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

27 January 2021
148-21

Supporting document 1

Risk and technical assessment – Application A1210

Maltogenic alpha amylase enzyme from GM *Saccharomyces cerevisiae*

Executive summary

The purpose of the application is to amend Schedule 18 – Processing Aids of the Australia New Zealand Food Standards Code (the Code) to include a protein engineered maltogenic alpha-amylase (EC 3.2.1.133) from a genetically modified (GM) strain of *Saccharomyces cerevisiae.* The source organism for the enzyme gene is *Geobacillus stearothermophilus*. The proposed use of maltogenic alpha-amylase is as a processing aid in the manufacture of bakery products. It assists in limiting staling of baked products and so improves the quality and shelf life of the baked product, which is clearly articulated in the application.

The evidence presented to support the proposed use of the enzyme provides adequate assurance that the enzyme, in the quantity and form proposed to be used, is technologically justified and has been demonstrated to be effective in achieving its stated purpose. The enzyme meets international purity specifications and has been authorised for use in the USA.

The safety assessment concluded that the use of the enzyme under the proposed conditions is safe. The host is neither pathogenic nor toxigenic and has a long history of safe use in food. The gene donor organism has a history of safe use for the production of food enzymes and raises no public health concerns. The GM production strain was confirmed to contain the inserted DNA and this DNA was shown to be inherited across several generations. While there is a lack of history of safe use of this specific enzyme, the alpha-amylase extracted directly from the source organism has a long history of safe use. The enzyme shows no significant homology to any known toxins. A degree of homology between the protein engineered maltogenic alpha-amylase and several respiratory allergens was found. However, respiratory allergens are generally not food allergens, and since the enzyme is completely degraded under the conditions of the human stomach, the risk of food allergy from the proposed uses of the enzyme is considered to be negligible.

Based on the reviewed toxicological data it is concluded that, in the absence of any identifiable hazard, an acceptable daily intake (ADI) ‘not specified’ is appropriate. A dietary exposure assessment was therefore not required.

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1 Introduction

Lallemand Baking Solutions has applied to FSANZ, seeking permission for use of a new source of maltogenic alpha-amylase (EC 3.2.1.133) as a processing aid in baking. This enzyme is produced by a genetically modified strain of *Saccharomyces cerevisiae* expressing a protein engineered variant of the maltogenic alpha-amylase gene from *Geobacillus stearothermophilus*. The protein engineered variant confers improved thermostability of the enzyme for baking purposes.

The function of the enzyme is to catalyse the hydrolysis of starch polysaccharides in dough during the baking process, reducing crumb firmness and staling. If permitted following a pre-market assessment, the maltogenic alpha-amylase will provide an additional option for manufacturers of baked products.

1.1 Objectives of the assessment

The objectives of this Risk and Technical Assessment for maltogenic alpha amylase were to:

- determine whether the proposed purpose is clearly stated and that the enzyme achieves its technological function in the quantity and form proposed to be used as a food processing aid

- evaluate any potential public health and safety issues that may arise from the use of this enzyme protein, produced by a GM 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), and
  - safety of the enzyme protein.

2 Food technology assessment

2.1 Characterisation of the enzyme

2.1.1 Identity and properties of the enzyme

The production microorganism of the enzyme is a GM strain of *S. cerevisiae*. The donor microorganism of the maltogenic alpha amylase gene is *G. stearothermophilus* (see Section 3).

Details of the identity of the enzyme are provided in Table 1.

Table 1: Identity and relevant details of the enzyme maltogenic alpha-amylase

Generic common name:	Maltogenic alpha-amylase
Accepted IUBMB[1] name:	Glucan 1,4-alpha-maltohydrolase
Systematic name:	4-alpha-D-glucan alpha-maltohydrolase

[1] International Union of Biochemistry and Molecular Biology

EC number:	3.2.1.133
CAS[2] registry number:	160611-47-2
Reaction:	Hydrolysis of (1→4)-α-D-glucosidic linkages in polysaccharides so as to remove successive α-maltose residues from the non-reducing ends of the chains
Optimal temperature (°C):	80
Optimal pH:	5.5

2.2 Manufacturing process

2.2.1 Production of the enzyme

The enzyme is produced by a submerged fermentation process, which is the common production method of producing food enzymes. The specific processes are provided in the application which is summarised briefly here as these are very well known processes. They are fermentation, separation of the yeast after completion of fermentation, autolysis of the yeast to release the enzyme, separation, purification and concentration of the enzyme using filtration processes and then formulation after spray drying using carriers such as maltodextrin to the appropriate product specification and packaging.

