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Te Mana Kounga Kai – Ahitereiria me Aotearoa

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Supporting document 1

Risk and technical assessment – Application A1282
Subtilisin from GM *Bacillus subtilis* as a processing aid

Executive summary

Food Standards Australia New Zealand (FSANZ) received an application from Danisco Australia (IFF) to vary the Australia New Zealand Food Standards Code (the Code) to permit the use of subtilisin from genetically modified (GM) *Bacillus subtilis* as a processing aid for protein processing. Subtilisin is derived by submerged fermentation of B. subtilis containing the gene for subtilisin from Bacillus clausii.

The proposed use of subtilisin as an enzyme processing aid in the quantity and form proposed is consistent with its typical function of hydrolysing proteins. Subtilisin performs its technological purpose during the production of the nominated foods and is not performing a technological purpose in the final food.. The enzyme meets relevant identity and purity specifications in the Code.

There are no safety concerns from the use of subtilisin from a GM strain of *B. subtilis* containing the subtilisin gene from *Bacillus clausii*. Subtilisin from other sources has a long history of safe use in food. The production organism is neither pathogenic nor toxigenic. Analysis of the GM production strain confirmed the presence and stability of the inserted DNA.

In addition, the applicant provided a 90-day toxicity study in rats in which the subtilisin enzyme that is the subject of this application caused no adverse effects. The No Observed Adverse Effect Level (NOAEL) was 480.6 mg total organic solids (TOS)/kg bw/day, the highest dose tested. The theoretical maximum daily intake (TMDI) of the enzyme was calculated to be 2.13 mg TOS/kg bw/day.

A comparison of the NOAEL and the TMDI results in a margin of exposure (MoE) of approximately 200. Based on the reviewed data we concluded that in the absence of any identifiable hazard, an acceptable daily intake (ADI) of ‘not specified’ is appropriate.

FSANZ concludes there are no health and safety concerns for consumers.

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1 Introduction

IFF (trading as Danisco Australia Pty Ltd) applied to Food Standards Australia New Zealand (FSANZ) to amend the Australia New Zealand Food Standards Code (the Code) to permit the use of a protein engineered variant of the enzyme subtilisin from a genetically modified (GM) strain of *Bacillus subtilis*. This subtilisin enzyme is derived by submerged fermentation from *B. subtilis* containing the gene for subtilisin from *Bacillus clausii*.

The enzymatic cleavage of protein bonds with the help of subtilisin can be of benefit in the processing of foods and food ingredients that naturally contain proteins and peptides. Target food groups include dairy processing, egg processing, meat and fish processing, and plant and meat protein processing. Subtilisin would be used at minimum levels necessary and following Good Manufacturing Practice (GMP).

The Code already permits the use of subtilisin from *B. licheniformis* as well as other enzymes (e.g., α-acetolactate decarboxylase, α-amylase, serine protease) from *B. subtilis*..

1.1 Objectives of the Assessment

The objectives of this safety assessment are to evaluate any potential public health and safety concerns that may arise from the use of subtilisin produced by submerged fermentation of *B. subtilis*, carrying the subtilisin gene from *B. clausii*.

Some information relevant to this assessment is commercially confidential information (CCI), therefore some details cannot be provided in a public report.

2 Food Technology Assessment

2.1 Specifications for identity and purity

2.1.1 Identity

Subtilisin is a serine endopeptidase (IUBMB 2020), a type of protease (Campbell-Platt 2018; Nagodawithana and Reed 1993). According to the literature, protein engineering substantially altered subtilisin catalysis, substrate specificity, pH/rate profile, and stability to oxidative, thermal, and alkaline inactivation (Wells and Estell 1988).

The applicant described the identity of subtilisin. FSANZ has verified this information using the scientific literature and the International Union of Biochemistry and Molecular Biology (IUBMB) enzyme nomenclature reference (IUBMB 2020). The identity is summarised in Table 1.

