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Supporting document

Risk and Technical Assessment – A1219

Alpha-amylase from GM *Bacillus licheniformis* as a processing aid

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Executive summary

Danisco New Zealand Limited (Danisco) applied to Food Standards Australia New Zealand (FSANZ) to amend the Australia New Zealand Food Standards Code (the Code) to permit the use of alpha-amylase produced by a genetically modified (GM) *Bacillus licheniformis* strain M1584 as an enzyme processing aid. The alpha-amylase enzyme is produced by submerged fermentation of the *B. licheniformis* strain carrying the alpha-amylase gene originating from an isolate of *Cytophaga* species (sp.).

FSANZ has undertaken an assessment to determine whether the enzyme achieves its technological purpose 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 alpha-amylase as an enzyme in starch processing, brewing of beverages and production of potable alcohol is consistent with its typical function of catalysing the hydrolysis of starch. Analysis of the evidence provides adequate assurance that the proposed use of the enzyme, at a level not higher than necessary to achieve the desired enzyme reaction under Good Manufacturing Practice (GMP), is technologically justified.

Alpha-amylase performs its technological purpose during the production of food and is not performing a technological purpose in the final food. It is therefore appropriately categorised as a processing aid as defined in the Code. There are relevant identity and purity specifications for the enzyme in the Code.

No public health and safety concerns were identified in the assessment of alpha-amylase produced by this GM *B. licheniformis* strain JML1584 under the proposed use conditions. The host organism *B. licheniformis* Bra7 from which *B. licheniformis* JML1584 was derived, is neither pathogenic nor toxigenic and has a long history of safe use in food. Analysis of the modified production strain confirmed the presence and stability of the inserted DNA.

Toxicity testing of the enzyme showed no evidence of genotoxicity *in vitro* and the no observed adverse effect level (NOAEL) in a 90-day oral gavage study in rats was the highest

dose tested, 500 mg total organic solids (TOS)/kg bw/day. The theoretical maximum daily intake (TMDI) was calculated to be up to 0.79 mg TOS/kg bw/day. Comparison of the NOAEL with the TMDI gives a margin of exposure (MOE) of approximately 600.

Bioinformatics analysis indicated that the enzyme shows no significant homology with any known toxins or allergens.

Based on the reviewed 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

Danisco New Zealand Limited (Danisco), applied to Food Standards Australia New Zealand (FSANZ) to amend the Australia New Zealand Food Standards Code (the Code) to permit the use of alpha-amylase by a genetically modified (GM) *Bacillus licheniformis* strain as an enzyme processing aid. The alpha-amylase enzyme is produced by submerged fermentation of the *B. licheniformis* strain JML1584 carrying the alpha-amylase gene originating from an isolate of *Cytophaga* species (sp.). The enzyme is intended to be used in brewed beverages, potable alcohol production and starch processing at the minimum level required to achieve the desired effect, in accordance with the principles of current Good Manufacturing Practice (GMP).

There are permissions in the Code for alpha-amylase to be used as a processing aid, including from plant sources, in malted cereals (S18—4(4)), and from various microbial origins including *Bacillius licheniformis* (S18—4(9) and S18—9). However, there is no permission for alpha-amylase from *B. licheniformis* carrying the alpha-amylase gene originating from *Cytophaga* sp. as requested by the applicant. If permitted following a pre-market assessment, the alpha-amylase that is the subject of this application would provide an additional option for manufacturers seeking to use alpha-amylase as a processing aid in brewed beverages, potable alcohol production and starch processing.

The applicant markets different liquid enzyme preparations containing the enzyme concentrate under various names including 'Spezyme SL' and 'GC 126'. The carriers and stabilisers used in the enzyme preparations differ depending on the intended use of the preparation.

1.1 Objectives of the assessment

The objectives of this risk and technical assessment were to:

- determine whether the proposed purpose is a solely technological purpose (function) and that the enzyme achieves its technological purpose as a processing aid in the quantity and form proposed to be used
- evaluate potential public health and safety issues that may arise from the use of this enzyme, produced by a genetically modified organism, as a processing aid, specifically by considering the:
  - history of use of the host and gene donor organisms
  - characterisation of the genetic modification(s)
  - safety of the enzyme.