The manufacturing processes are such to ensure the production microorganism is removed from the final enzyme preparation. The source of the maltodextrin used as a carrier for the enzyme preparation is not sourced from wheat but from corn starch so it is not a potential allergen source. The final enzyme preparation is produced to ensure it complies with international purity specifications of enzymes, being the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2006) and the Food Chemicals Codex (FCC) (USP, 2018) as discussed in the next section.

2.2.2 Specifications

There are international specifications for enzyme preparations used in food production (JECFA 2006; USP 2018). Both of these specification sources are primary sources listed in section S3—2 of the Code. Enzyme preparations must meet these specifications.

Table 2 provides a comparison of representative batch analysis of three non-sequential batches of the enzyme preparation with the international specifications established by JECFA and FCC, as well as those detailed in the Code (being section S3—4, as applicable). Analytical results for heavy metals (lead, arsenic, cadmium and mercury) confirm that representative batches meet the requirements of S3—4 of the Code.

[2] Chemical Abstracts Service

Table 2: Product specifications for commercial enzyme preparation

Analysis	Enzyme batch analysis	Specifications		
		JECFA	FCC[1]	Code
Lead (mg/kg)	<0.061, <0.250, <0.250	≤ 5	≤ 5	≤2
Arsenic (mg/kg)	<0.012, <0.025, 0.247	-	-	≤1
Cadmium (mg/kg)	<0.075, <0.050, <0.025	-	-	≤1
Mercury (mg/kg)	<0.025, <0.100, <0.050	-	-	≤1
Total coliforms (cfu/g)	0, 0, <10	≤30	≤30	-
Salmonella (in 25 g)	Absent	Absent	Negative	-
Enteropathic *E. coli* (in 25 g)	Absent	Absent	-	-
Antimicrobial activity	Absent	Absent	-	-
Production organism[2]	Absent	-	-	-
Recombinant DNA[2]	Absent	-	-	-

1. FCC – Food Chemical Codex; 2. Stated in the application to comply with European Food Safety Authority (EFSA) guidelines

The application provided analytical results indicating that representative samples of the commercial enzyme preparations do not contain any of the production source microorganism or recombinant DNA. This is indicated to comply with EFSA guidelines, but these are not official specification requirements.

Based on the above results, the enzyme preparation meets international and Code specifications for enzymes used in food production.

2.3 Technological purpose of the enzyme

The technological purpose of this enzyme is similar to that of other already permitted forms of the enzyme, in that it will be used in the manufacture of bakery products. Its purpose as a processing aid during the baking process is to reduce crumb firmness and staling in bread and other bakery products, thereby improving the quality and shelf life of these products.

As identified by the IUBMB (2017) maltogenic alpha-amylase catalyses the hydrolysis of 1-4-alpha-glucosidic linkages in polysaccharides to remove successive alpha-maltose residues from the non-reducing ends of these chains. In the baking process the action of the enzyme produces smaller molecules, being mainly maltose. The formation of molecules of smaller chain lengths interrupts the usual staling process of the formation of a stable network structure that increases crumb firmness, as an indicator of staling.

2.4 Technological justification of the enzyme

Information was provided in the confidential commercial information section of the application supporting the benefits and technological justification of using the enzyme in the baking industry to reduce staling of the produced bread (or other bakery products). The details of a study assessing the impact on reducing staling of the produced bread using the applicant's enzyme compared to a control without using any enzyme and an alternative version of the enzyme were reviewed. The parameters assessed were crumb firmness and crumb resilience. The assessment concludes that both enzymes provided improved performance compared to the control produced without use of an enzyme. The results for all the different time points and the two parameters indicated the applicant's enzyme had comparable performance to the competitor's enzyme, some were slightly better and others slightly worse.

However after the full 2 weeks storage the applicant's performance was slightly better than the competitor's.

Ultimately it would be up to the end food producers to determine if the enzyme is of value to their production process and for their products. Various commercial considerations will also be important for any decisions.

2.3 Food technology conclusion

The proposed use is as a processing aid in the manufacture of bakery products to assist in limiting staling of the produced product and so improve the quality and shelf life of the baked product. FSANZ concludes that the evidence presented to support the proposed use provides adequate assurance that the enzyme, in the form and prescribed amounts, is technologically justified and has been demonstrated to be effective in achieving its stated purpose. The enzyme performs its technological purpose during production and manufacture of foods after which it is inactivated thereby not performing a technological function in the final food. It is therefore appropriately categorised as a processing aid and not a food additive. The enzyme preparation meets international purity specifications.

