FOOD STANDARDS Australia New Zealand
Te Mana Kounga Kai – Ahitereiria me Aotearoa

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Supporting document 1

Risk and technical assessment report – Application A1206

Subtilisin from GM *Bacillus licheniformis* as a processing aid (enzyme)

Executive summary

Novozymes applied to Food Standards Australia New Zealand (FSANZ) to amend Schedule 18 – Processing Aids of the Australia New Zealand Food Standards Code (the Code) to permit the use of the enzyme subtilisin from a genetically modified (GM) strain of *Bacillus licheniformis* containing the subtilisin gene from *Pyrococcus furiosus*, as a processing aid in the production of potable alcohol. Subtilisin would be used at the minimum levels necessary to achieve the desired effect and according to requirements for normal production following Good Manufacturing Practice (GMP).

FSANZ has undertaken an assessment to determine whether the enzyme achieves its technological function in the quantity and form proposed to be used and to evaluate public health and safety concerns that may arise from the use of this enzyme.

FSANZ concludes that the proposed use of this subtilisin as an enzyme processing aid in potable alcohol production, in the quantity and form proposed to be used, is consistent with its typical function of hydrolysing proteins. Subtilisin performs its technological purpose during production of potable alcohol and is not performing a technological function in the final food, therefore functioning as a processing aid as defined in the Code. The enzyme meets relevant identity and purity specifications.

The safety assessment concluded that the use of the enzyme under the proposed conditions is safe. *B. licheniformis* has a long history of safe use as a source microorganism of enzyme processing aids, including several that are already permitted in the Code. The bacterium is neither toxigenic nor pathogenic. Characterisation of the GM production strain confirmed both presence and stable inheritance of the inserted subtilisin gene.

The applicant provided a 13-week toxicity study in rats and *in vitro* genotoxicity studies with an α-amylase produced by a closely related predecessor strain of the subtilisin production strain. This α-amylase was chosen as most appropriate for assessment of subtilisin, because the recipient strain of the α-amylase production strain is identical to the one for the subtilisin production strain, and the method used to insert the DNA is identical to the one described for the construction of the subtilisin production strain. The α-amylase was not genotoxic *in vitro* and caused no adverse effects in a 13-week toxicity study in rats. The no observed adverse effect level (NOAEL) was 796 mg/kg bw/day total organic solids (TOS), the highest dose tested.

A comparison of the NOAEL of the closely related α-amylase with the estimated theoretical maximum daily intake (TMDI) (0.03 mg/kg bw/day TOS) indicates that the Margin of Exposure between the NOAEL and TMDI is more than 26,500.

A degree of amino acid sequence homology with a food allergen from melon was identified, but the enzyme is considered unlikely to pose an allergenicity concern because a study with another subtilisin from *B. licheniformis* found no evidence of food allergenicity, exposure is expected to be very low and there is a long history of safe use of subtilisin enzymes from other sources with no reports of food allergy identified.

Based on the reviewed toxicological data, it is concluded that in the absence of any identifiable hazard an Acceptable Daily Intake (ADI) ‘not specified’ is appropriate.

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1 Introduction

Novozymes applied to Food Standards Australia New Zealand (FSANZ) for permission to use the enzyme subtilisin from a genetically modified (GM) strain of *Bacillus licheniformis* containing the subtilisin gene from *Pyrococcus furiosus* as a processing aid in the production of potable alcohol. Subtilisin would be used at minimum levels necessary to achieve the desired effect and according to requirements for normal production following Good Manufacturing Process (GMP).

Subtilisin is a serine proteinase. There is currently permission in the Australia New Zealand Food Standards Code (the Code) to use serine proteinase of microbial origin (EC 3.4.21.14) as a processing aid including with *B. licheniformis* as the source, but not containing the gene for subtilisin from *P. furiosus*. The current permission is for general use in food rather than a specific use for potable alcohol as requested in this application. Assessment of the enzyme is limited to use in potable alcohol.