2 Food technology assessment

2.1 Characterisation of the enzyme

The production microorganism of the enzyme is a GM strain of *B. licheniformis,* i.e. *B. licheniformis strain JML1584.* The donor microorganism of the alpha-amylase gene is an isolate of *Cytophaga* sp. Further details are provided in section 3.

The applicant provided relevant information regarding the identity of the alpha-amylase enzyme. FSANZ verified this using an appropriate enzyme nomenclature reference (IUBMB 2022). Details of the identity of the enzyme are provided in Table 1.

Table 1 Identity of alpha-amylase

Accepted IUBMB[1] name:	α-amylase
Systematic name:	4-α-D-glucan glucanohydrolase
Other names:	glycogenase; endoamylase; Taka-amylase A; 1,4-α-D-glucan glucanohydrolase
EC number:	3.2.1.1
Reaction:	Endohydrolysis of (1→4)-α-D-glucosidic linkages in polysaccharides containing three or more (1→4)-α-linked D-glucose units

For the representation of the endohydrolysis reaction of (1→4)-α-D-glucosidic linkages catalysed by alpha-amyalse, refer to BRENDA[2] (Chang et al., 2021).

2.2 Manufacturing process

2.2.1 Production of the enzyme

The enzyme is produced by submerged fermentation of the genetically modified strain of *B. licheniformis.* The fermentation steps are propagation of the culture (inoculation), seed fermentation and main fermentation. A recovery stage follows fermentation, to separate the biomass and to purify, concentrate and stabilise the enzyme. The enzyme is concentrated via ultrafiltration to remove low molecular weight compounds. The enzyme concentrate is then formulated into the enzyme preparation[3] and packaged. The applicant states that production of the enzyme is done in accordance with Good Manufacturing Practice (GMP). A manufacturing flow-chart was provided in an appendix to the application. Further details on the manufacturing process, raw materials and ingredients used in the production of the enzyme preparations were provided as Confidential Commercial Information (CCI).

The applicant provided an allergen declaration for the enzyme concentrate as an appendix to the application. The declaration indicates that wheat and soybeans could be present in the enzyme concentrate, from dextrose and soy flour used during fermentation.

2.2.2 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 (FAO/WHO 2006) and in the Food Chemicals Codex (FCC 2008). 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. Schedule 3 of the Code also includes specifications for arsenic and heavy metals (section S3—4) if they are not already detailed within specifications in sections S3—2 or S3—3.

[1] International Union of Biochemistry and Molecular Biology
[2] BRENDA is available at www.brenda-enzymes.org
[3] 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.

The applicant provided the results of analysis of three different batches of their enzyme preparation Spezyme SL. Table 2 provides a comparison of the analyses 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 met all relevant specifications for arsenic and metals and the microbiological criteria.

Table 2 *Comparison of the applicant's Spezyme SL enzyme preparation compared to JECFA, Food Chemicals Codex, and Code specifications for enzymes*

Analysis	Analysis provided by manufacturer*	Specifications		
		JECFA (2006)	*Food Chemicals Codex (FCC, 2020)*	*Australia New Zealand Food Standards Code (section S3—4)*
Lead (mg/kg)	<0.01-0.1	≤ 5	≤ 5	≤2
Arsenic (mg/kg)	<0.01	-	-	≤1
Cadmium (mg/kg)	<0.001	-	-	≤1
Mercury (mg/kg)	<0.005	-	-	≤1
Coliforms (cfu/g)	<1	≤30	≤30	-
***Salmonella* (in 25 g)**	Negative	Absent	Negative	-
***E. coli* (in 25 g)**	Negative	Absent	-	-
Antibiotic activity	Negative	Absent	-	-

* across three samples

Whilst the manufacturing processes ensure the production microorganism is removed from the final enzyme preparation, the food enzyme is a biological isolate of variable composition, containing the enzyme protein, as well as organic and inorganic material derived from the microorganism and fermentation process. Refer to section 3.4 below for the total organic solids (TOS) value.