3 Safety assessment

3.1 History of use

3.1.1 Host organism

Saccharomyces cerevisiae, also known as Baker's yeast, has a long history of use in food production. *S. cerevisiae* is a non-pathogenic and non-toxigenic species. The unmodified parent yeast strain is used in the commercial baking industry for bread production.

The production strain taxonomy has been confirmed as *S. cerevisiae* using whole genome sequencing (WGS) analysis. WGS was also used to confirm the absence of virulence factors, antimicrobial resistance genes and plasmids. Additional phenotypic AMR testing was performed to confirm the WGS findings using a set of four antibiotics: hygromycin, zeocin, geneticin and nourseothricin.

S. cerevisiae is listed as a source for an enzyme of microbiological origin (β-fructofuranosidase (EC 3.2.1.26)) in Schedule 18 – Processing aids of the Code. The *S. cerevisiae* production strain is maintained at the applicant's internal yeast culture collection.

3.1.2 Gene donor organism(s)

The maltogenic alpha-amylase gene was sourced from *Geobacillus stearothermophilus*, formerly known as *Bacillus stearothermophilus*. While *G. stearothermophilus* has been associated with food spoilage (André et al. 2017), it is not classified as a risk agent for human pathogenicity[3]. Furthermore, enzymes obtained from this organism have previously been approved and are listed in Schedule 18 of the Code, demonstrating a history of safe use for enzymes obtained from this microorganism.

[3] https://my.absa.org/Riskgroups

3.2 Characterisation of the genetic modification(s)

3.2.1 Description of DNA to be introduced and method of transformation

The gene that encodes the maltogenic alpha-amylase enzyme was chemically synthesised based on the sequence from *G. stearothermophilus.* The gene sequence has been codon-optimised to allow efficient expression in yeast. Modifications were also made to the amino acid sequence to improve thermostability. The expression cassette was generated with the enzyme gene flanked by specific promoters and terminators and was designed to allow targeted integration into the host genome. The transformation method was a standard method for the host species.

3.2.2 Characterisation of inserted DNA

A range of methods were used to characterise the insertion of the expression cassette. The data provided showed that the enzyme gene has been integrated into the targeted site, has the expected sequence and has not undergone rearrangement.

3.2.3 Genetic stability of the inserted gene

A genotypic analysis was performed in triplicate, comparing presence of the inserted DNA before and after a typical fermentation run. The data provided by the applicant shows that the expression of the gene is consistent across several generations, indicating the production strain is genetically stable.

3.3 Safety of maltogenic alpha amylase

The enzyme that is the subject of this application meets the specifications of JECFA and the Food Chemicals Codex.

3.3.1 History of safe use of the enzyme

Maltogenic alpha-amylase produced directly from the source organism, *G. stearothermophilus*, has been used since the mid-1990s in baking (Derde et al. 2012; Goesaert et al. 2009), and is approved for use in Australia, New Zealand and other countries. The maltogenic alpha-amylase that is the subject of this assessment is protein engineered and has not been the subject of assessment by any national or international regulatory agency. The US FDA responded with a “No Questions” letter to a GRAS Notification (GRN 842; January 2020) for this maltogenic alpha-amylase.

3.3.2 Bioinformatics concerning potential for toxicity

After generating a custom database of known toxins in UniProtKB[4] in November 2018, the applicant conducted a bioinformatics search using the amino acid sequence of this maltogenic alpha-amylase. There were no hits with a significant E-value, indicating a lack of similarity to any known toxin.

3.3.3 Stability of the enzyme in a simulated digestion assay

Simulated gastric digestion assay of the enzyme (unpublished study by Marzorati et al. 2020). Regulatory status: Not GLP

The test article for this assay was the enzyme that is the subject of the application. The

[4] https://www.uniprot.org/

assay was conducted using a Simulator of the Human Microbial System (SHIME®) as described by Molly *et al* (1993). The assay was conducted in triplicate, simulating the upper gastrointestinal tract under fed conditions. The test article was administered at 175 mg/reactor at the beginning of the gastric phase, a quantity that simulates a dose of 2.5 mg powdered enzyme/kg bw/day, or 0.368 mg Total Organic Solids (TOS)/kg bw/day for a 70 kg person. The gastric phase involved incubation with stirring for 2h at 37°C, in a reactor in which a sigmoidal decrease of pH was carried out from 5.5 to 2.0, and to which pepsin, phosphatidylcholine, nutritional medium and salts were added. The contents of the reactor were sampled at 0 and 120 min. After 2 h the small intestinal phase was initiated with increased pH from 2.0 to 5.5 within 5 min, from 5.5 to 6.5 in the first hour, 6.5 to 7 in the second hour and maintained at a constant 7.0 for a third hour. Pancreatic enzyme release was simulated by addition of raw animal pancreatic extract, trypsin and chymotrypsin, and bile release was simulated by addition of bovine bile extract. Sampling was conducted at 60, 120 and 180 min of the small intestinal phase.