2.1.2 Purity

Appropriate GMP controls and processes are used in the manufacture of subtilisin to ensure that the finished product does not contain any impurities that pose a risk to public health. The specification for impurities and microbial limits for the subtilisin product can be found in Table 2. FSANZ has assessed the certificates of analysis for three lots of product and agrees that the product meets the specification and the requirements for enzyme preparations of the Food Chemical Codex (2015) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA 2006).

Table 1: Identity of the subtilisin assessed.

Systematic name:	Subtilisin
Marketing Name	Will depend on the application. An example is FoodPro® PXT.
Appearance	Off white powder
Other names	Alcalase, bacillopeptidase, alkaline proteinase, protease, thermoase, subtilopeptidase
EC number[1]	3.4.21.62
CAS RN[2]**:**	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.
Biological source	Produced by submerged fermentation of *B. subtilis*, carrying the subtilisin gene from *B. clausii*.
Molecular and Structural Formula	Subtilisin is a protein. The complete amino acid sequence is known (Smith *et al.* 1966, Jacobs *et al.* 1985)

Table 2: Product specifications

Type	Measure	Application	JECFA[3]	FCC[4]
Metals mg/kg	Arsenic	≤3.0	≤5	≤5
	Cadmium	≤0.5		
	Mercury	≤0.5		
	Lead	≤5.0		
Microbiological	Total viable count CFU/g	<10,000		
	Total coliforms CFU/g	<30	<30	<30
	E. coli in 25 g	absent	absent	
	Salmonella in 25 g	absent	absent	negative
	Antibiotic activity by test	negative		
	Production strain by test	negative		

[1] The Enzyme Commission number (EC number), as implemented by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB), is a numerical classification scheme for enzymes, based on the chemical reactions they catalyse.
[2] A CAS Registry Number (CAS RN) is a unique identification number assigned by the Chemical Abstracts Service (CAS) to every chemical substance described in the open scientific literature.
[3] JECFA (2006)
[4] Food Chemicals Codex (FCC, 2018)

2.2 Manufacturing process

The enzyme is produced by a submerged fermentation process using appropriate substrate and nutrients. When fermentation is complete, the biomass is removed by centrifugation/filtration. The remaining fermentation broth containing the enzyme is filtered and concentrated. The concentrated enzyme solution is then standardised and stabilised with diluents. Finally, a polish filtration is applied.

Full details on the raw materials used for the production are available for this assessment. This information is proprietary CCI and cannot be disclosed in this report. After an assessment of all the information (including confidential information), FSANZ agrees that the manufacture of subtilisin is monitored and controlled adequately to ensure the finished preparation complies with specifications and is suitable as a processing aid in food applications.

2.3 Technological function and justification

Subtilisin would be used at minimum levels necessary and following Good Manufacturing Practice (GMP). The applicant states that the technological purpose of the subtilisin preparations is to act as a processing aid in food to degrade proteins into peptides and amino acids. This enzymatic conversion produces protein hydrolysates with improved food functional properties in a wide range of food groups including dairy eggs, meat and fish, and plant and meat proteins. This considerably broadens the target of the enzyme from the current permission for degrading proteins to lower viscosity and aid yeast growth in alcohol production.

The subtilisin enzyme preparation will be used as a processing aid. After its action, the enzyme is typically deactivated or removed during subsequent production and refining processes (EFSA CEP Panel 2023). Subtilisin is therefore not present or active in the final food or present in negligible amounts with no technical function.

FSANZ agrees that subtilisin hydrolyses proteins and peptide amides (IUBMB 2020). Proteases are used in the manufacture of a range of foods to catalyse the degradation of proteins (Nagodawithana and Reed,1993, Gomaa, 2018). The technological purpose of the enzyme in degrading proteins and producing protein hydrolysates is well established in the literature (e.g., Markland and Smith 1971, Azrin *et al.* 2022).

2.4 Food Technology conclusion

- The proposed use of this subtilisin as an enzyme processing aid fulfils a technological function consistent with its typical purpose of hydrolysing proteins.
- After its action, the enzyme is deactivated or removed during subsequent production and refining processes. Subtilisin is therefore not present in the final food or present in negligible amounts with no technical function.
- This enzymatic conversion produces protein hydrolysates with improved food functional properties in a wide range of food. This broadens use in a wider range of foods for the enzyme from the current subtilisin permissions.
- The enzyme meets the specification and the requirements for enzyme preparations of the Food Chemical Codex and JECFA

3 Safety Assessment

The objective of this safety assessment is to evaluate any potential public health and safety concerns that may arise from the use of subtilisin, produced by this *B. subtilis*, as a processing aid.

