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

**30 November 2021**
**181-21**

## Supporting document 1

# Risk and technical assessment report – Application A1231 Maltogenic alpha amylase from GM *Escherichia coli* as a processing aid (enzyme)

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# 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 the enzyme maltogenic alpha amylase (EC 3.2.1.133), sourced from a genetically modified (GM) strain of *Escherichia coli (E. coli* BLASC*)*. This production organism contains the maltogenic alpha amylase gene from *Geobacillus stearothermophilus*. Maltogenic alpha amylase is proposed as a processing aid in baking, brewing and starch processing.

The available evidence provides adequate assurance that the proposed use of this enzyme is technologically justified and effective in achieving its stated purpose. The enzyme meets international purity specifications and has already been approved for use by the European Food Safety Authority (EFSA).

No public health and safety concerns were identified in the assessment of this maltogenic alpha amylase produced from a GM strain of *E. coli* under the proposed use conditions. The *E. coli* host is neither pathogenic nor toxigenic and analysis of the GM production strain (*E. coli* BLASC) confirmed the presence and stability of the introduced DNA. Bioinformatic analysis indicated that the enzyme shows no significant homology with any known toxins.

Maltogenic alpha amylase was not genotoxic *in vitro*. The no observed adverse effect level (NOAEL) determined in a 90-day oral gavage study in rats was 1000 mg/kg bw/day total protein, equivalent to 838 mg/kg bw/day Total Organic Solids (TOS). The theoretical maximum daily intake (TMDI) was calculated to be 1.06 mg/kg bw/day TOS. A comparison of the NOAEL and the TMDI gives a Margin of Exposure (MOE) of approximately 790.

Bioinformatic analysis showed that the enzyme has a degree of homology with several known allergens. None were food allergens. No report of sensitisation to any form of maltogenic alpha amylases was found in a search of the scientific literature and maltogenic alpha amylases from the same source organism are already permitted in the Code. On that basis, the enzyme was considered to be of low allergenic risk to consumers when used as a processing aid in food.

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

Advanced Enzyme Technologies Ltd. applied to Food Standards Australia New Zealand (FSANZ) to permit use of the enzyme maltogenic alpha amylase (EC 3.2.1.133) as a processing aid in in baking, brewing and starch processing. This enzyme is sourced from a genetically modified (GM) strain of *Escherichia coli,* with the strain name *Escherichia coli* BLASC (*E. coli* BLASC)*,* containing the maltogenic alpha amylase gene from *Geobacillus stearothermophilus.*

Currently, Schedule 18 of the Australia New Zealand Food Standards Code (the Code) includes permission for two maltogenic α-amylases – one produced by a GM *Bacillus subtilis* containing the maltogenic α-amylase gene from *G. stearothermophilus,* and one by a GM strain of *Saccharomyces cerevisiae*, engineered to express an optimised variant of the maltogenic alpha amylase gene, also from *G. stearothermophilus*[1]. Therefore, this particular maltogenic alpha amylase enzyme produced by a GM *E. coli* needs a pre-market assessment before permission can be given for its use as a processing aid. If permitted, the enzyme will provide an additional option for food and beverage manufacturers that produce baked products, or are involved in starch processing or brewing.

## 1.1 Objectives of the assessment

The objectives of this risk and technical assessment 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 potential public health and safety concerns that may arise from the use of this enzyme, produced by a GM microorganism, as a processing aid, specifically by considering the:

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

# 2 Food technology assessment

## 2.1 Characterisation of the enzyme

### 2.1.1 Identity of the enzyme

The applicant provided relevant information regarding the identity of the enzyme, and this has been verified using an appropriate enzyme nomenclature reference (IUBMB 2021 – accessed 27 September 2021).

