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Te Mana Kounga Kai – Ahitereiria me Aotearoa

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**210-22**

## Supporting document

Risk and Technical Assessment – Application A1220

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

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# Executive summary

Novozymes Australia Pty Limited applied to Food Standards Australia New Zealand (FSANZ) to amend Schedule 18 of the Australia New Zealand Food Standards Code (the Code) to include beta-amylase (EC 3.2.1.2) as a processing aid for use in starch processing for the manufacture of maltose syrup. This enzyme is sourced from a genetically modified (GM) strain of *Bacillus licheniformis* containing the beta-amylase gene from *Priestia flexa* (basionym *Bacillus flexus*).

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 the enzyme in starch processing in the manufacture of maltose syrup is consistent with its typical function of catalysing the hydrolysis of starch to maltose. Analysis of the evidence provides adequate assurance that the proposed use of the enzyme, in the requested amount (a level not higher than necessary to achieve the desired enzyme reaction under good manufacturing practice (GMP), is technologically justified.

Beta-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 and the applicant provided evidence that the enzyme meets these specifications.

No public health and safety concerns were identified in the assessment of beta-amylase from GM *Bacillus licheniformis* under the proposed use conditions. *Bacillus licheniformis* has a long history of safe use as a source of enzyme processing aids, including several that are already permitted in the Code. The *Bacillus licheniformis* host is neither pathogenic nor toxigenic. Analysis of the GM production strain confirmed the presence and stability of the inserted DNA.

The enzyme does not show any appreciable sequence homology with known toxins. In a 90-day repeat dose oral gavage study in Sprague Dawley rats, conducted under good laboratory

practice (GLP), there were no treatment-related effects on any of the parameters measured and the NOAEL was the highest dose tested, 1.199 g TOS/kg bw/day. Results of two GLP-compliant genotoxicity assays, a bacterial reverse mutation assay and a micronucleus assay, were negative. Results of a sequence homology assessment against known allergens, together with the processes used to produce the enzyme, lead to the conclusion that risk of allergenicity of the enzyme to consumers is negligible.

The theoretical maximum daily intake (TMDI) based on FSANZ's calculations is 0.81 mg TOS/kg body weight/day. Comparison of the NOAEL (1.199 g TOS/kg bw/day) and the TMDI results in a Margin of Exposure (MOE) of approximately 1500.

In the absence of any identifiable hazard, an Acceptable Daily Intake (ADI) of 'not specified' is appropriate for beta-amylase from *P. flexa*, expressed in a GM strain of *Bacillus licheniformis*.

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# 1 Introduction

Novozymes Australia Pty Limited (Novozymes) applied to Food Standards Australia New Zealand (FSANZ) to permit the use of the enzyme beta-amylase (EC 3.2.1.2) as a processing aid for use in starch processing in the production of maltose syrups.

This enzyme is sourced from a genetically modified (GM) strain of *Bacillus licheniformis* containing the beta-amylase gene from *Priestia flexa* (basionym *Bacilus Flexus*). The gene donor host named in the application is *Bacillus flexus* (DSM 1320$^T$). *Bacillus flexus* is however, now named as *Priestia flexa* on The List of Prokaryotic names with Standing in Nomenclature (Parte et al., 2020).

## 1.1 Objectives of the assessment

Currently, Schedule 18 of the Australia New Zealand Food Standards Code (the Code) includes permission for beta-amylase to be used as a processing aid, including from specified plant sources (sweet potato *Ipomoea batatas,* soybean *Glycine max,* malted cereals) and specified unmodified microbial sources (*Bacillus amyloliquefaciens, Bacillus subtilis*). This beta-amylase produced by a GM strain of *B. licheniformis* requires a pre-market assessment before permission can be given for its use as a processing aid.

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 concerns that may arise from the use of this enzyme 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 production microorganism of the beta-amylase enzyme is a GM strain of *B. licheniformis*. The applicant provided relevant information regarding the identity of the beta-amylase enzyme. FSANZ verified this using the IUBMB[1] enzyme nomenclature database (McDonald et al 2009). Details of the identity of the enzyme are provided in Table 1.