3.1 History of use of the organisms

3.1.1 Host organism

B. subtilis is a Gram-positive, rod-shaped, endospore-forming, facultative anaerobic bacterium that is widely distributed in the environment (Galano et al., 2021). The species has a long history of safe use to produce medicinal proteins, industrial enzymes, and food processing aids (Olempska-Beer et al., 2006). *B. subtilis* has a well-defined genetic background, is known to be nonpathogenic to humans and is generally not toxigenic (de Boer and Diderichsen, 1991).

B. subtilis has been granted Qualified Presumption of Safety (QPS) status by the European Food Safety Authority (EFSA) with the qualification that there is absence of cytotoxic activity (EFSA BIOHAZ Panel et al., 2023). QPS assess the taxonomic identity of the microorganism, the related body of knowledge, potential safety concerns and antimicrobial resistance. In addition to EFSA's accreditation, *B. subtilis* has a Tier 1 exemption under United States Environmental Protection Agency (EPA) regulations (EPA, 1997). Processing aids derived from *B. subtilis* have previously been granted the status of generally recognized as safe (GRAS) by the US FDA. The Joint Expert Committee on Food Additives (JECFA) has conducted a technical review of *B. subtilis*, confirming its use as a safe strain for enzyme production (JECFA, 2006).

FSANZ has previously assessed the safety of *B. subtilis* as the production organism for several food processing aids. Within the Code, Schedule 18 to Standard 1.3.3 currently permits the following enzymes derived from *B. subtilis*: α-Acetolactate decarboxylase, α-Amylase, β-Amylase, β-Galactosidase, Aqualysin 1, Asparaginase, Endo-1,4-beta-xylanase, β-Glucanase, Hemicellulase multicomponent enzyme, Maltogenic α-amylase, Metalloproteinase, Pullulanase and Serine proteinase.

The production strain was identified as *B. subtilis* CF520B and was derived from the host strain *B. subtilis* BG125. The host strain BG125 has been used to produce two food processing enzymes approved by FSANZ: β-Galactosidase (A1218) and Lactase (A1167). The production strain from BG125 have previously been confirmed as *B. subtilis* based on 100% identity of the 16s RNA sequence (A1218).

The applicant applied the safe strain lineage concept of Pariza and Johnson (2001). Using this concept, the information provided by the applicant showed that the risk of toxin production was very low. Information provided by the applicant demonstrated that *B. subtilis* strains from this lineage are non-cytotoxic. The stability of the production strain was demonstrated phenotypically through consistent batch parameters and application of suitable microbiological controls through production. Additionally, the organism was not detected within the final enzyme product in three independent fermentation batches.

No public health and safety concerns were identified, and the production organism is neither pathogenic nor toxigenic.

3.1.2 Gene donor organism

The subtilisin gene of the production strain was sourced from *Bacillus clausii* with American Type Culture Collection number 21536. The taxonomy of this organism was confirmed via an identification certification from the University of Bristol and Pyrolysis-Mass Spec analysis. *B. clausii* has been granted QPS status by the EFSA (EFSA BIOHAZ Panel et al., 2023). The nomenclature for the donor species was recently updated to *Shouchella clausii*, however this name change does not affect the safety assessment of the organism (Joshi et al., 2021). The continued use of the basonym, *B. clausii*, is appropriate.

3.2 Characterisation of the genetic modifications

3.2.1 Description of DNA to be introduced and method of transformation.

Multiple copies of a protein engineered variant of the subtilisin gene[5] from *B. clausii* were introduced into the genome of the host *B. subtilis* strain using standard methodologies. Data provided by the applicant and analysed by FSANZ confirmed the identity of the protein engineered subtilisin enzyme.