Accepted IUBMB[2] name: glucan 1,4-α-maltohydrolase

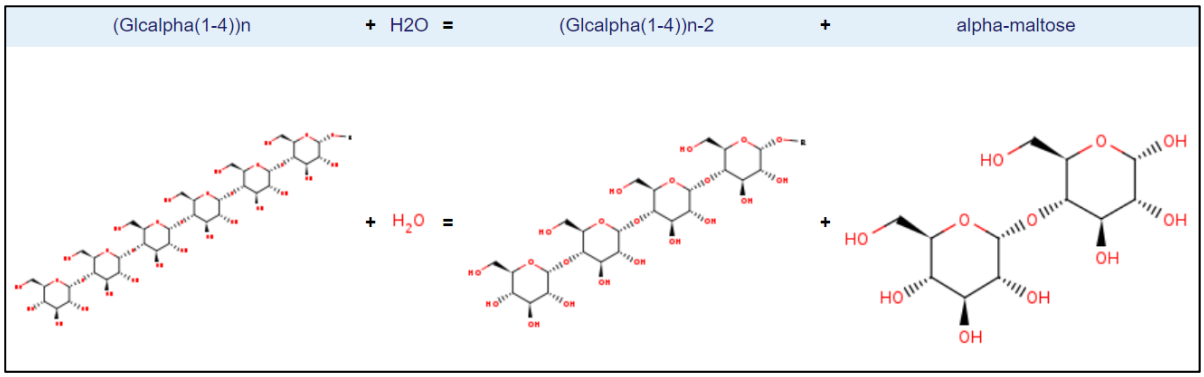
Systematic name: 4-α-D-glucan α-maltohydrolase

[1] Application A1210 at https://www.foodstandards.gov.au/code/applications/Pages/A1210.aspx
[2] International Union of Biochemistry and Molecular Biology.

| | |
|---|---|
| Other names: | maltogenic α-amylase[3]; 1,4-α-D-glucan α-maltohydrolase |
| IUBMB enzyme nomenclature: | EC 3.2.1.133 |
| CAS[4] number: | 160611-47-2 |
| Reaction: | Hydrolysis of (1→4)-alpha-D-glucosidic linkages in polysaccharides removing successive alpha-maltose residues from the non-reducing ends of the chains |

### 2.1.2 Technological purpose of the enzyme

Maltogenic alpha amylase catalyses the hydrolysis of (1→4)-alpha-D-glucosidic linkages in amylose, amylopectin and related polysaccharides in starch. As the name implies, the enzyme successively removes maltose units from the non-reducing end of the polysaccharide chain (see Figure 1).




Source: BRENDA:EC3.2.1.133 (https://www.brenda-enzymes.org/enzyme.php?ecno=3.2.1.133)

***Figure 1*** *Representation of hydrolysis reaction of a polysaccharide catalysed by maltogenic alpha amylase, producing maltose*

The stated technological purpose of maltogenic alpha amylase in baking, brewing and starch processing is consistent with the scientific literature (Marc et al. 2002; Slominska 1989). In addition, it is supported by the results of studies provided by the applicant that investigated the effects of the enzyme in bread making; the production of maltose syrup with corn starch as a substrate; and its action on the starch of barley malt and millet in brewing.

Specifically, in baking, the enzyme catalyses the selective hydrolysis of starch which helps prevent retrogradation, imparting anti-staling properties in bread and other baked goods (Marc et al. 2002). In starch processing for the production of maltose/glucose syrups, the enzyme's action yields elevated maltose levels through either prolonged hydrolysis of starch or by multiple hydrolytic episodes on numerous bonds before dissociating from the substrate's active site (Daute et al. 1999). In brewing, the enzyme is added at the mashing step, where it hydrolyses the starch containing substrates to release high amounts of simple sugars that support yeast growth during fermentation.

Table 1 includes a summary of the physical and chemical properties of the enzyme.

[3] Although the term that is used throughout the application, this document and the Call for Submissions is 'maltogenic alpha amylase', the term that will be used in the proposed draft variation to the Code for this enzyme is 'maltogenic α-amylase', as this will ensure consistency with existing permissions in Schedule 18 of the Code.
[4] Chemical Abstracts Service.

***Table 1*** *Maltogenic alpha amylase enzyme physical/chemical properties*

| Physical/chemical properties | |
|---|---|
| Enzyme activity | Not less than 100,000 MAN U/g* |
| Appearance | Light brown to brown coloured powder with characteristics odour |
| Temperature optimum | 60°C |
| Temperature stability | Stable between 25-65°C for 2 hours |
| pH optimum | 5.0 |
| pH stability | Stable between 4.0-5.5 for 4 hours |

*One unit of enzyme activity is defined as the amount of enzyme that cleaves 1 µmol of maltotriose per minute under the given assay conditions.

The applicant provided the results of a characterisation study of the enzyme, indicating that it is stable up to 65°C and that it exhibits good activity and stability in acidic conditions. This is advantageous to food manufacturers because the enzyme can be used in processing conditions that are at lower risk of microbial contamination, which is useful from a food safety perspective.