[1] International Union of Biochemistry and Molecular Biology

***Table 1*** *Identity*

| | |
|---|---|
| ***Generic common name:*** | Beta-amylase |
| ***Accepted IUBMB name:*** | β-amylase |
| ***Systematic name:*** | 4-α-D-glucan maltohydrolase |
| ***Other names:*** | (1-4)-alpha-D-glucan maltohydrolase, 1,4-alpha-D-glucan malto-hydrolase, alpha-1,4-glucan maltohydrolase, saccharogen amylase; glycogenase, 1,4-α-D-glucan maltohydrolase |
| ***EC number:*** | 3.2.1.2 |
| ***Reaction:*** | Hydrolysis of (1→4)-α-D-glucosidic linkages in polysaccharides to remove successive maltose units from the non-reducing ends of the chains |

For a graphical representation of the hydrolysis reaction catalysed by beta-amylase, refer to its record in the enzyme database BRENDA[2] (Chang et al 2021).

## 2.2 Manufacturing process

### 2.2.1 Production of the enzyme

Novozymes' beta-amylase is produced by submerged fermentation of the GM strain of *B. licheniformis*. The fermentation steps are inoculum, seed fermentation and main fermentation. A recovery stage follows fermentation, involving primary and liquid separation, germ filtration, concentration to achieve the desired enzyme activity, evaporation and stabilisation to provide a concentrated enzyme solution free of the production strain and insoluble substances. The recovery stage is followed by formulation of the enzyme into an enzyme preparation.[3] Novozymes' beta-amylase enzyme preparation is sold as a liquid product consisting of glycerol, potassium sorbate, sodium benzoate, and sorbitol. The applicant states that the enzyme is manufactured in accordance with current Good Manufacturing Practice for Food (cGMP) and the principles of Hazard Analysis and Critical Control Point (HACCP).

The applicant states that all raw materials used in the fermentation and recovery processes are standard ingredients of food grade quality that meet predefined quality standards. The raw materials conform to either specifications set out in the Food Chemical Codex, 12th edition, 2020 or regulations applying in the European Union.

Details on the manufacturing process, raw materials and ingredients used in the production of the beta-amylase enzyme preparation were provided in the application or as Confidential Commercial Information (CCI).

[2] https://www.brenda-enzymes.org/enzyme.php?ecno=3.2.1.2

[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.

### 2.2.2 Allergen considerations

The applicant provided the Product Data Sheet for the enzyme preparation. This states that certain allergens are not present including: cereals containing gluten, crustaceans, egg, fish, lupin, milk (including lactose), molluscs, nuts, peanuts, sesame, soy, sulphur dioxide/sulphites. The applicant also sent additional information to FSANZ as CCI, providing supporting information regarding the absence of the relevant allergens in their enzyme preparation and in maltose syrup prepared using their enzyme preparation.

### 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 (FAO/WHO 2006) and in the Food Chemicals Codex (FCC 2008). These specifications are included in earlier publications of 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. The applicant states that the final enzyme preparation is consistent with the requirements in both 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.

The applicant provided the results of analysis of a representative batch of the beta-amylase preparation. Table 2 provides a comparison of this analysis 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 manufacturer's beta-amylase preparation compared to JECFA, Food Chemicals Codex, and Code specifications for enzymes*

| *Analysis* | *Analysis provided by manufacturer* | *JECFA (2006)* | *Specifications Food Chemicals Codex (FCC, 2020)* | *Australia New Zealand Food Standards Code (section S3—4)* |
|---|---|---|---|---|
| **Lead (mg/kg)** | <0.5 | ≤5 | ≤5 | ≤2 |
| **Arsenic (mg/kg)** | <0.3 | - | - | ≤1 |
| **Cadmium (mg/kg)** | <0.05 | - | - | ≤1 |
| **Mercury (mg/kg)** | <0.05 | - | - | ≤1 |
| **Coliforms (CFU/g)** | <4 | ≤30 | ≤30 | - |
| ***Salmonella* (in 25 g)** | Not detected | Absent | Negative | - |
| ***E. coli* (in 25 g)** | Not detected | Absent | - | - |
| **Antibiotic activity** | Not detected | Absent | - | - |

While 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

Novozymes' beta-amylase is intended for use in starch processing in the production of maltose syrup.