In addition to the subtilisin gene, the inserted expression cassette also contained a chloramphenicol resistance gene derived from the Bacillus plasmid pC194. Chloramphenicol resistance allows for the selection of transformants containing the cassette. Data provided by the applicant (Section 2.1.2) indicates that the centrifugation/filtration steps would remove the production strain, containing the chloramphenicol resistance gene, from the final subtilisin enzyme preparation.

3.2.2 Characterisation of the inserted DNA

Data provided by IFF and analysed by FSANZ confirmed the presence of the inserted DNA in the production strain, at the site of the endogenous alkaline protease *aprE* in *B. subtilis*.

3.2.3 Stability of the introduced DNA

The results of whole genome sequencing confirmed that the inserted subtilisin gene is integrated into the genome of the production strain and does not have the ability to replicate autonomously. The inserted gene is therefore considered to be genetically stable.

3.3 Safety of subtilisin

3.3.1 History of use of subtilisin

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 include subtilisin from a non-GM *B. licheniformis*, are currently permitted enzymes in the Code. (Federal Register 1999).

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[5] In *Bacillus* species, subtilisins are a class of alkaline serine proteases (Harwood and Kikuchi 2022). The terms ‘subtilisin’, alkaline protease’ and ‘serine protease/proteinase’ have been used interchangeably in this application.

3.3.2 Bioinformatic assessment of subtilisin toxicity

A BLAST search for homology, using the amino acid sequence of the subtilisin as the query sequence, was performed on proteins marked as toxins in the UniProt[6] database (release: 2022-12-14). In addition, a specific BLAST search for homology of the mature subtilisin sequence was also performed. Search results provided to FSANZ as CCI showed that the subtilisin enzyme does not show significant homology to any protein sequence identified as a toxin.

3.3.3 Evaluation of toxicity studies

The applicant assessed the safety of subtilisin according to the decision tree of Pariza and Johnson (2001). Pariza and Johnson (2001) and Pariza and Cook (2010) have published guidelines for the safety assessment of microbial enzyme preparations. The safety assessment of a given enzyme preparation is based upon an evaluation of the toxigenic potential of the production organism. The applicant followed a decision tree pathway with the outcome that the subtilisin enzyme is “accepted” as safe for its intended use (due to demonstrated safe strain lineage of the production strain), provided that an adequate margin of exposure can be demonstrated.

The applicant provided commercial-in-confidence information to support the safe strain lineage of the production strain *B. subtilis*. Safety studies on *B. subtilis* strains and enzyme preparations derived from recombinant production strains showed that, regardless of the production organism strain, all enzyme preparations were found to be non-pathogenic, non-mutagenic, and non-clastogenic. Studies conducted on strains from the safe strain lineage support other production strains pertaining to the same safe strain lineage. The safe strain lineage concept has been discussed by Pariza and Johnson (2001) and is consistent with Food and Agriculture Organization/World Health Organization guidance on risk assessment of food enzymes (FAO/WHO 2020).

In addition to demonstration of the safe strain lineage, the applicant submitted a 90-day oral toxicity study on the specific subtilisin enzyme that is the subject of this application.

3.3.3.1 Animal studies with subtilisin

90-day repeated dose oral toxicity study in rats ([Redacted], 2011). Regulatory status: GLP; conducted in accordance with OECD TG 408 (1998)

B. subtilis subtilisin was administered daily by oral gavage to Sprague Dawley (Ntac:SD strain) rats (10/sex/group) at doses of 0, 120.2, 240.3, or 480.6 mg TOS/kg bw/day, for 90 days. The vehicle/negative control was saline, and the dose volume was 5 mL/kg bw/day. The animals were pair housed (same sex) under standard laboratory conditions of environment and husbandry.

Animals were observed daily for clinical condition and behavioural changes. Detailed clinical observations, body weights and feed consumption were recorded weekly. A functional battery of tests was performed on each animal in the last 2 weeks of dosing, including sensory reactivity, grip strength, and motor activity assessments. Ophthalmic examination was conducted on all animals of the control and high-dose groups at study termination. Prior to necropsy, blood and urine samples were collected for haematology, clinical chemistry, and urinalysis. All animals underwent a detailed necropsy at study termination, including full macroscopic examination of an extensive range of organs and tissues.