The applicant claims and FSANZ agrees that maltogenic alpha amylase performs its primary technological function during food processing and, as such, meets the definition of a processing aid. The applicant states that the highest recommended dose for its intended uses is 33.90 mg Total Organic Solids (TOS)[5] per kilogram of raw material.

### 2.1.3 Technological justification for the enzyme

The specific benefits of the action of the maltogenic alpha amylase in baking, brewing and starch processing are described below.

Baking

In baking, maltogenic alpha amylase is added to the raw materials during dough preparation to catalyse the rapid hydrolysis of amylopectin forming oligosaccharides with different degrees of polymerization (DP)[6] i.e. maltose, glucose, maltotriose and maltotetraose. This selective hydrolysis of starch prevents retrogradation, imparting anti-staling benefits in baked products such as breads, thereby extending shelf life. The applicant's study at Annex M2 of the application examined the effect of maltogenic alpha amylase on a number of characteristics of bread including shelf life. It indicated that loaf bread baked with the enzyme had a shelf life of up to 8 days more than that of the control baked without the addition of maltogenic alpha amylase (measured by day of first appearance of mould) (p 9 of Annex M2).

Other benefits relate to the sensory properties of baked products and include enhanced crumb softness, crumb moisture retention, resilience, loaf bread volume, and crumb texture, which together contribute to a superior quality product. The results of the above-mentioned study on loaf bread also support these claims.

Starch processing

[5] A compositional analysis of three batches determined TOS (%) values of 83.79, 84.82 and 85.57.
[6] The number of monomer units in the polymer.

In starch processing for the production of maltose/glucose syrups, the enzyme is added to the saccharification step for more efficient processing. Other benefits include:

- improved yields of maltose and glucose, for example, through the hydrolysis of maltotriose
- reduced risk of microbial contamination due to the high saccharification temperature that can be achieved (noting also the pH optima of 5.0)
- improved filterability owing to a reduction in long chain dextrins
- increased levels of glucose and maltose in starch hydrolysates that have a positive effect on the clarity of the hydrolysates.

The applicant provided a study examining the action of maltogenic alpha amylase on corn starch that supported these claims and concluded that the enzyme was effective in producing high maltose syrup.

Brewing
In brewing, maltogenic alpha amylase is added during mashing, before lautering or mash filtration. There it hydrolyses the starch containing substrates to release high amounts of simple sugars that support yeast growth during fermentation. Specifically, benefits of the action of the enzyme in brewing include:

- decreased mashing time owing to its ability to efficiently hydrolyse the starch containing substrates
- improved yields due to the release of high amounts of maltose, for the subsequent action of the enzymes in the yeast, producing ethanol
- wider choice of raw materials, due to its ability to hydrolyse starch from various grains into maltose
- improved rates of filtration of the wort (in combination with other enzymes)
- a synergistic effect on improving the fermentable nitrogen content of the hydrolysed extract.

The results of a study provided by the applicant on the action of maltogenic alpha amylase in brewing using a combination of barley malt and millet support these claims, noting the overall effect of using the enzyme is an increase to yield and productivity.

## 2.2 Manufacturing process

### 2.2.1 Production of the enzyme

The enzyme is produced by fed batch fermentation of the GM strain of *E. coli.* Briefly, it comprises the processes of fermentation, recovery and purification, and formulation and packaging, including quality control of the finished product.

The fermentation process begins with injecting the stock culture suspension into the inoculum flask containing fermentation medium. When a sufficient amount of biomass is obtained, a suspension of cells is transferred from the inoculum flask to the seed fermentation tank, and eventually to the main fermentation tank. The applicant has provided information regarding the raw materials used in fermentation and FSANZ has confirmed that these are appropriate for use in food production.

After desired growth is achieved in the fermenter, isopropyl-β-D-thiogalactopyranoside (IPTG) is aseptically added to induce enzyme production; the enzyme is produced and retained intracellularly. The fermentation process is continued until the desired level of enzyme production is achieved, after which the fermentation is considered complete. During

all stages of the fermentation process, samples are taken to ensure the absence of microbial contamination.

The recovery process is a multi-step operation to separate the biomass from the enzyme-containing culture medium. As maltogenic alpha amylase is produced intracellularly and retained inside the cells of the production microorganism, the fermentation broth is first briefly subjected to an elevated temperature and passed through a cell homogenizer to lyse the cells. This results in the release of the enzyme from the bacterial cells into the fermentation medium.