Amylases break down starch molecules and other polysaccharides to convert complex carbohydrates to simple sugars. The 'beta' designation indicates the location of the targeted glycosidic bond; as identified by the IUBMB (IUBMB 2022), beta-amylase hydrolyses 1,4-

alpha-D-glucosidic bonds. This releases maltose molecules from the non-reducing end of the starch chain which can be used to manufacture maltose syrups.

Beta-amylases are commonly found in plants (including sweet potato, barley, and rye seeds) and microbes (including *B. cereus*, *B. megaterium*, *B. polymyxa*, and *Clostridium thermosulfurogenes*) (Das et al 2019). Microbial beta-amylase has been shown to be more productive than enzymes derived from wheat or barley in terms of maltose yield, and can be used at a slightly higher temperature, which reduces the risk of microbial contamination (Kojima 2010). Maltose syrups are commonly used in brewing, baking, canning, and the manufacture of soft drinks and confectionery (Adeyanju et al 2012).

The stated technological purpose of the beta-amylase enzyme is supported by scientific literature (e.g. Das et al 2019, Kojima 2010).

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

***Table 3*** *Physical and chemical properties of beta-amylase enzyme preparation*

| Physical and chemical properties of commercial enzyme preparation | |
|---|---|
| Enzyme activity | 5000 BAMU/g |
| Appearance | Brown coloured liquid |
| Storage conditions | 0–25°C |
| Density | 1.20 g/mL |

*BAMU/g: beta-amylase units per gram*

The enzyme preparation is available as a liquid concentrate, standardised in beta-amylase units (BAMU) to 5000 BAMU/g. The application includes a description of Novozymes' method used to determine beta-amylase activity. In summary, beta-amylase is used to hydrolyse maltohexaose to maltotetraose and maltose. The maltotetraose is then oxidised to produce hydrogen peroxide. The hydrogen peroxide is further reacted with other compounds to form a purple product, proportional to the amount of maltohexaose used originally. The increase in absorbance at 540 nm correlates to the amount of product formed, which is proportional to the enzyme activity. The highest level used in food manufacturing is 10,000 BAMU per kilogram dry starch matter.

The Codex guideline, *Guidelines on Substances used as Processing Aids* (CAC/GL 75-2010) sets out general principles for the safe use of substances used as processing aids. The Guideline states that substances used as processing aids shall be used under conditions of good manufacturing practice (GMP). Therefore, use of commercial enzyme preparations should follow GMP, where use is at a level that is not higher than that necessary to achieve the desired enzymatic reaction. The applicant requested use of the enzyme at GMP levels.

Beta-amylase (from two plant sources, two non-GM microbial sources, and one GM source) is approved for use in the manufacture of foods as listed in subsections S18—4(4), S18—4(5) and S18—9(3) respectively.

## 2.4 Technological justification

As outlined above, the technological purpose of beta-amylase is in starch processing, where it hydrolyses starch to produce maltose for syrups. Its use as requested by the applicant is therefore technologically justified.

The enzyme performs its function of catalysing the hydrolysis of 1,4-alpha-D-glycosidic bonds in starch to produce maltose during the processing of starch. It is therefore functioning as a processing aid for the purposes of the Code.

The Code already permits beta-amylase (from other sources) to be used in the manufacture of certain foods. The specific benefits of the action of beta-amylase in starch processing, as summarised from the application, are described below.

Beta-amylase produces a consistent level of maltose in syrups, at a higher level than other enzymes used to degrade starch. The amount of glucose in the syrup produced is proportionately lower. Beta-amylase produced from microbial sources can be used at a higher operating temperature than that derived from plant sources such as wheat and barley, reducing the risk of contamination.