Prior to conducting the assay, the separation of the relevant proteins by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted. The enzyme eluted as a single band just below 75 kDa. It was degraded if spiked into stomach medium which contained pepsin, but no other components of the stomach or small intestinal media interfered with the elution of the enzyme.

Under the conditions of the assay, the enzyme was completely degraded at the 120 min (2 h) sampling time-point of the gastric phase, and therefore could not be detected at any sampling time-point of the small intestinal phase.

3.3.4 Toxicology studies in animals

No toxicology studies conducted in animals with the maltogenic alpha-amylase that is the subject of this application were submitted. Under the Application Handbook, such studies are not required based on the results of the bioinformatic analysis for similarity to known toxins, digestion assay and the history of safe use of the host and gene donor organisms. However, as supporting information, reviews by EFSA and JECFA of similar maltogenic alpha-amylases were considered.

EFSA has reviewed five 90-day repeat-dose studies in rats of similar maltogenic alpha-amylases, including maltogenic alpha-amylases produced in strains of *Bacillus subtilis* genetically modified to express the maltogenic alpha amylase from *G. stearothermophilus*. All studies were conducted under good laboratory practice (GLP) and in accordance with OECD Guideline 408. No Observed Adverse Effect Levels (NOAELs) in 13-week studies ranged from 318.4 to 986 mg TOS/kg bw/day. In three of the 13-week studies, all of which were conducted using Sprague Dawley rats, the NOAEL was the middle dose of three, because of adverse effects observed at the high dose. Adverse effects observed at the high dose were as follows:

- *90-day study of maltogenic alpha-amylase from a genetically modified strain of Bacillus subtilis NZYM-OC (EFSA CEP Panel 2018a, EFSA reference EFSA-Q-2014-00922)* Statistically significant decreases in group mean values for total leucocyte count, and counts of lymphocytes, eosinophils, monocytes and large unstained cells, in high-dose females relative to controls. NOAEL 371 mg TOS/kg bw/d.
- *90-day study of maltogenic alpha-amylase from a genetically modified strain of Bacillus subtilis NZYM-SO (EFSA CEP Panel 2018b, EFSA-Q2015-00046)* Group mean value for mean corpuscular haemoglobin in high-dose males was significantly higher than that of male controls, and group mean values for total leucocyte count and lymphocyte count in high-dose females were significantly lower than those of female controls. In high-dose groups of both sexes, minimal hyperplasia/hyperkeratosis was observed at the limiting

ridge of the stomach, but similar changes were not observed in control rats or at lower doses. NOAEL 318.4 mg TOS/kg bw/d.

- *90-day study of maltogenic alpha-amylase from a genetically modified strain of Bacillus subtilis NZYM-SM (EFSA CEF Panel 2018a, EFSA-Q2015-00046)* Significant changes, relative to those of control animals of the same sex, in group mean values for clinical chemistry parameters relevant to renal function were observed at the high dose. NOAEL 320 mg TOS/kg bw/day.

In summary, findings at the highest dose were not consistent between the three studies, with the exceptions of the decreases in group mean total leucocyte count and lymphocyte count in high-dose females, relative to controls, in two studies. The other two 90-day studies reviewed by EFSA (EFSA CEF Panel 2018b, EFSA CEP Panel 2019) were conducted using Wistar rats. The test articles were maltogenic alpha-amylase from genetically modified *B. subtilis* MAM and genetically modified *E. coli* BLASC respectively, and the NOAEL was the highest dose tested, 986 mg TOS/kg bw/d and 838 mg TOS/kg bw/d respectively. The relevance of the adverse findings observed in one strain of rat is uncertain, since the test articles were not the same enzyme as the subject of the current application. In all the EFSA assessments, it was concluded that there were no safety concerns under the intended conditions of use, because there was a sufficiently high margin of exposure between the rodent NOAEL and the estimated dietary exposure of human consumers.