Subsequent steps in the recovery process involve primary separation of the biomass and insoluble/unused media from the liquid, concentration, centrifugation, pre-filtration and micro-filtration (germ filtration), and spray drying. Diafiltration is used for enzyme concentration in the recovery process to remove low molecular weight impurities such as the IPTG inducer (added to induce enzyme production as outlined above). The enzyme-containing liquid concentrate obtained after micro-filtration may be stabilised by the addition of glycerol. Spray drying may involve the addition of an approved food-grade stabiliser (e.g. maltodextrin), if required, to obtain the unformulated concentrate.

The maltogenic alpha amylase is sold as a powder preparation, with enzyme activity of not less than 100,000 MAN U/g. If required, the spray-dried unformulated concentrate may be formulated with an approved food-grade formulating agent (e.g. maltodextrin), and adjusted to a declared enzyme activity.

The applicant states that fermentation is carried out in accordance with current Good Manufacturing Practice (cGMP) and the principles of Hazard Analysis and Critical Control Points (HACCP). The manufacturing facility is ISO 9001, ISO 22000 (Food Safety Management System) and GMP (Good Manufacturing Practice) certified.

### 2.2.2 Allergen considerations

The applicant has stated that materials used in the fermentation process do not contain ingredients based on any of the top eight allergens[7] (FALCPA 2004). In addition, no allergenic ingredients are added to the enzyme preparation post fermentation, during recovery, purification or formulation. The applicant has provided documentation to support this claim, which also substantiates the nil presence of substances additional to those listed by FALCPA, namely, sesame seeds (and products thereof), sulphur dioxide and sulphites at concentrations of not more than 10 mg/kg, lupin (and products thereof), and molluscs (and products thereof) – FSANZ notes that mandatory allergen labelling requirements in the Code also extend to these foods. See also section 3.3.5 regarding the allergen homology search results.

### 2.2.3 Specifications

The JECFA Combined Compendium of Food Additive Specifications (FAO/WHO 2006) and the Food Chemicals Codex 12th edition (The United States Pharmacopeia 2020) are international specifications for enzymes used in the production of food. These are primary sources of specifications listed in section S3—2 of Schedule 3 of the Code. Enzymes need to meet these specifications. Schedule 3 of the Code also includes specifications for heavy metals (section S3—4) if they are not already detailed within specifications in sections S3—2 or S3—3.

Table 2 provides a comparison of the analysis of three different batches of the maltogenic

[7] Milk, eggs, fish, crustacean shellfish, tree nuts, peanuts, wheat and soybeans.

alpha amylase product with international specifications established by JECFA and Food Chemicals Codex, as well as those in the Code (as applicable). Based on these results, the enzyme preparation meets all relevant specifications.

***Table 2*** *Analysis of enzyme maltogenic alpha amylase compared to JECFA, Food Chemicals Codex, and Code specifications for enzymes (3 batches)*

| Analysis | Analytical results provided by the applicant | Specifications | | |
|---|---|---|---|---|
| | | *JECFA* | *Food Chemicals Codex* | *Australia New Zealand Food Standards Code (section S3—4)* |
| Lead (mg/kg) | <0.1 | ≤ 5 | ≤ 5 | ≤2 |
| Arsenic (mg/kg) | <0.1 | - | - | ≤1 |
| Cadmium (mg/kg) | <0.1 | - | - | ≤1 |
| Mercury (mg/kg) | <0.025 | - | - | ≤1 |
| Coliforms (cfu/g) | <10 | ≤30 | ≤30 | - |
| *Salmonella* (in 25 g) | Absent | Absent | Negative | - |
| *E. coli* (in 25 g) | Absent | Absent | - | - |
| Antimicrobial activity | Absent by test | Absent | - | - |
| Mycotoxins | Below detection limits | No toxicologically significant levels | | |

## 2.3 Food technology conclusion

FSANZ concludes that the use of this maltogenic alpha amylase in baking, brewing and starch processing is clearly described in the application and is consistent with its known technological functions in starch hydrolysis. Analysis of the evidence provides adequate assurance that the use of this enzyme, in the quantity and form proposed to be used, which must be consistent with GMP controls and processes, is technologically justified. The enzyme meets international purity specifications.

# 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

*Escherichia coli* BL21 (DE3) strain was engineered to integrate the native *ccdB* gene (from *E. coli* F plasmid) into the genome, which encodes a cytotoxin specific to bacterial cells. The ccdA/ccdB antitoxin/toxin system ensures stability of plasmid-based expression vectors (Szpirer and Milinkovitch, 2005). The recipient strain is identified as SAML27.