1.1 Objectives of the assessment

The objectives of this risk and technical assessment were to:

- determine whether the proposed purpose of the enzyme is clearly stated and that the enzyme achieves its technological function in the quantity and form proposed to be used as a processing aid specifically in the production of potable alcohol
- evaluate public health and safety concerns that may arise from the use of this enzyme, produced by a genetically modified microorganism, as a processing aid, specifically by considering the:
  - history of use of the gene donor and production microorganisms
  - characterisation of the genetic modification(s)
  - safety of the enzyme.

2 Food technology assessment

2.1 Characterisation of the enzyme

2.1.1 Identity of the enzyme

The applicant provided relevant information regarding the identity of the subtilisin enzyme, and this has been verified using the International Union of Biochemistry and Molecular Biology (IUBMB) enzyme nomenclature reference (IUBMB 2020).

Accepted IUBMB[1] name:	subtilisin
Other names:	alcalase, bacillopeptidase A, bacillopeptidase B, protease S, subtilopeptidase, thermoase, and other names listed by IUBMB (IUBMB 2020)
IUBMB enzyme nomenclature:	EC 3.4.21.62
CAS[2] number:	9014-01-1
Reaction:	Hydrolysis of proteins with broad specificity for peptide bonds, and a preference for a large uncharged residue in P1. Hydrolyses peptide amides.

Subtilisin is a serine endopeptidase (IUBMB 2020), which are commonly referred to as a type of protease (Campbell-Platt, 2018; Nagodawithana and Reed,1993). Subtilisin was originally part of a group of enzymes numbered by the IUBMB as EC 3.4.21.14. The IUBMB has since transferred this to individual entries, including for subtilisin (EC 3.4.21.62), instead of the original EC number, EC 3.4.21.14 (IUBMB 2020).

Subtilisin with *B. licheniformis* as the source, containing the gene for subtilisin from *P. furiosus* as requested in this application, is not currently permitted for use as a processing aid in the Code.

2.1.2 Technological purpose and justification of the enzyme

Subtilisin hydrolyses proteins and peptide amides (IUBMB 2020). Proteases are commonly used in the manufacture of a range of foods to catalyse the degradation of proteins (Nagodawithana and Reed,1993).

The applicant states that subtilisin preparations are used as a processing aid in potable alcohol production, to degrade proteins into peptides and amino acids. The benefits of this as stated by the applicant are lowering viscosity and fast and complete fermentation from released peptides and amino acids used as nutrients for yeast.

The technological purpose of the enzyme in degrading proteins which lowers viscosity and aids yeast growth in alcohol production, as proposed in the application, is supported by the literature (Gomaa, AM, 2018).

[1] International Union of Biochemistry and Molecular Biology.
[2] Chemical Abstracts Service.

As noted by the applicant, the benefits of using enzymes in the production of potable alcohol can also be obtained by other means (without the use of enzymes or reduced use of enzymes), such as modified production processes or recipe changes. However they suggest that using enzymes to hydrolyse protein in the production of potable alcohol provides the opportunity to improve the yield of potable alcohol production under environmentally friendly and cost-efficient production conditions. The use of enzymes to improve processing and provide economic benefits is supported by the literature (Campbell-Platt, G, 2018; Chaudhary, S et al, 2015).

Use of commercial enzyme preparations should follow Good Manufacturing Practice (GMP), where use is at a level that is not higher than that necessary to achieve the desired enzymatic reaction. The conditions of use of the enzyme during potable alcohol production will depend on a number of factors including the nature of the application and the individual food manufacturers' production processes. The optimum use level should be assessed and adjusted using trials that reflect their particular processes. The applicant states that the highest recommended use level of subtilisin during potable alcohol production is up to 90 PROT(A)[3] per kg starch dry matter, which corresponds to 0.3 g of subtilisin enzyme preparation per kg starch dry matter, equivalent to 9 mg total organic solids (TOS) per kg starch dry matter[4].

2.2 Manufacturing process

2.2.1 Production of the enzyme

The enzyme is produced using a standard manufacturing process, which is comprised of a fermentation process, a purification process and a formulation process, followed by quality control of the finished product, as outlined by Anstrup, 1979. Detail is provided in section A.4 of the application.