## 2.5 Food technology conclusion

FSANZ concludes that the proposed use of this beta-amylase as an enzyme in starch processing in the manufacture of maltose syrups is consistent with its typical function of catalysing the hydrolysis of starch to produce maltose units. FSANZ concludes that the evidence presented to support the proposed use provides adequate assurance that the use of the enzyme, in the form and requested amount (i.e. at a level consistent with GMP) is technologically justified and has been demonstrated to be effective in achieving its stated purpose.

Beta-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 and the applicant provided evidence that the enzyme meets these specifications.

# 3 Safety assessment

Some information relevant to this section is CCI, so full details cannot be provided in this public report.

## 3.1 History of use

### 3.1.1 Host organism

*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* 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, serine proteinase and subtilisin.

The production strain relevant to this application was developed from the applicant's *B*. *licheniformis* Si3 lineage which was derived from a wild-type Ca63 strain. The same lineage has been assessed in applications for the serine proteinase (A1098) and subtilisin (A1206) processing aids.

The Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) confirmed the species designation using molecular characterisation. The name *B*. *licheniformis* is validly published under the International Code of Nomenclature of Bacteria.

*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).

The production microorganism is removed from the enzyme product by primary separation and filtration unit operations.

### 3.1.2 Gene donor organisms

The gene donor host named in the application is *Bacillus flexus* (DSM 1320$^T$). DSM 1320 is the type strain for *Bacillus flexus*. Gupta et al. (2020) has recently reassessed the taxomony of the *Bacillus* species. A new name, *Priestia flexa*, was proposed and accepted on The List of Prokaryotic names with Standing in Nomenclature (Parte et al., 2020). DSM 1320 is now named as *P*. *flexa*. The applicant was notified about the name change and has accepted the donor species as *P*. *flexa*.

## 3.2 Characterisation of the genetic modification(s)

### 3.2.1 Description of the DNA to be introduced and method of transformation

The gene that encodes the beta-amylase enzyme was synthesised *in vitro* based on the sequence from *P. flexa* available in public databases. Data provided by Novozymes and analysed by FSANZ confirmed the expected beta-amylase amino acid sequence.

The beta-amylase gene was introduced into the genome of the host strain, *B. licheniformis* and placed under the control of an engineered *Bacillus* hybrid promoter and *Bacillus* terminator. A native *B. licheniformis* gene encoding a chaperon protein was introduced alongside the beta-amylase gene to improve production yield. The beta-amylase gene was integrated at specific integration sites in the host genome and the final production strain was selected based on rapid growth and high beta-amylase activity.

### 3.2.2 Characterisation of inserted DNA

Data provided by Novozymes confirmed the presence of the inserted DNA in the production strain. The applicant also provided the results of genome sequencing which confirmed the absence of antibiotic resistance genes in the production strain.

### 3.2.3 Genetic stability of the inserted gene

The assessment confirmed the inserted 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.

To provide further evidence of the stability of the introduced beta-amylase gene, the applicant provided phenotypic data from large-scale fermentation of the production strain. These data confirmed that the beta-amylase gene is expressed over multiple generations and is stable.

## 3.3 Safety of the enzyme

### 3.3.1 History of safe use

The enzyme has been approved for use in Denmark since 2015, France since 2016, Brazil

since 2018 and Mexico since 2019. Confidential sales volume data were provided by the applicant to confirm that it has been sold for commercial use in France, Brazil and Mexico.

### 3.3.2 Bioinformatics concerning potential for toxicity

A recent (2020) sequence homology assessment of the beta-amylase to known toxins was conducted. Amino acid sequences of known protein toxins were extracted from UNIPROT. The sequence alignment program ClustalW 2.0.10 was used to align each sequence to that of the beta-amylase. A summary file containing the length of each sequence and number of identical residues was also created. From this, the identity percentage to the beta-amylase sequence or the compared toxin sequence was calculated, whichever was longest. This approach was chosen because the toxin sequences have many different lengths, both much shorter and much longer than that of the beta-amylase. By always using the longest sequence, artificial high scores from very short or very long toxins are avoided. The largest homology encountered was 16.1%, indicating that the homology to any toxin sequence in this database is random and very low.