Other studies of similar maltogenic alpha-amylases, originating from *B. stearothermophilus* but expressed in strains of *B. subtilis,* were included in GRAS Notifications (GRN 746 and 751) to which the US FDA responded with “No Questions” letters.

JECFA reviewed alpha-amylase from *B. stearothermophilus* and concluded that there were no adverse effects in a 13-week dog study, or in a one-generation reproduction study in rats (JECFA 1990).

3.3.5 Genotoxicity assays

No genotoxicity assays conducted with the maltogenic alpha-amylase that is the subject of this application were submitted. Under the Application Handbook, such studies are not required based on the results of the bioinformatic analysis for similarity to known toxins, digestion assay and the history of safe use of the host and gene donor organisms. However, as supporting information, reviews by EFSA of similar maltogenic alpha-amylases were considered.

Genotoxicity assays of a number of similar maltogenic alpha-amylases, including maltogenic alpha-amylases produced by *B. subtilis* genetically engineered to express the maltogenic alpha-amylase of *B. stearothermophilus*, have been recently reviewed by EFSA (EFSA CEP Panel 2018a,b, 2019; EFSA CEF Panel 2018a,b). Assays included bacterial reverse mutation assays, *in vitro* chromosomal aberration tests, and an *in vitro* micronucleus test in human peripheral blood lymphocytes. The assays were conducted under GLP and in compliance with the relevant OECD guidelines. The weight of evidence supports the conclusion that the tested maltogenic alpha-amylases are not genotoxic.

3.3.6 Potential for allergenicity

A sequence homology search was conducted by the applicant in 2020, using the AllergenOnline[5] database using a sliding window of 80 amino acids of the full-length amino acid sequence. Six identity matches >35% were found; two for proteins from *Aspergillus*

[5] AllergenOnline is curated by the Food AllergyResearch and Resource Program of the University of Nebraska, and is available at http://www.allergenonline.org/

oryzae, one for a protein from *Schizophyllum commune*, one for a protein from *Aedes aegypti* and two for proteins from *Aspergillus fumigatus*. All the allergenic proteins are sensitisers by inhalation rather than food allergens.

Additional homology searches of the same database conducted by the applicant in 2020 were the highly conservative 8-mer search, and the full length FASTA36 alignment of the amino acid sequence (using the default settings of E value cut-off = 1 and maximum alignments of 20). No alignment with allergenic proteins at or near the 35% identity threshold was found with either search.

FSANZ repeated the same three searches in September 2020, with the same results. The AllergenOnline database at the time of FSANZ's searches was version 20 (February 10, 2020).

FSANZ notes that the complete degradation of the enzyme under conditions simulating those of the human stomach, means that the enzyme is unlikely to have allergenic potential. Furthermore there are no reports of food allergy from the maltogenic alpha-amylase extracted from the source organism, *G. stearothermophilus*.

3.3.7 Assessments by other regulatory agencies

There are no safety assessment reports, prepared by international or other national government agencies, for the protein engineered maltogenic alpha-amylase that is the subject of this application.

The US FDA responded with a "No Questions" letter to a GRAS Notification, GRN 842, in January 2020. However this does not constitute a safety assessment report by a regulatory agency.

4 Conclusions

The safety assessment determined the use of the protein engineered maltogenic alpha-amylase produced by the genetically modified *S. cerevisiae* as a food processing aid at GMP levels in bakery products is safe.

The host organism, *S. cerevisiae* (baker's yeast and brewer's yeast) is considered a safe strain, with a long history of use in food and no evidence of toxicity or pathogenicity. The gene donor organism, *G. stearothermophilus*, also raises no public health concerns and has a history of safe use for the production of food enzymes. Characterisation of the GM production strain confirmed both presence and stable inheritance of the inserted maltogenic alpha-amylase gene.

Although there is a lack of history of safe use of this specific enzyme, maltogenic alpha-amylase produced directly from *G. stearothermophilus* has a substantial history of safe use in baking. Animal and genotoxicity studies of maltogenic alpha-amylases from different sources do not indicate reason for concern.

No similarity was found to any known toxin in the UniProtKB database. No sequence homology to known food allergens was identified. A degree of homology between the protein engineered maltogenic alpha-amylase and several respiratory allergens was found. Respiratory allergens are generally not food allergens (Dauvrin et al. 1998, Bindslev-Jensen et al. 2006), and since the enzyme is completely degraded under the conditions of the human stomach, the risk of food allergy from the proposed uses of the enzyme is considered to be negligible.

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. A dietary exposure assessment was therefore not required.

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