2.3 Technological purpose of the enzyme

Danisco's alpha-amylase is intended for use in brewed beverages, potable alcohol production and starch processing.

Alpha-amylase belongs to the hydrolase enzyme class and is a type of glycosidase (which hydrolyse O- and S-glycosyl compounds). Specifically, alpha-amylase acts on starch, glycogen and related polysaccharides and oligosaccharides, catalysing the endohydrolysis of (1→4)-α-D-glucosidic linkages in polysaccharides containing three or more (1→4)-α-linked D-glucose units, releasing free sugar groups (IUBMB 2022). Alpha-amylase produced from *B. licheniformis* is more heat stable than some other alpha amylases and can therefore be used in situations where a higher heat is used (Nagodawithana and Reed,1993, Anstrup)

The applicant states that in brewing the enzyme would be used in the liquefaction and saccharification of starch (mashing) from malted cereal and other plant sources, such as barley, wheat, sorghum, rice, potato and tapioca. The resultant process liquors (worts) are fermented, to produce ethanol. As identified by the applicant and supported by the literature (Damodaran 2008), alpha-amylase is used to maximise the conversion of starchy substrates to fermentable carbohydrate. This is in addition to relying on amylases present in malted

grains. The enzyme would be added during the mashing of cereal or other plant sources in the initial stages of the brewing process.

In potable alcohol production, alpha-amylase can be used to hydrolyse the starch in raw materials such as malt, rye, and potatoes to sugars that are fermented, forming ethanol (Nagodawithana and Reed,1993).

In relation to starch processing, as stated by the applicant and supported by literature (Nagodawithana and Reed,1993, Damodaran 2008), alpha-amylase from the *Bacillus* species can be used in combination with other enzymes to produce sugar syrups. The alpha-amylase is added to a starch slurry at the start of the process to liquefy the slurry to a viscosity which enables efficiencies for starch processing and end use purposes. According to the applicant, the resulting substance would be used for the manufacture of starch syrups or could be further treated as glucose-rich syrups that can be further processed to meet various specifications: crystallised to produce dextrose, isomerised to produce high fructose corn syrup, or fermented to produce organic acids or amino acids. Alpha-amylase sourced from the *Bacillus* species can withstand the temperatures needed during that process (Nagodawithana and Reed,1993, Damodaran 2008).

The applicant provided information on the physical and chemical properties of the enzyme preparation. Table 3 summarises this information.

Table 3 *Physical and chemical properties of alpha amylase*

Physical and chemical properties of commercial enzyme preparation	
Enzyme activity	27150 – 31850 DLU/g[1]
Appearance	Subject to formulation of final enzyme preparation
Temperature range	Optimum 60-70°C
Storage stability	Stable for 20 months at room temperature (>80% activity remaining)[2]

1 Example only (Spezyme SL). Enzyme activity is subject to the enzyme preparation. Determined using the Applicant's assay method.
2 Example only (Spezyme SL), subject to formulation of final enzyme preparation

As alpha-amylase performs it's technological function during the production of the foods mentioned above it would be regarded by FSANZ as functioning as a processing aid for the purposes of the Code.

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 applicant stated that the enzyme would be used at GMP levels.

2.4 Technological justification

As outlined above, alpha-amylase is used to catalyse the breakdown of starch to free sugar groups. It can be used to liquefy starch during the production of sugar syrups made from starch. In brewed beverages and potable alcohol it is used to maximise the conversion of starchy substrates to fermentable carbohydrate. Its use as requested by the applicant is therefore technologically justified.

2.5 Food technology conclusion

FSANZ concludes that the proposed use of this alpha-amylase in starch processing, brewing of beverages and production of potable alcohol is consistent with its typical function of catalysing the hydrolysis of starch.