Enzymes are generally sold as enzyme preparations, which consist of the enzyme(s) and other ingredients, to facilitate their storage, sale, standardisation, dilution or dissolution. Novozymes' enzyme preparation is composed of the enzyme solids, water, glycerol as a stabiliser and sodium benzoate and potassium sorbate as preservatives. The enzyme preparation is a light to dark brown liquid. Novozymes has confirmed that their subtilisin enzyme preparation is only provided in a liquid form.

2.2.2 Allergen considerations

Glucose syrup may be used as a raw material in the fermentation process in the manufacture of the enzyme. The glucose syrup could potentially be sourced from wheat. FSANZ has previously concluded that wheat-derived glucose syrups that have been purified and contain 10-20 mg gluten/kg glucose are likely to present a negligible risk to the majority of wheat allergic individuals (FSANZ 2016).

The applicant has provided a data sheet for their enzyme preparation which indicates that the major allergens (those requiring declaration under Standard 1.2.3 of the Code, including wheat) are not present in the enzyme preparation.

[3] protease unit, enzyme activity determined relative to an enzyme standard

[4] To distinguish the proportion of the enzyme preparation derived from the source material and manufacturing process from that contributed by intentionally added formulation ingredients, the content of total organic solids (TOS) is calculated as follows: % TOS = 100 - (A + W + D) where: A = % ash, W = % water and D = % diluents and/or other formulation ingredients.

2.2.3 Specifications

There are international specifications for enzyme preparations used in the production of food. These have been established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in its Compendium of Food Additive Specifications and in the Food Chemicals Codex (FCC). These specifications are included in the primary sources listed in section S3—2 of Schedule 3 of the Code and enzymes used as a processing aid must meet either of these specifications.

With respect to those specifications, Novozymes states that their subtilisin enzyme preparation complies with the purity criteria for Enzyme Preparations in Food in Food Chemicals Codex (FCC 2018) and conforms to the JECFA specification for enzyme preparations (JECFA 2006).

Schedule 3 also includes specifications for heavy metals (section S3—4) if they are not already detailed within specifications in sections S3—2 or S3—3.

Table 1 below provides a comparison of a representative batch of the subtilisin enzyme preparation with international specifications established by JECFA and Food Chemicals Codex, as well as those in the Code (as applicable). Based on these results, the enzyme preparation meets all relevant specifications for metals and the microbiological criteria. As noted above, Novozymes states that their enzyme product conforms to the specifications for identity and purity set by FCC and JECFA. These are broader than the specifications for metals and microbiological limits referred to here.

Table 1 *Comparison of Novoymes' subtilisin compared to JECFA, Food Chemicals Codex, and Code specifications for enzymes*

Analysis	Novozymes' analysis*	Specifications		
		JECFA (2006)	*Food Chemicals Codex (FCC, 2018)*	*Australia New Zealand Food Standards Code (section S3—4)*
Lead (mg/kg)	Not detected	≤ 5	≤ 5	≤2
Arsenic (mg/kg)	Not detected	-	-	≤1
Cadmium (mg/kg)	Not detected	-	-	≤1
Mercury (mg/kg)	Not detected	-	-	≤1
Coliforms (cfu/g)	<4	≤30	≤30	-
***Salmonella* (in 25 g)**	Not detected	Absent	Negative	-
***E. coli* (in 25 g)**	Not detected	Absent	-	-

*representative batch

2.3 Food technology conclusion

FSANZ concludes that the proposed use of this subtilisin as an enzyme processing aid in potable alcohol production, in the quantity and form proposed to be used (which must be consistent with GMP controls and processes), is to achieve a technological function consistent with its typical effect of hydrolysing proteins. Subtilisin hydrolyses proteins into peptides and amino acids which lowers the viscosity of the fermentation solution and provides nutrients for yeast for faster and more complete fermentation compared to other options for fermentation.

Approval of subtilisin with *B. licheniformis* as the source and containing the gene for subtilisin from *P. furiosus* would provide manufacturers of potable alcohol with an alternative source of subtilisin.

There are relevant identity and purity specifications for the enzyme in the Code.

Subtilisin performs its technological purpose during production of potable alcohol and is not performing a technological function in the final food, therefore functioning as a processing aid as defined in the Code.