Selected organs and tissues from the control animals, high-dose group animals and unscheduled deaths were subjected to microscopic examination.

Four animals were found dead – two males and one female from the mid-dose group on days 20, 87, and 42, respectively; and one male from the high-dose group on day 60. No

[6] http://www.uniprot.org

adverse clinical signs were observed prior to death in any of these animals. All animals showed blood, blood clots, and/or reddish-watery fluid in the chest cavity at necropsy, indicative of a dosing error. These mortalities were therefore considered to be gavage errors and not treatment related. One mid-dose female was killed in a moribund condition and microscopic examination revealed inflammation of the lungs and larynx, suggestive of a dosing error. This mortality was therefore also considered to be a gavage error and not treatment related.

A slight decrease in body weight gain was observed in the high dose males, however as this finding was only slight and within the range of historical reference data, it was considered to not be of toxicological significance.

There was no treatment related adverse effects on clinical examinations, sensory activity, grip strength, motor activity, food consumption, ophthalmoscopy, haematology, blood chemistry, urinalysis, organ weights or macroscopic/microscopic pathology findings.

It was concluded that the NOAEL in this study was 480.6 mg TOS/kg bw/day, the highest dose tested.

3.3.4 Potential for allergenicity

The applicant provided details of recent searches (2021) for amino acid sequence homology of the subtilisin enzyme to known allergens, using the FARRP allergen protein database[7], using four sequence alignments: the full-length protein (more than 35% identity), an 80 mer sliding window (more than 35% identity), a scaled 80 mer sliding window (more than 35% identity), and an 8 mer sliding window (100% identity).

No homology to sequences of known food allergens was identified using these search parameters. The applicant concluded that that oral intake of subtilisin is not anticipated to pose any food allergenic concern.

3.3.5 Safety assessments by overseas agencies

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

Subtilisin from various microorganisms is considered safe by international agencies and *B. subtilis* is widely accepted as a safe production organism. The US FDA has responded with a "No Questions" letter to GRAS Notifications (GRN) 714 (Subtilisin from *B. amyloliquefaciens* produced in *B. subtilis*) and 905 (use of *B. subtilis* strain SG188) (FDA 2018, 2020). In addition, the applicant provided information on another GRAS Notification (989) that is pending (Subtilisin from *B. clausii*). However, these are not assessments by the FDA and are not accepted by FSANZ as an assessment by an international agency.

[7] AllergenOnline: http://www.allergenonline.org/

3.4 Toxicology conclusions

There are no safety concerns from the use of subtilisin from a GM strain of *B. subtilis* containing the subtilisin gene from *B. clausii*. Subtilisins from other sources have a long history of safe use in food. Subtilisin was assessed according to the safe strain lineage concept. The applicant provided commercial-in-confidence information to support the safe strain lineage of the production strain *B. subtilis*. Studies conducted on strains from the safe strain lineage support other production strains pertaining to the same safe strain lineage. In addition, the applicant provided a 90-day toxicity study in rats in which subtilisin caused no adverse effects. The NOAEL was 480.6 mg TOS/kg bw/day, the highest dose tested.

3.5 Dietary Exposure

The objective of the dietary exposure assessment was to review the budget method calculation presented by the applicant as a ‘worst-case scenario’ approach to estimating likely levels of dietary exposure if all the TOS from the subtilisin preparation remain 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 be compared to an acceptable daily intake or a NOAEL to estimate a margin of exposure (MOE) for risk characterisation purposes. Whilst the budget method was originally developed for use in assessing food additives, it is also appropriate to use for estimating the TMDI for processing aids (FAO/WHO 2020b). The method is used by international regulatory bodies and the FAO/WHO Joint Expert Committee on Food Additives (JECFA) (FAO/WHO 2021) for dietary exposure assessments for processing aids.