Analysis of the evidence provides adequate assurance that the proposed use of the enzyme, at a level not higher than necessary to achieve the desired enzyme reaction (i.e. according to GMP levels), is technologically justified.

Alpha-amylase performs its technological purpose during the production of food and is not performing a technological purpose in the final food. It is therefore appropriately categorised as a processing aid as defined in the Code.

There are relevant identity and purity specifications in the Code that enzyme preparations containing this enzyme would need to meet if its use is approved.

3 Safety assessment

3.1 History of use

Some information relevant to this section is Confidential Commercial Information (CCI), so full details cannot be provided in this public report.

3.1.1 Host organism

There is a long history of safe industrial use of *B. licheniformis* strains as a microorganism to produce enzymes for food processing (de Bore et al., 1994; Sewalt et al., 2018; Muras et al., 2021). From literature available, there are no published reports suggesting any invasive properties of this microorganism to human health.

Bacillus licheniformis is a gram-positive, endospore-forming, mesophilic and facultative anaerobic bacterium, present in soil and the marine environment. Its optimal growth temperature is around 50°C and it can survive at much higher temperatures. *B. licheniformis* is classified under the genus of *Bacillus* within the family of *Bacillaceae* in the order of *Bacillales* of the class of *Bacilli* within phylum XIII of *Firmicutes* (Whitman 2009).

B. licheniformis isolates have been identified as the cause of foodborne illness associated with cooked meats, ice cream, cheese, raw milk, infant feed, prawns (Salkinoja-Salonen et al. 1999). However, the incidence of human infections and pathogenicity is rare and tends to be limited to immune-compromised individuals (Haydushka et al, 2012; Logan, 2012).

Industry strains of *B. licheniformis* have been generally considered as non-pathogenic due to the absence of invasive traits (Muras et al., 2021). Industry strains of *B. licheniformis* have been regarded as safe as a biological agent by the US Environmental Protection Agency (2017) and the European Food Safety Authority (2010) provided that they do not produce toxins and do not harbour any acquired antimicrobial resistance genes to clinically relevant antibiotics. Industry strains of *B. licheniformis* have been safely used to produce industrial enzymes as processing aids since 1972 (de Bore et al., 1994; Sewalt et al., 2018; Muras et al., 2021). Using the safe strain concept, the information provided by the applicant (CCI) showed that the risk of toxin production by the production strain was very low.

FSANZ has previously assessed the safety of enzymes as processing aids produced by *B.*

licheniformis. Schedule 18 of Standard 1.3.3 of the Code permits the use of the following enzymes produced by *B. licheniformis*: alpha-amylase, chymotrypsin, endo-1,4-beta-xylanase, beta-galactosidase, glycerophospholipid cholesterol acyltransferase, maltotetraohydrolase, pullulanase, and serine proteinase.

The production strain referred to in this application, *B. licheniformis* strain JML1584, was derived from proprietary strain *B. licheniformis* Bra7 by incorporating a synthetic alpha-amylase variant gene based on the sequence of the alpha-amylase gene of an isolate of *Cytophaga* sp.

B. licheniformis Bra7 is an industrial strain used for alpha-amylase production by the applicant since 1989. FSANZ has accepted a number of enzymes produced by *B. licheniformis* Bra7 as processing aids in the past, such as maltogenic alpha-amylase from GM *B. licheniformis* (2021); pullulanase from *B. licheniformis* (2019); and acyltransferase from *B. licheniformis* (2011). FDA has approved a number of enzymes produced by *B. licheniformis* Bra7 for GRAS status, such as alpha-amylase *B. licheniformis* carrying an alpha-amylase gene from *Geobacillus stearothermophilus* (2015); alpha-amylase enzyme preparation produced by *B. licheniformis* carrying a modified alpha-amylase gene from *Cytophaga* sp. (2016).