3 Safety assessment

3.1 Objectives for safety assessment

The objectives of this safety assessment for subtilisin are to evaluate any potential public health and safety concerns that may arise from the use of this enzyme, produced by a GM microorganism, as a processing aid. Specifically this will be by considering:

- history of use of the host and gene donor microorganisms
- characterisation of the genetic modification(s)
- safety of the enzyme.

3.2 History of use of host and gene donor microorganisms

3.2.1 Host organism

Bacillus licheniformis

B. licheniformis is widely used to produce food-grade enzymes and other food products (Schallmey et al. 2004). FSANZ has previously assessed the safety of *B. licheniformis* as the source organism for a number of food processing aids (both GM and non-GM). Schedule 18 to Standard 1.3.3 of the Code currently permits the use of the following enzymes derived from *B. licheniformis*: α-amylase, chymotrypsin, endo-1,4-beta-xylanase, β-galactosidase, glycerophospholipid cholesterol acyltransferase, maltotetraohydrolase, pullulanase and serine proteinase.

Asporogenic forms of *B. licheniformis* are designated as Risk Group 1 agents, which are not associated with disease in healthy adult humans (NIH, 2013). Virulence is also not generally associated with *B. licheniformis.* There are, however, strains *of B. licheniformis* that have been implicated in human infection in immunocompromised individuals and neonates (EPA, 1997). Toxin-producing isolates of *B. licheniformis* have been isolated from raw milk, commercially-produced baby food and other foods involved in food poisoning incidents (Salkinoja-Salonen et al. 1999).

The production strain relevant to this application is derived from the *B. licheniformis* Si3 lineage, and the applicant has used other *B. licheniformis* production strains derived from this lineage safely for many years. Data provided with the application confirmed the identity of the parental strain and the production strain as *B. licheniformis.*

The absence of cytotoxicity has been demonstrated for the Si3 strain lineage. Data provided by the applicant described the analysis of cytotoxicity following the current guidelines for the assessment of toxigenic potential of *Bacillus* species (EFSA, 2014). The absence of toxigenic activity of the parental strain and a predecessor strain of the production strain were

demonstrated in Vero[5] cells. The lack of cytotoxicity for the same strains has also been demonstrated in Chinese hamster ovary cells (Pedersen et al. 2002).

Data was also provided with the application demonstrating that the *B. licheniformis* production strain is not detectable in the final enzyme preparation to be used as a food processing aid. The manufacturing process involves appropriate controls to prevent microbial contamination, and the microbial quality of the final enzyme preparation meets the specifications required by JECFA (2006) (see section 2.2.3).

To prepare the host organism for the expression of subtilisin, a series of genetic modification steps were undertaken. A description of these changes has been assessed and includes silencing of unnecessary proteins that would impact the yield and purity of subtilisin and to increase the ability and efficiency to transform the host. During this process, an antibiotic resistance gene was integrated into the host genome. After successful transformation of the subtilisin gene sequence was achieved, the antibiotic resistance gene was removed.

3.2.2 Gene donor organism(s)

The gene that encodes the subtilisin enzyme was chemically synthesised based on the sequence from *Pyrococcus furiosus. P. furiosus* is a bacterial extremophile isolated from volcanic marine sediment (Fiala and Stetter, 1986), and the sequence was synthesised from a Biosafety Level 1 strain.

A range of regulatory sequences and other non-coding sequences were used to prepare the expression cassettes. Full details about these sequences were provided for assessment but cannot be disclosed because it is confidential commercial information (CCI). The microbiological assessment determined that the gene donor organisms were all Risk Group 1 or Biosafety Level 1 organisms.

3.3 Characterisation of the genetic modification(s)

3.3.1 Description of DNA to be introduced and method of transformation

The chemically synthesised gene (*aprPFU2*) encoding the subtilisin enzyme was inserted into two distinct *aprPFU2* expression cassettes, designed to allow targeted integration into the host genome. Each expression cassette was transformed independently into the recipient strain. No vector backbone sequence was included in the transformations.

3.3.2 Characterisation of inserted DNA

Data from phenotypic analyses was provided, demonstrating the presence of the *aprPFU2* expression cassettes in the production strain.