### 3.3.3 Toxicity studies

All the submitted toxicity studies provided were conducted using a batch of the beta-amylase synthesized in the same way as the commercial product, but without stabilization or standardization for commercial sale.

#### *3.3.3.1 Animal Studies*

*90-day repeat-dose oral gavage study of beta-amylase in Sprague Dawley rats (Webley et al 2014; unpublished study). Regulatory status: GLP; in general accordance with OECD test guideline 408*

The vehicle and control article for this study was reverse osmosis water, in which the enzyme concentrate was completely miscible. The highest dose used was undiluted enzyme concentrate, 1.199 g TOS/kg bw/day. Low and middle doses were 0.120 and 0.396 g TOS/kg bw/day respectively. Dose formulations were sampled for enzyme concentration in Weeks 1, 6 and 13 of the in-life phase. Rats were received at 42 to 48 days old, and acclimatized for at least one week and subject to ophthalmological examinations before being assigned to groups, 10/sex/group. Rats were group-housed, 5/cage, under standard laboratory conditions of environment and husbandry. Food and water were provided *ad libitum,* except prior to blood collection.

Parameters determined during the study included survival, clinical observations, bodyweight changes, food consumption and water consumption. In Week 12, all rats were assessed for sensory reactivity, grip strength and motor activity, and ophthalmological examinations were performed on control and 1.199 g TOS/kg bw/day (high dose) rats. In Week 13, rats were anaesthetised for collection of blood for haematology, measurement of coagulation times, and clinical chemistry. Rats were then killed and detailed necropsy was performed. Fresh organ weights, as sex-appropriate, were recorded of adrenals, brain, epididymides, heart, kidneys, liver, ovaries, spleen, testes, thymus, and uterus. A comprehensive list of organs and tissues was preserved for histopathology.

All rats survived to the end of the in-life phase and there were no treatment-related effects on any of the parameters measured. It was concluded that the no observed adverse effect level (NOAEL) was the highest dose tested, 1.199 g TOS/kg bw/day.

### *3.3.3.2 Genotoxicity*

*Bacterial reverse mutation assay of beta-amylase (Lund et al 2014; unpublished study). Regulatory status: GLP; in general accordance with OECD test guideline 471*

The bacterial reverse mutation assay was conducted under GLP conditions and in general accordance with OECD guideline 471, although the exposure in liquid culture (the “treat and plate” method) used is not described in any Guideline. This method was used because an enzyme concentrate is likely to contain free histidine and tryptophan, which could cause a “feeder effect” on bacterial colony growth, and result in false positive results. Bacterial test strains used in the assay were *Salmonella enterica* ser. Typhimurium strains TA1535, TA100, TA1537, and TA98, and *Escherichia coli* WP2uvrApKM101. The solvent and negative control article was deionised water. Bacteria were exposed to beta-amylase in a phosphate-buffered nutrient broth for 3 hours, at concentrations of 156, 313, 625, 1250, 2500 and 5000 µg TOS/mL. The test article was removed by centrifugation prior to plating of the bacteria. Two separate experiments were conducted, each in triplicate, run in the absence and presence of S9 mix for metabolic activation. Tests with appropriate positive control articles were run in parallel.

The enzyme was slightly toxic to some of the bacterial strains at concentrations ≥ 1250 µg TOS/mL. Slight to moderate growth stimulation, that was not of significant magnitude to meet the criteria for a positive or equivocal response, was demonstrated by increases in the viable count of some exposed cultures compared to the solvent control, and was attributed to the “feeder effect”. No treatments of any of the bacterial strains resulted in increases in revertant colonies that meet the criteria for a positive or equivocal response. The positive control articles induced the expected significant increases in revertant colonies compared to the solvent control plates, confirming the validity of the assay. It was concluded that the enzyme is not mutagenic under the conditions of this assay.