3.1.2 Gene donor organism(s)

The gene donor, *Cytophaga* sp., was isolated by plating suspensions of soil and starch samples onto a media containing raw corn starch as the main carbon source (Jeang et al., 1995). *Cytophaga* sp. is a gram-negative, unicellular, non-spore forming bacterium commonly found in soil and sediments of lakes and oceans and is especially proficient in degrading various biopolymers such as cellulose, chitin, and pectin (Mayberger, 2011). Recent literature classified *Cytophaga* as a genus in the family of *Cytophagaceae* within the order of *Cytophgales* under the class of *Cytophagia* in the bacteria phylum of *Bacteroidota* (Garcia-López et al., 2019). The gene donor is referred to as *Cytophaga* sp. in the application as the classification of the species is incomplete.

Literature indicate that some isolates of *Cytophaga* sp. have been reported to be fish pathogens (Stewalt et al., 2018), however FSANZ is not aware of foodborne illnesses associated with *Cytophaga* bacteria.

3.2 Characterisation of the genetic modification(s)

3.2.1 Description of DNA to be introduced and method of transformation

The alpha-amylase enzyme is encoded by the *amy* gene derived from an isolate of *Cytophaga* sp. Data provided by Danisco and analysed by FSANZ confirmed the identity of the alpha-amylase enzyme. The alpha-amylase enzyme has been protein engineered and contains seven amino acid changes compared to the wild type alpha-amylase enzyme.

The *amy* gene was inserted into the genome of the host strain *B. licheniformis* and placed under the control of the native *amyL* gene regulatory sequences and the *aprE* gene leader sequence from *B. subtilis*. The native *cat* gene was used as a selectable marker enabling selection of positive transformants by growth on media supplemented with chloramphenicol. The *amy* gene was integrated at a specific integration site in the host's genome and was amplified through increasing concentrations of chloramphenicol to produce the final production strain, *B. licheniformis* JML1584.

3.2.2 Characterisation of inserted DNA

Data provided by Danisco confirmed the presence of the inserted DNA in the production strain. No bacterial vector DNA was introduced during the genetic modification, hence antibiotic resistance genes are not found in the *B. licheniformis* JML1584 production strain.

3.2.3 Genetic stability of the inserted gene

The stability of the introduced DNA in the production strain was examined by genome sequencing. DNA extracted from cultures after prolonged fermentation and stock culture prior to fermentation as a control were analysed. These data confirmed that the *amy* gene is expressed over multiple generations and is stable.

3.3 Safety of maltogenic alpha-amylase

3.3.1 History of safe use of the enzyme

There are multiple alpha-amylase enzymes from microbial sources and from malted cereals that are currently permitted as processing aids in Schedule 18 of the Code. However, *Cytophaga* sp. alpha-amylase is not permitted and does not have a history of safe use in Australia or New Zealand. The applicant stated that the enzyme is approved for use in Denmark and France.

There are no known reports of adverse effects arising from the consumption of alpha-amylase used as a food processing aid in Australia or New Zealand, or any other jurisdiction where these enzymes have been approved as a processing aids.

3.3.2 Bioinformatics concerning potential for toxicity

A BLAST search was performed using the mature amino acid sequence of alpha-amylase against the complete UniProt database[4]. With a conservative E-value[5] threshold of 0.1, none of the top 1000 matches were toxins or venoms.

In addition, A BLAST search was performed using the mature amino acid sequence of alpha-amylase against the UniProt animal toxin database[6]. No matches to toxins or venoms were found.

3.3.3 Evaluation of enzyme toxicity studies

The *Cytophaga* sp. alpha-amylase test item used in the following toxicity studies was produced using *B. licheniformis* JML1584 and was representative of the material that is the subject of this application.

90-day repeated dose oral toxicity study in rats (MPI Research, 2014). Regulatory Status: GLP; conducted according to OECD Test Guideline (TG) 408.

The alpha-amylase test item was administered to Crl:CD(SD) rats (10/sex /group) at doses of 0, 100, 250 and 500 mg Total Organic Solids (TOS)/kg body weight (bw)/day by oral gavage

[4] UniProt database: https://www.uniprot.org/
[5] The E value (or Expect value) indicates the significance of a match found when searching a sequence database. The closer an E value gets to zero, the less likely an alignment could have been produced by chance.
[6] UniProt toxins database: https://www.uniprot.org/program/Toxins

for 13 weeks. The vehicle control was distilled water.