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

- all solid foods and non-milk beverages contain the maximum use level of 569 mg TOS/kg in the raw material (proteins from various sources)
- the maximum physiological requirement for solid food (including milk) is 25 g/kg bw/day.
- 25% of solid food are processed high protein products.
- protein bars contain a ratio of raw material (proteins from various sources) to the final food of 0.3.
- protein hydrolysates used in e.g., soups, bouillons, dressings contain a ratio of raw material (proteins from various sources) to the final food of 0.17.
- among all solid foods, protein bars produced the highest theoretical maximum level in the final food when each solid food was assessed individually. Therefore, the enzyme preparation use level for protein bars was used in the budget method calculation to represent all processed solid foods.
- the maximum physiological requirement for liquid is 100 mL/kg bw/day (the standard level used in a budget method calculation for non-milk beverages)
- 10% of non-milk beverages are processed high protein products.
- sports drinks contain a ratio of raw material (proteins from various sources) to the final food of 0.3.
- among all the non-milk beverages, use in sports drinks was the only use presented for beverages, therefore the enzyme preparation use level for sports drinks was used in the budget method calculation for all processed non-milk beverages.

- all the TOS from the enzyme preparation remains in the final food.
- all producers use this subtilisin preparation at the highest use level.
- the final foods containing the theoretical amount of the subtilisin preparation would be consumed daily over the course of a lifetime.

Based on these assumptions, the applicant calculated the TMDI of the TOS from the enzyme preparation to be 2.73 mg TOS/kg bw/day.

As assumptions made by the applicant differ from those that FSANZ would have made in applying the budget method, FSANZ independently calculated the TMDI using the following assumptions that are conservative and reflective of a first tier in estimating dietary exposure:

- The maximum physiological requirement for solid food (including milk) is 50 g/kg bw/day (the standard level used in a budget method calculation where there is potential for the enzyme preparation to be in baby foods or general-purpose foods that would be consumed by infants).
- FSANZ would generally assume 12.5% of solid foods contain the enzyme based on commonly used default proportions noted in the FAO/WHO Environmental Health Criteria (EHC) 240 Chapter 6 on dietary exposure assessment (FAO/WHO 2009). However, the applicant has assumed a higher proportion of 25% based on the nature and extent of use of the enzyme and therefore FSANZ has also used this proportion for solid foods as a worst-case scenario.
- FSANZ would generally assume 25% of non-milk beverages contain the enzyme based on commonly used default proportions noted in the FAO/WHO Environmental Health Criteria (EHC) 240 Chapter 6 on dietary exposure assessment (FAO/WHO 2009).

A further refinement was undertaken, using the following assumptions that are also conservative but more representative of actual food consumption patterns, and are a second tier in estimating dietary exposure:

- The mean amount consumed of solid food (including milk) is 20 g/kg bw/day (based on the Australian 2011-12 National Nutrition and Physical Activity Survey (2011-12 NNPAS) consumption data[8]).
- The mean amount consumed of non-milk beverages is 30 g/kg bw/day (based on the 2011-12 NNPAS consumption data).
- FSANZ would generally assume 12.5% of solid foods contain the enzyme based on commonly used default proportions noted in the FAO/WHO Environmental Health Criteria (EHC) 240 Chapter 6 on dietary exposure assessment (FAO/WHO 2009).

[8] Australian Health Survey: Nutrition and Physical Activity | Australian Bureau of Statistics (abs.gov.au) (accessed 15 November 2023)

- However, the applicant has assumed a higher proportion of 25% based on the nature and extent of use of the enzyme and therefore FSANZ has also used this proportion for solid foods as a worst-case scenario.
- FSANZ would generally assume 25% of non-milk beverages contain the enzyme based on commonly used default proportions noted in the FAO/WHO Environmental Health Criteria (EHC) 240 Chapter 6 on dietary exposure assessment (FAO/WHO 2009).

All other inputs and assumptions used by FSANZ remained as per those used by the applicant. The TMDI of the TOS from the enzyme preparation based on FSANZ's calculations was 6.40 mg TOS/kg bw/day for the first-tier calculation using consumption data based on physiological requirements. The TMDI for the second tier refined calculation based on actual consumption amounts was 2.13 mg TOS/kg bw/day.