3.3.3 Genetic stability of the inserted gene

A phenotypic analysis was performed over several fermentation runs to show genetic stability of the production strain. Evidence from an activity assay, purity profile and other analyses of the enzyme preparation was consistent across fermentation runs. The data show that the production strain is genetically stable.

[5] Vero cells are derived from the kidney of an African green monkey, and are continuous cell lines commonly used in microbiological research.

3.4 Safety of subtilisin

3.4.1 History of use

Subtilisin enzymes have a history of use in food in Australia and New Zealand, as well as overseas (Pariza and Foster 1983; Pariza and Johnson 2001). Serine proteinases of microbial origin, which includes subtilisin from a non-GM *B. licheniformis*, are currently permitted enzymes in the Code. The US FDA has affirmed a protease enzyme preparation containing subtilisin obtained from *B. subtilis* or *B. amyloliquifaciens* as Generally Recognized as Safe (GRAS), based on a history of common use in food since before 1958 (Federal Register 1999).

The applicant provided a 13-week toxicity study in rats and *in vitro* genotoxicity studies with an α-amylase (PPY34423) produced by a closely related predecessor strain of the production strain. This α-amylase was chosen as most appropriate for assessment of subtilisin, because the recipient strain of the α-amylase production strain is identical to the one for the subtilisin production strain, and the method used to insert the DNA is identical to the one described for the construction of the subtilisin production strain.

The applicant also provided supporting information consisting of a 14-day oral toxicity study in rats on subtilisin (batch PPA34557) produced by a host strain closely related to the production strain, and a bacterial reverse mutation assay on subtilisin (batch PPA48131) produced by an optimised version of the strain used to produce batch PPA34557.

3.4.2 Bioinformatic assessment of enzyme toxicity

The amino acid sequence of subtilisin was compared to sequences present in the UniProt database (http://www.uniprot.org/) containing entries from Swiss-Prot and TrEMBL and using the term 'toxin' to refine the search. The comparison method used a ClustalW 2.0.10 sequence alignment program (http://www.clustal.org/clustal2/) (Larkin et al. 2007) to align each sequence from the database with the subtilisin sequence.

The greatest homology found was 18.7%, indicating that the subtilisin is unlikely to be toxigenic.

Further evidence that the subtilisin is unlikely to be toxigenic comes from the absence of cytotoxicity of the parent strain and a predecessor strain of the production strain in Vero cells, as discussed in section 3.2.1.

3.4.3 Toxicology studies in animals

Studies with α-amylase PPY34423

13-week repeated dose oral toxicity study in rats (Huntingdon Life Sciences, 2013).
Regulatory status: GLP; conducted in accordance with OECD TG 408 (1998)

The test item in this study was an amylase (batch number PPY34423) from *B. licheniformis* of the Si3 strain lineage. Sprague-Dawley [Crl:CD(SD)] rats (10/sex/group, 6-7 weeks old at the start of treatment) were administered amylase at doses of 0, 80, 263 or 796 mg/kg bw/day total organic solids (TOS) for 13 weeks. Clinical signs were monitored daily, with detailed physical examination and arena observations performed weekly. Sensory reactivity, grip strength and motor activity were assessed in all animals in Study Week 12. Body weight and food consumption were recorded weekly. Animals in the control and high dose groups underwent ophthalmoscopy examinations in Week 12. Blood samples were collected from all animals in Week 13 for haematology and clinical chemistry analysis. Animals were subjected to detailed necropsy at the end of the study and organ weights were recorded.

Histopathology investigations were performed on tissues from all animals in the control and high dose groups, and on any tissues from the mid and low dose groups in which abnormalities were observed.

No deaths occurred during the study and no abnormal clinical signs were observed. There were no treatment related adverse effects on sensory activity, grip strength, motor activity, bodyweight gains, food consumption, ophthalmoscopy, haematology, blood chemistry, organ weights or macroscopic/microscopic pathology findings.

The no observed adverse effect level (NOAEL) in this study was 796 mg/kg bw/day TOS, the highest dose tested.