Animals were observed daily. Body weight, food consumption and detailed clinical examinations for signs of toxicity were recorded weekly. Ophthalmological examination was conducted on all test animals prior to treatment and at study termination. Functional performance and sensory reactivity tests were performed in week 13. Gross pathology, haematology, clinical chemistry and measurement of organ weights was conducted on all animals at study termination, and a histopathological examination was conducted on organs and tissues from the control and high-dose group animals.

No mortality occurred during the study. No treatment-related effects were observed on feed consumption, body weights, haematology, clinical chemistry, ophthalmology, or functional observations functional performance or sensory reactivity. No treatment-related macroscopic abnormalities or histopathological findings were observed in any of the test animals at necropsy.

The No Observed Adverse Effect Level (NOAEL) was set at 500 mg TOS/kg bw/day, which was the highest dose tested.

Genotoxicity

Bacterial reverse mutation test (BioReliance, 2014). Regulatory Status: GLP; conducted according to OECD TG 471.

The potential mutagenicity of alpha-amylase was evaluated in *Salmonella enterica* ser. Typhimurium strains TA98, TA100, TA1535, TA1537 and *Escherichia coli* strain WP2 *uvrA*, with and without metabolic activation using rat liver homogenate (S9). A complete mutation test was undertaken at a wide dose-range (1.5 – 5000 µg protein/plate), which was followed by an additional confirmatory mutation test using a refined dose-range (15 – 5000 µg protein/plate). Bacterial cultures were treated for 1 hour with the test item, before treatment was removed and cells plated (treat and plate method).

Positive controls in the absence metabolic activation were *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (WP2uvrA, TA100 and TA1535), ICR-191 (TA1537) and 2-nitrofluorene (TA98). The positive control in the presence of metabolic activation was 2-aminoanthracene (all strains). Distilled water was used as the vehicle control.

No concentration-related increases in revertant colonies were observed in cultures treated with the test item, relative to vehicle controls, with or without metabolic activation. All positive control treatments showed the anticipated increases in mutagenic activity demonstrating the validity of the assay. It was concluded that alpha-amylase test item was not mutagenic under the conditions of this test.

In vitro mammalian chromosomal aberration test (DuPont Haskell Global Centers, 2014). Regulatory status: GLP; conducted according to OECD TG 473.

The potential of alpha-amylase to cause chromosomal aberrations in mammalian cells was tested using human lymphocytes isolated from peripheral blood, collected from a healthy volunteer. Treatment with the alpha-amylase test item was either a 4 hour pulse exposure with or without S9, followed by an 18 hour recovery; or 22 hours of continuous exposure without S9. Positive control assays were conducted in parallel using mitomycin C in the absence of S9 and cyclophosphamide in the short-term treatment with S9.

As a result of dose-selection experiments, the dose range for the 24-hour continuous

treatment was adjusted to 10 – 100 µg/mL total protein to keep the mitotic index above 50% at the high dose treatments. The dose range used for the 4 hour treatments with and without S9 was retained at 250 – 5000 µg/mL total protein.

There were no treatment related increases in chromosomal aberrations observed in peripheral blood lymphocytes following exposure to the alpha-amylase test item, relative to the vehicle controls, under any of the conditions tested. The positive controls demonstrated a statistically significant increase in chromosomal aberrations, validating the sensitivity of the experimental methodology. It was concluded that alpha-amylase did not cause chromosome aberrations in peripheral blood lymphocytes, under the conditions of the study.

3.3.4 Potential for allergenicity

A FASTA search was performed using the mature amino acid sequence of *Cytophaga* sp. alpha-amylase using the AllergenOnline[7] database (queried in February 2020) using three sequence alignments: full length protein (E-value 0.1 and >35% identity), an 80-mer sliding window (>35% identity) and an 8-mer sliding window search (100% identity). No sequence matches to known allergens were identified using these parameters.