The second tier refined TMDI is closer to actual dietary exposure over a long period of time, or over a lifetime given it is based on actual total food and beverage consumption amounts from nutrition survey data.

Both the FSANZ and applicant's estimates of the TMDI will be overestimates of the dietary exposure given the conservatisms in the budget method. This includes the assumption that all the TOS from the enzyme preparation remains in the final foods and beverages whereas the applicant has stated that the enzyme is likely to either be inactivated or removed during processing. If any inactivated enzyme remained after processing, it would be present in insignificant quantities and perform no function in the final food.

4 Safety assessment conclusion

Subtilisins from a number of sources have a long history of safe use in food.

There are no safety concerns from the use of subtilisin from a GM strain of *B. subtilis* containing the subtilisin gene from *B. clausii*. The production organism is neither pathogenic nor toxigenic. Analysis of the GM production strain confirmed the presence and stability of the inserted DNA. The available data supports the safe strain lineage of the production strain *B. subtilis.*

A comparison of the NOAEL and the TMDI results in an MOE of approximately 200. Based on the reviewed data, it is concluded that in the absence of any identifiable hazard, an acceptable daily intake 'not specified' is appropriate.

5 References

[Redacted] (2011) 90-Day oral toxicity study in rats.

Azrin, N.A.M., Ali, M.S.M., Rahman, R.N.Z.R.A., Oslan, S.N. and Noor, N.D.M., 2022. Versatility of subtilisin: A review on structure, characteristics, and applications. Biotechnology and applied biochemistry, 69(6), pp.2599-2616.

Campbell-Platt, G (2018) Food Science and Technology (second edition), John Wiley and Sons, West Sussex

de Boer AS, Diderichsen B. (1991) On the safety of Bacillus subtilis and B. amyloliquefaciens: a review. Applied Microbiology and Biotechnology. 36(1):1-4.

Douglass JS, Barraj LM, Tennant DR, Long WR, Chaisson CF (1997) Evaluation of the Budget Method for screening food additive intakes. Food Additives and Contamination 14:791–802

EFSA BIOHAZ Panel, Koutsoumanis, K., Allende, A., Alvarez-Ordonez, A., Bolton, D., Bover-Cid, S., Chemaly, M., De Cesare, A., Hilbert, F., Lindqvist, R., Nauta, M., Peixe, L., Ru, G., Simmons, M., Skandamis, P., Suffredini, E., Cocconcelli, P. S., Fernández Escámez, P. S., Maradona, M. P., … Herman, L. (2023). Updated list of QPS-recommended microorganisms for safety risk assessments carried out by EFSA [Data set]. Zenodo.

EFSA CEP Panel (EFSA Panel on Food Contact Materials, Enzymes and Processing Aids) 2023. Safety evaluation of the food enzyme subtilisin from the non-genetically modified Bacillus paralicheniformis strain DP-Dzx96. EFSA Journal, 21(8), 1–11. https://doi.org/10.2903/j.efsa.2023.8155

EPA (1997) Final risk assessment of Bacillus subtilis. US Environmental Protection Agency

FAO/WHO (2009) Environmental Health Criteria 240. Principles and Methods for the Risk Assessment of Chemicals in Food Chapter 6 – Dietary exposure assessment of chemicals in food, WHO, Geneva.

FAO/WHO (2020) Environmental Health Criteria 240. Principles and Methods for the Risk Assessment of Chemicals in Food. Section 9.1.4.2: Enzymes. WHO, Geneva https://cdn.who.int/media/docs/default-source/food-safety/publications/section9-1-4-2-enzymes.pdf?sfvrsn=e238e86e_2

FAO/WHO (2020b) Environmental Health Criteria 240. Principles and Methods for the Risk Assessment of Chemicals in Food. Chapter 6: Dietary exposure assessment of chemicals in food. Second Edition 2022. WHO, Geneva. https://www.who.int/docs/default-source/food-safety/publications/chapter6-dietary-exposure.pdf?sfvrsn=26d37b15_6

FAO/WHO (2021) Evaluation of certain food additives: eighty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives. WHO Technical Report Series, No. 1027