Studies with PPA34557 subtilisin

14-day repeated dose oral toxicity study in rats (Jai Research Foundation, 2013). Regulatory status: GLP; study design based on OECD Test Guideline (TG) 407 (2008)

The test item in this study was subtilisin (batch number PPA34557) from *B. licheniformis* of the Si3 strain lineage. Wistar rats of the RccHan: WIST strain (5/sex/group, 8-9 weeks old at the start of treatment) were administered subtilisin PPA43557 by oral gavage at doses of 0, 103.2, 340.5 or 1032 mg/kg bw/day TOS. The vehicle and negative control was reverse-osmosis water. Clinical signs were monitored daily. Bodyweight and food consumption were recorded twice weekly. Blood was collected from all animals at termination on Day 15, the samples were evaluated for haematology (including assessment of coagulation factors) and clinical chemistry. At necropsy a macroscopic examination was performed and selected organ weights were recorded. Urinalysis was not performed and no tissues were preserved for histopathological examination.

There were no deaths, no abnormal clinical signs, and no toxicologically important effects on bodyweight gains, food consumption, haematology, blood chemistry, organ weights or gross pathology findings.

The NOAEL in this study was 1032 mg/kg bw/day TOS, the highest dose tested.

3.4.4 Genotoxicity assays

Studies with α-amylase PPY34423

Bacterial reverse mutation assay using a treat and plate modification (Novozymes, 2013) Regulatory status: GLP; in accordance with OECD Guideline 471 (1997).

The test item in this study was an amylase from *B. licheniformis* of the Si3 strain lineage (batch number PPY34423). Test concentrations were reported in terms of dry matter of the test item. The vehicle and negative control was water. Test systems for the assay were *S. typhimurium* strains TA100, TA98, TA1537, TA1535 and *E. coli* WP2*uvr*A pKM101. Concentrations of 156, 313, 625, 1250, 2500 and 5000 µg/plate amylase were used in both independent main tests, which were conducted following the ‘treat and plate’ method. Both tests were conducted in the presence and absence of metabolic activation (S9 mix). All assays were conducted in triplicate.

There was no evidence of mutagenicity, cytotoxicity or precipitation at any of the dose levels investigated. Results obtained with the negative and positive controls were compatible with historical control values for the laboratory, confirming the validity of the assays. It was concluded that the amylase was not mutagenic under the conditions of the study.

Micronucleus test in cultured human lymphocytes (Covance Laboratories 2013) Regulatory status: GLP; conducted in accordance with OECD TG 487 (2010).

The test item was an amylase from *B. licheniformis* (batch number PPY34423) of the Si3 strain lineage and the vehicle control was water. The test system comprised human peripheral blood lymphocytes collected from two healthy non-smoking donors with no known recent exposure to genotoxic chemicals and radiation. Lymphocytes were pre-cultured in the presence of the mitogen phytohaemagglutinin for 48 hours prior to the start of exposure.

Following a preliminary cytotoxicity test, two experiments were conducted. In experiment I cells were exposed to the test substance in the presence or absence of metabolic activation (S9 mix) for 3 hours, then washed and cultured in fresh medium for a further 21 hours. In experiment II cells were exposed to the test substance for 24 hours in the absence of S9, then washed and cultured in fresh medium for a further 24 hours. Cells were cultured in duplicate. Concentrations of the test item ranged from 3000-5000 µg/mL in experiment I and 1000-5000 µg/mL in experiment II.

Mitomycin C and vinblastine were used as clastogenic and aneugenic positive controls respectively in the absence of S9. Cyclophosphamide was the clastogenic positive control in the presence of S9. Cytochalasin B was added to the culture medium after removal of the test item to inhibit cell division (cytokinesis) but not nuclear division (karyokinesis). At the end of the study cells were fixed on slides and scored for micronuclei. Initially 2000 cells per concentration were analysed for micronuclei. For clarification, an additional 1000 binucleate cells were analysed per culture from the 5000 µg/mL concentration in experiment II.

In experiment I, with or without S9, the frequencies of micronucleated binucleate (MNBN) cells following treatment with amylase were similar to the vehicle controls. In experiment II a statistically significant increase in frequency of MNBN cells was seen at 5000 µg/mL compared with the vehicle control, although only one of the two replicates had a MNBN frequency that exceeded the historical control range. A further 1000 cells per culture were scored at this concentration which resulted in the overall cell frequency falling within normal values. All positive controls induced significant increases in the frequency of MNBN cells, confirming the validity of the test system.