Respiratory sensitisation of occupationally exposed individuals to some food enzyme processing aids, such as alpha-amylases from other species have been reported (Baur & Posch, 1998). However, food enzyme processing aids that are respiratory allergens are not usually food allergens (Poulsen 2004, Bindslev-Jensen et al. 2006), and there are no reports of sensitisation to *Cytophaga* sp. alpha-amylase in the scientific literature.

It is concluded that the presence of *Cytophaga* sp. alpha-amylase produced using modified *B. licheniformis* JML1584 in food is unlikely to pose an allergenicity concern to consumers.

3.3.5 Assessments by other regulatory agencies

The applicant stated that *Cytophaga* sp. alpha-amylase produced using GM *B. licheniformis* is approved for use in Denmark and France. Only the approval letter for Denmark was provided to FSANZ for verification. No written assessments were provided.

The US FDA responded with a “No Questions” letter to a GRAS Notification (GRN) 664. However, this is not an assessment by the FDA and not accepted by FSANZ as an assessment by an international agency.

3.4 Dietary exposure assessment

The objective of the dietary exposure assessment was to review the budget method calculation presented by the applicant as a ‘worse-case scenario’ approach to estimating likely levels of dietary exposure assuming all added alpha-amylase enzyme from GM *Bacillus licheniformis* 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 be compared to an acceptable daily intake (ADI) or a NOAEL to estimate a margin of exposure (MOE) for risk characterisation purposes.

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

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

- the maximum physiological requirement of solid foods (including milk) is 25 g/kg body weight/day
- the maximum physiological requirement for non-milk beverages is 100 mL/kg body weight/day (the standard level used in a budget method calculation)
- 50% of solid foods and 25% of non-milk beverages contain alpha-amylase
- the maximum alpha-amylase level in final solid foods was 0.723 mg TOS/kg food and for non-milk beverages was 30.91 mg TOS/kg food (i.e. the highest use level from all uses within each group)
- all of the enzyme remains in the final food.

Based on these assumptions, the applicant calculated the TMDI of alpha-amylase to be 0.78 mg TOS/kg body weight/day.

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

- the maximum physiological requirement for solid foods (including milk) is 50 g/kg body weight/day. This is the standard level used in a budget method calculation where there is potential for the enzyme to be in baby foods or general purpose foods that would be consumed by infants (Hansen, 1966), which for this enzyme would be from modified starch and syrups in breads, dairy products and drinks.

- 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 50% based on the nature and extent of use of the enzyme and therefore FSANZ has also used this proportion as a worst case scenario.

All other inputs and assumptions used by FSANZ remained as per those used by the applicant. The TMDI based on FSANZ's calculations for solid foods and non-milk beverages were 0.02 mg TOS/kg body weight/day and 0.77 mg TOS/kg body weight/day respectively, resulting in a total of 0.79 mg TOS/kg bw/day.

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 that it was assumed that the enzyme remains in the final foods and beverages. The applicant has stated that the enzyme is inactivated or removed during the subsequent production and refining processes and does not have a function in the final food.

4 Discussion

No public health and safety concerns were identified in the assessment of alpha-amylase produced by this GM *B. licheniformis* strain JML1584 under the proposed use conditions. The host organism *B. licheniformis* Bra7 from which *B. licheniformis* JML1584 was derived has a long history of safe use in food. Analysis of the GM production strain confirmed the presence and stability of the introduced DNA.

Bioinformatics analysis indicated that the enzyme shows no significant homology with any

known toxins or allergens.

Toxicity testing of the enzyme showed no evidence of genotoxicity *in vitro* and the NOAEL in a 90-day oral gavage study in rats was the highest dose tested, 500 mg TOS/kg bw/day. The TMDI was calculated to be up to 0.79 mg TOS/kg bw/day. Comparison of the NOAEL with the TMDI gives a MOE of approximately 600.

5 Conclusions

Based on the reviewed 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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