FCC (2018). Enzyme preparations. In: Food Chemicals Codex, 11th edition. Rockville (MD): United States Pharmacopeial Convention

FDA (2018) Agency response letter to GRAS Notice No. GRN 714: Subtilisin from *Bacillus amyloliquefaciens* produced in *Bacillus subtilis*. US Food and Drug Administration, Center for Food Safety and Applied Nutrition, Office of Food Additive Safety, Maryland, USA

FDA (2020) Agency response letter to GRAS Notice No. GRN 905: Bacillus subtilis SG188. US Food and Drug Administration, Center for Food Safety and Applied Nutrition, Office of Food Additive Safety, Maryland, USA

Federal Register (1999) Carbohydrase and protease enzyme preparations derived from Bacillus subtilis or Bacillus amyloliquefaciens; Affirmation of GRAS status as direct food ingredients. Federal Register Vol. 64, No. 78. Friday April 23, 1999. https://www.govinfo.gov/content/pkg/FR-1999-04-23/pdf/99-10011.pdf

Galano M., van den Dungen MW., van Rij T., Abbas HE. (2021) Safety evaluation of food enzymes produced by a safe strain lineage of Bacillussubtilis. Regulatory Toxicology and Pharmacology, 125:105030.

Gomaa, AM (2018) Application of Enzymes in Brewing. Journal of Nutrition and Food Science Forecast, vol 1 ed 1.

Harwood CR, Kikuchi Y (2022) The ins and outs of *Bacillus* proteases: activities, functions, and commercial significance. FEMS Microbiol Rev. 46(1):fuab046

International Union Of Biochemistry And Molecular Biology (IUBMB) 2020, https://www.qmul.ac.uk/sbcs/iubmb/enzyme/EC34/3421.html

Jacobs, M., Eliasson, M., Uhlén, M. and Flock, J.I., 1985. Cloning, sequencing and expression of subtilisin Carlsberg from Bacillus licheniformis. Nucleic Acids Research, 13(24), pp.8913-8926.

JECFA (2006) General Specifications and Considerations for Enzyme Preparations. In: Combined Compendium of Food Additive Specifications [Online Edition]. World Health Organization, Geneva, Switz. Available at: http://www.fao.org/food/food-safety-quality/scientific-advice/jecfa/jecfa-additives/en/.

Joshi, A., Thite, S., Karodi, P., Joseph, N., Lodha, T. (2021). *Alkalihalobacterium elongatum* gen. nov. sp. nov.: An Antibiotic-Producing Bacterium Isolated from Lonar Lake and Reclassification of the Genus *Alkalihalobacillus* into Seven Novel Genera. Frontiers in Microbiology, 12:722369.

Markland Jr, F.S. and Smith, E.L., 1971. 16 Subtilisins: Primary structure, chemical and physical properties. *The enzymes*, *3*, pp.561-608.

Nagodawithana T and Reed G (1993) Enzymes in Food Processing (3rd ed.) Academic Press, Inc. London.

Olempska-Beer, Z.S., Merker, R.I., Ditto, M.D., DiNovi, M.J. (2006) Food-processing enzymes from recombinant microorganisms - A review. Regulatory Toxicology and Pharmacology 45:144-158

Pariza MW, Cook M (2010) Determining the safety of enzymes used in animal feed. Regulatory Toxicology and Pharmacology 56: 332-342.

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

Pariza MW, 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.

Smith, E.L., Markland, F.S., Kasper, C.B., DeLange, R.J., Landon, M., and Evans, W.H., 1966. The complete amino acid sequence of two types of subtilisin, BPN'and Carlsberg. Journal of Biological Chemistry, 241(24), pp.5974-5976.

United States Pharmacopeial Convention. Council of Experts and United States Pharmacopeial Convention. Food Ingredient Expert Committee, 2015. Food Chemicals Codex. US Pharmacopeia Conv.

Wells, J.A. and Estell, D.A., 1988. Subtilisin—an enzyme designed to be engineered. Trends in biochemical sciences, 13(8), pp.291-297.