It was concluded that the amylase did not induce micronuclei in cultured human peripheral blood lymphocytes under the study conditions.

Studies with PPA48131 subtilisin from the Si3 strain lineage

Bacterial reverse mutation assay using a treat and plate modification (Covance Laboratories, 2018) Regulatory status: GLP; conducted in accordance with OECD TG 471 (1997)

The test item in this study was subtilisin (batch number PPA48131) from *B. licheniformis* of the Si3 strain lineage. Test concentrations were reported in terms of TOS of the test item subtilisin. The vehicle and negative control was water. Test systems for the assay were *Salmonella typhimurium* strains TA100, TA98, TA1537, TA1535 and *Escherichia coli* WP2*uvr*A pKM101. Concentrations of 16, 50, 160, 500, 1600 and 5000 µg/plate subtilisin were used in the first of two independent main tests. The second test used 160, 300, 625, 1250 and 5000 µg/plate. Both tests were conducted in the presence and absence of metabolic activation (S9 mix). A 'treat and plate' procedure was used for all treatments in this study as the test item may contain free amino acids (i.e. histidine and tryptophan) which may cause artefacts through growth stimulation in a standard plate-incorporation test. As the test article is a protease, which is likely to reduce S9 activity due to degradation of the S-9 enzymes, the test article formulations used in the presence of S9 were autoclaved in order to denature and inactivate the protease activity of the test article. Additional positive control treatments in the presence of S9 and the autoclaved test item were included to confirm the

inactivation of the protease activity of the test item. All assays were conducted in duplicate.

There was no evidence of mutagenicity, cytotoxicity or precipitation at any of the dose levels investigated. Results obtained with the negative and positive controls were compatible with historical control values for the laboratory, confirming the validity of the assays. It was concluded that the subtilisin was not mutagenic under the conditions of this study.

3.4.5 Potential for allergenicity

The applicant performed searches for homology of the amino acid sequence of the subtilisin enzyme that is the subject of this application to known allergens in the Allergen Online database of the University of Nebraska's Food Allergy Research and Resource Program (FARRP) and the World Health Organisation and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee. The following searches were conducted:

- A search for full-length sequence alignment for matches of > 35% identity
- A search for > 35% identity over 80 amino acids
- A search for > 35% identity over 80 amino acids with scaling enabled
- A search for 100% identity over 8 contiguous amino acids

The search identified a number of matches to known allergens, however only one match was classed as a food allergen. The others were either respiratory, aero or contact allergens, which are not usually food allergens (Dauvrin et al. 1998, Bindslev-Jensen et al. 2006).

The food allergen was Cuc m 1 (cucumisin) from muskmelon (*Cucumis melo*), which had up to 39% identity with the subtilisin produced by *B. licheniformis* across an 80 amino acid window. In contrast, the searches for full-length sequence alignment > 35% identity and search for 100% identity over 8 amino acids did not identify matches with cucumisin or any other food allergens. The full-length sequence comparison only showed up to 19.1% identity between cucumisin and subtilisin produced by *B. licheniformis*.

Similarity between cucumisin and microbial subtilisin enzymes has been reported in the scientific literature and a GRAS notice from 2017[6] (Yamagata et al. 1994; Cuesta-Herranz et al. 2003).

No evidence of food allergy to another subtilisin from *B. licheniformis* was found in a study of 400 individuals with allergy to inhalation, food, bee or wasp allergens (Bindslev-Jensen *et al.* 2006). The application subtilisin has a higher identity over the full sequence length with the subtilisin used in the study by Bindslev-Jensen *et al.* than with cucumisin (46% versus 19.1%, respectively). A literature search also found no reports of food allergy associated with consumption of subtilisin enzymes.

The applicant has indicated that the harsh conditions during potable alcohol processing will completely denature the subtilisin. In addition, the enzyme is likely to be removed from the final product by the distillation process.

Based on the available information, subtilisin is unlikely to pose an allergenicity concern.

