mg TOS/kg/day

The safety margin can thus be calculated to be 1032/0.03 or 34,400.

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

In the estimates on human consumption given in F.2.1 above, it is assumed that all raw materials are processed using the subtilisin enzyme preparation 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 raw material is used as an ingredient in a variety of food products and beverages.

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.

LIST OF REFERENCES

Aunstrup K (1979) Production, Isolation, and Economics of Extracellular Enzymes in Applied Biochemistry and Bioengineering, Volume 2, Enzyme Technology, Eds. Wingard, L.B., Katchalski-Katzir, E. and Goldstein, L, pp. 28-68

Bindslev-Jensen C, Skov PS, Roggen EL, Hvass P, Brinch DS (2006) Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry. Food Chem. Toxicol. 44, 1909-1915.

Cressman RF, Ladics G (2009). Further evaluation of the utility of “sliding window” FASTA in predicting cross-reactivity with allergenic proteins. Regulatory Toxicology and Pharmacology, 54(3), S20-S25.

Douglass JS, Barraj LM, Tennant DR, Wesley RL, Chaisson CF (1997). Evaluation of the Budget Method for screening food additive intakes. Food Additives and Contaminants. 14 (8), 791-802.

EFSA (2007). Introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA. Opinion of the Scientific Committee. The EFSA Journal, 587, 1-16.

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP). (2014). Guidance on the assessment of the toxigenic potential of Bacillus species used in animal nutrition. EFSA Journal, 12(5), 3665.

EFSA Panel on Biological Hazards (BIOHAZ) (2018). Update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA 7: suitability of taxonomic units notified to EFSA until September 2017. EFSA Journal, 16(1), e05131.

FDA (Food and Drug Administration) (1983) Direct Food Substances Affirmed as GRAS, Mixed Carbohydrase and Protease Enzyme Product. 21 CFR Part 184. Federal Register, 48 (2), 239-240.

Goodman RE, Vieths S, Sampson HA, Hill D, Ebisawa M, Taylor SL, van Ree R (2008) Allergenicity assessment of genetically modified crops—what makes sense? Nature Biotechnology, 26 (1), 73-81.

Gryczan TJ, Contente S, Dubnau D (1978) Characterization of Staphylococcus aureus Plasmids Introduced by Transformation into Bacillus subtilis. Journal of Bacteriology, 134 (1), 318-329.

Hansen SC (1966) Acceptable Daily Intake of Food Additives and Ceiling on Levels of Use. Fd. Cosmet. Toxicol. 4, 427-432.

Horinouchi S, Weisblum B (1982) Nucleotide Sequence and Functional Map of pE194, a Plasmid that Specifies Inducible Resistance to Macrolide, Lincosamide, and Streptogramin Type B Antibiotics. Journal of Bacteriology, 150 (2), 804-814.

ILSI (1997) An evaluation of the budget method for screening food additive intake. Summary report prepared under the responsibility of ILSI Europe Food Chemical Intake Task Force, 1-12.

JECFA (1987) Carbohydrase (alpha-amylase) from Bacillus licheniformis. WHO Food Additives Series, 20 (592). http://www.inchem.org/documents/jecfa/jecmono/v20je02.htm (Last visited 16 January 2015)

JECFA (2004) Alpha-amylase from Bacillus licheniformis containing a genetically engineered alpha-amylase gene from B. licheniformis. WHO Food Additives Series, 52.

Ladics GS, Bannon GA, Silvanovich A, Cressman RF (2007). Comparison of conventional FASTA identity searches with the 80 amino acid sliding window FASTA search for the elucidation of potential identities to known allergens. Molecular Nutrition & Food Research, 51(8), 985-998.

Madsen GB, Norman BE, Slott S (1973) A New, Heat Stable Bacterial Amylase and its Use in High Temperature Liquefaction. Stärke/Starch, 25 (9), 304-308.

OECD (1986) Recombinant DNA Safety Considerations. Safety considerations for industrial, agricultural and environmental applications.

Pariza MW, Foster, EM (1983) Determining the Safety of Enzymes Used in Food Processing. Journal of Food Protection, 46 (5), 453-468.

Pariza MW and Johnson EA (2001) Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing: Update for a New Century. Regulatory Toxicology and Pharmacology, 33, 173-186.

Pedersen PB, Bjørnvad ME, Rasmussen MD and Petersen JN, 2002. Cytotoxic potential of industrial strains of Bacillus sp. Regulatory Toxicology and Pharmacology. 36, 155–161.

SCAN (2000) Opinion of The Scientific Committee on Animal Nutrition on the safety of use of Bacillus species in animal nutrition (expressed on 17 February 2000).

Schallmey M, Singh A, Ward OP (2004) Developments in the use of Bacillus species for industrial production. Can. J. Microbiol. 50, 1-17.

Singh R, Kumar M, Mittal A, Mehta PK (2016). Microbial enzymes: industrial progress in 21st century. 3 Biotech, 6(2), 174.

Sumantha A, Larroche C, Pandey A (2006). Microbiology and industrial biotechnology of food-grade proteases: a perspective. Food Technology and Biotechnology, 44(2), 211.