In vitro *micronucleus assay of beta-amylase in human peripheral lymphocytes (Whitwell et al 2014; unpublished study). Regulatory status: GLP; in compliance with OECD test guideline 487*

Lymphocytes were harvested from the peripheral blood from two healthy non-smoking female volunteers. The solvent and negative control article was sterile water. Appropriate positive control articles were used; mitomycin C and vinblastine as clastogenic and aneugenic positive control chemicals respectively in the absence of S9 mix, and cyclophosphamide as a clastogenic positive control chemical in the presence of S9 mix. Cultures were exposed to the test substance for three3 hours in the presence and absence S9 mix) and harvested 24 hours after the beginning of treatment. In addition, a continuous 24-hour treatment without S9 mix was conducted with harvesting 48 hours after the beginning of treatment. For the three-hour exposure without S9 mix, test concentrations of the enzyme in the definitive experiment were 2000, 3000, 4000 and 5000 µg TOS/mL, whereas for the three-hour exposure with S9 mix, they were 3000, 4000 and 5000 µg TOS/mL. For the 24-hour exposure without S9 mix, the concentrations were 500, 2000, 3000 and 4000 µg TOS/mL. Negative control tests were conducted in quadruplicate while tests of the enzyme concentrate were conducted in duplicate. The cultures were treated with cytochalasin-B after removal of the test substance. The three highest concentrations were selected for scoring of micronuclei by evaluating the effect of the test substance on the replication Index (RI). One thousand binucleate cells from each culture (2000 per concentration) were analysed for micronuclei.

Treatment of cells with Beta amylase, batch PPY36295 in the absence and presence of S9 resulted in frequencies of MNBN cells which were similar to and not significantly ($p \leq 0.05$) higher than those observed in concurrent vehicle controls for the majority of all

concentrations analysed  Exceptions to this were noted at an intermediate concentration analysed (3000 µg TOS/mL) following three-hour treatment without S9 and at the highest concentration (4000 µg TOS/mL following the 24-hour treatment. However, these increases were small. Mean MNBN cell values fell within normal ranges for all concentrations, with the exception of a single replicate culture at 4000 µg TOS/mL for 24 hours' exposure. This result was not observed in the replicate culture or following a second scoring of a separate prepared slide from the culture. The MNBN cell frequency of all treated cultures fell within the 95th percentile of the current observed historical vehicle control (normal) ranges). The small statistical increases were not considered to be of biological importance. The positive control articles induced the expected increases in MNBN cells, confirming the validity of the assay. It was concluded that the beta-amylase enzyme did not induce micronuclei in human peripheral blood lymphocytes.

### 3.3.4 Potential for allergenicity

A sequence homology assessment of the beta-amylase enzyme to known allergens was conducted by comparing the amino acid sequence of the enzyme to allergens from the FARRP allergen protein database[4].

Homology above the threshold of 35 % across an 80 amino acid window was identified to one known food allergen, Tri a 17. This allergen is from wheat (*Triticum aestivum*). The allergen had a 44.7% identity with the beta-amylase across a window of 76 amino acids. However Tri a 17 had only a 25.7 % identity over the full-length sequence. Full-length comparison produces fewer false positives compared to the 80 amino acid window comparison, and full-length comparison has been recommended to be used to compare identities of proteins to allergens (Ladics et al, 2007).

The intended use of the beta-amylase that is the subject of this assessment is to hydrolyse starch in order to produce maltose syrups, and during the production of the syrup, > 99% of the enzyme is removed by processes that include filtration, ion exchange chromatography, carbon treatment and crystallisation. As a result, the presence of residual amounts of enzyme TOS is negligible. It is concluded that the beta-amylase is unlikely to pose any allergenic concern in food.