3.4.6 Safety assessments by overseas agencies

Safety assessments of the subtilisin enzyme preparation by international agencies or other national government agencies are not available.

[6] GRAS notice 714

The US FDA has evaluated and affirmed a protease enzyme preparation containing subtilisin obtained from *B. subtilis* or *B. amyloliquifaciens* as GRAS, based on evidence of a substantial history of safe consumption of the enzyme preparations in food by a significant number of consumers prior to 1958, and corroborated by other available evidence (Federal Register 1999).

3.5 Dietary exposure assessment

The objective of this dietary exposure assessment is to review the budget method calculation presented by the applicant as a 'maximum worse case' approach to estimating likely levels of dietary exposure assuming all added subtilisin remained in the food.

The budget method is a valid screening tool for estimating the theoretical maximum daily intake (TMDI) of a food additive (Douglass et al 1997). The calculation is based on physiological food and liquid requirements, the food additive concentration in foods and beverages, and the proportion of foods and beverages that may contain the food additive. The TMDI can then compared to an ADI or a NOAEL to estimate a margin of exposure for risk characterisation purposes. In this case, given the application is only applicable to potable alcohol, the solid food component of the budget method has not been included in the calculation.

In their budget method calculation the applicant has made the following assumptions:

- maximum physiological requirement for liquids (other than milk) is 100 mL/kg body weight/day (the standard level used in a budget method calculation)
- daily consumption of processed non-milk beverages is 25 mL/kg body weight/day assuming 25% of non-milk beverages are processed
- all processed beverages contain 12% starch hydrolysates
- liquid density ~ 1
- all of the enzyme remains in the final food.

Providing daily consumption of processed non-milk beverages is 25 mL/kg body weight/day, this equates to a consumption of 3.0 g starch derived dry matter/kg body weight/day. The applicant calculates that as the highest proposed use level of subtilisin is equivalent to 9 mg TOS/kg starch dry matter, the estimated TDMI = 0.03 mg TOS/kg body weight/day. FSANZ has reviewed the assumptions and accepts the calculation. This is likely to be an overestimate of the dietary exposure given the inherence conservatisms in the budget method and that it was assumed that the enzyme remains in the final food, where as it is likely to be removed in the processing steps used to produce the potable alcohol.

4 Discussion

The safety assessment concluded that there are no safety concerns from the use of subtilisin from a GM strain of *B. licheniformis* containing the subtilisin gene from *P. furiosus*, as a processing aid at GMP levels in the production of potable alcohol. Subtilisins from other sources have a long history of safe use in food.

B. licheniformis has a long history of safe use as a source microorganism of enzyme processing aids, including several that are already permitted in the Code. The bacterium is neither toxigenic nor pathogenic. Characterisation of the GM production strain confirmed both presence and stable inheritance of the inserted subtilisin gene.

The applicant provided a 13-week toxicity study in rats and *in vitro* genotoxicity studies with

an α-amylase produced by a closely related predecessor strain of the subtilisin production strain. This α-amylase was chosen as most appropriate for assessment of subtilisin, because the recipient strain of the α-amylase production strain is identical to the one for the subtilisin production strain, and the method used to insert the DNA is identical to the one described for the construction of the subtilisin production strain. The α-amylase was not genotoxic *in vitro* and caused no adverse effects in a 13-week toxicity study in rats. The NOAEL was 796 mg/kg bw/day TOS, the highest dose tested.

A comparison of the NOAEL of the closely related α-amylase with the estimated TMDI (0.03 mg/kg bw/day TOS) indicates that the Margin of Exposure between the NOAEL and TMDI is more than 26,500.

A degree of amino acid sequence homology with a food allergen from melon was identified, but the enzyme is considered unlikely to pose an allergenicity concern because a study with another subtilisin from *B. licheniformis* found no evidence of food allergenicity, exposure is expected to be very low and there is a long history of safe use of subtilisin enzymes from other sources with no reports of food allergy identified.

5 Conclusion

Based on the reviewed toxicological data, it is concluded that in the absence of any identifiable hazard an Acceptable Daily Intake (ADI) 'not specified' is appropriate.

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