### 3.3.5 Assessments by other regulatory agencies

No safety assessment reports by other regulatory agencies are available. The enzyme has been approved for use in Denmark since 2015, France since 2016, Brazil since 2018 and Mexico since 2019.

## 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 beta-amylase enzyme 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 ADI or a NOAEL to estimate a margin of exposure for risk characterisation purposes.

[4] http://www.allergenonline.org

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

- the maximum physiological requirement for solid food (including milk) is 25 g/kg body weight/day
- 50% of solid food is processed
- the maximum physiological requirement for liquid is 100 mL/kg body weight/day (the standard level used in a budget method calculation)
- 25% of non-milk beverages are processed
- all processed solid food contains 25% starch or starch derived dry matter
- all processed non-milk beverages contain 12% starch hydrolysates
- the densities of non-milk beverages are ~ 1
- all of the enzyme remains in the final food
- all solid foods and non-milk beverages contain the highest use level of 88 mg TOS/kg starch dry matter.

Based on these assumptions, the applicant calculated the TMDI of the enzyme to be 0.53 mg TOS/kg body weight/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 body weight/day (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).
- 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 for solid foods 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 food and non-milk beverages is 0.81 mg TOS/kg body weight/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 whereas the applicant has stated that it is likely to either be reduced or removed during processing, or would be present in insignificant quantities. In addition the enzyme would be inactivated and perform no function in the final food to which the ingredient is added.

# 4 Discussion

FSANZ concludes that the proposed use of this beta-amylase as an enzyme in the processing of starch in the production of maltose syrups, is consistent with its typical function as an amylase. The evidence presented to support the proposed use provides adequate assurance that the use of the enzyme, in the form and requested amount (i.e., at a level consistent with GMP) is technologically justified and has been demonstrated to be effective in achieving its stated purpose.

Beta-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 and the applicant provided evidence that the enzyme meets these specifications.

No public health and safety concerns were identified in the assessment of beta-amylase from GM *B*. *licheniformis* under the proposed use conditions. *B*. *licheniformis* has a long history of safe use as a source of enzyme processing aids, including several that are already permitted in the Code. The *B*. *licheniformis* host is neither pathogenic nor toxigenic.

The gene that encodes the beta-amylase enzyme was synthesised *in vitro based* on the sequence from *P. flexa* available in public databases. Data provided by the applicant confirmed the presence of the inserted DNA in the production strain. The applicant also provided the results of genome sequencing which confirmed the absence of antibiotic resistance genes in the production strain.

The assessment confirmed that the inserted gene is integrated into the genome of the production strain and does not have the ability to replicate autonomously. To provide further evidence of the stability of the introduced beta-amylase gene, the applicant provided phenotypic data from large-scale fermentation of the production strain. These data confirmed that the beta-amylase gene is expressed over multiple generations and is stable.

The enzyme has been approved for use in Denmark since 2015, France since 2016, Brazil since 2018 and Mexico since 2019, and has been sold for commercial use. The enzyme does not show any appreciable sequence homology with known toxins. In a 90-day repeat dose oral gavage study in Sprague Dawley rats, conducted under good laboratory practice (GLP), there were no treatment-related effects on any of the parameters measured and the NOAEL was the highest dose tested, 1.199 g TOS/kg bw/day. Results of two GLP-compliant genotoxicity assays, a bacterial reverse mutation assay and a micronucleus assay, were negative. Results of a sequence homology assessment against known allergens, together with the processes used to produce the enzyme, lead to the conclusion that risk of allergenicity of the enzyme to consumers is negligible.

The TMDI based on FSANZ's calculations is 0.81 mg TOS/kg body weight/day. Comparison of the NOAEL (1.199 g TOS/kg bw/day) and the TMDI results in a Margin of Exposure (MOE) of approximately 1500.

# 5 Conclusion

In the absence of any identifiable hazard an Acceptable Daily Intake (ADI) of 'not specified' is appropriate for beta-amylase from *P. flexa*, expressed in a GM strain of *Bacillus llicheniformis*.

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