The identity of the host organism as *E. coli* was established by 16S RNA gene sequence

analysis of the BLASC strain using the NCBI nucleotide Basic Local Alignment Search Tool (NCBI BLAST). A 100% identity match to *E. coli* BL21 (DE3) strains was confirmed. An analysis of a complete genome for an *E. coli* BL21 (DE3) isolate (BioSample number SAMN14933839) using the NCBI pathogen detection tool for known virulence genes including the shiga-toxin ($stx_1$ and $stx_2$) or adherence (*eae* and *aggR*) genes was conducted. No matches were found.

The production strain, *Escherichia coli* (strain BLASC), was submitted to a culture collection for safe deposit.

### 3.1.2 Gene donor organisms

The gene donor organism, *Geobacillus stearothermophilus* has a long history of safe use for the production of industrial enzymes for food use including in Schedule 18 to Standard 1.3.3 of the Code.

## 3.2 Characterisation of the genetic modification(s)

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

The maltogenic alpha amylase enzyme is encoded by the *amyM* gene and was chemically synthesised based on the *Geobacillus stearothermophilus* sequence. Chemical synthesis allows for codon optimisation in *E. coli* without amino acid changes being made. Data provided by the applicant and analysed by FSANZ confirmed the expected maltogenic alpha amylase sequence. The nucleic acid sequence is devoid of the signal peptide sequence.

The *amyM* gene was introduced into the *E. coli* host via a plasmid expression vector. In addition to the *amyM* gene, this vector contains the native *ccdA* gene from the *E. coli* F plasmid. The *ccdA* gene is the antitoxin component of the a bacterial ccdA/ccdB toxin-antitoxin system (Jaffe et al. 1985). Its presence on the plasmid is intended to stabilise the plasmid-based expression system in the *E. coli* BLASC production strain without the need to use antibiotics (Szpirer and Milinkovitch, 2005).

### 3.2.2 Characterisation of inserted DNA

Data was provided showing the activity of maltogenic alpha amylase after three distinct fermentation runs. These data confirm the presence and functionality of the plasmid expression system in the *E. coli* BLASC production strain.

Further evidence that the production strain contains the *amyM* / *ccdA* expression plasmid is by virtue of its ability to produce the maltogenic alpha amylase enzyme and remain viable. The production strain requires the *ccdA* gene on the plasmid in order to survive. If the plasmid was not present, the ccdB bacteria-specific cytotoxin would not be repressed, the production strain will not survive and maltogenic alpha amylase would not be produced.

No antibiotic resistance genes were used during the genetic modification and hence, they are not found in the *E. coli* BLASC production stain.

### 3.2.3 Genetic stability of the inserted gene

Based on the use of the ccdA/ccdB antitoxin/toxin system and the activity of the maltogenic alpha amylase after three distinct fermentation runs, the *amyM* / *ccdA* expression plasmid is considered to be stable.

The applicant also provided Random Amplified Polymorphic DNA data that compared the

fingerprint profile of the production strain before and after fermentation. This data substantiates the stability of the production strain during fermentation and provides further evidence of the survival of the production strain. It can be inferred that the *ccdA*/*ccdB* antitoxin/toxin system is functional and the *amyM* / *ccdA* expression plasmid is stable.

## 3.3 Safety of maltogenic alpha amylase

### 3.3.1 History of safe use

Maltogenic alpha amylase isolated directly from *G. stearothermophilus* has been used since the mid-1990s in baking (Derde et al., 2012; Goesaert et al., 2009). Further, *G. stearothermophilus* maltogenic alpha amylase has been assessed for use in Australia and New Zealand when manufactured using genetically modified *B. subtilis*, genetically modified *Saccharomyces cerevisiae*[8] and genetically modified *Bacillus licheniformis*[9]. The subject *G. stearothermophilus* maltogenic alpha amylase enzyme of this application is produced in *E. coli* and the mature enzyme has an identical sequence to the *G. stearothermophilus* maltogenic alpha amylases already assessed by FSANZ.

There are no reports of adverse effects arising from the use of *G. stearothermophilus* maltogenic alpha amylase enzymes in Australia or any other jurisdiction where the enzyme has been approved.

### 3.3.2 Bioinformatics concerning potential for toxicity

A search was performed using the maltogenic alpha amylase protein sequence against the DBETH bacterial exotoxins database[10].and bacterial toxins in the UniProt database[11] (using an E-value[12] threshold of $10^{-4}$). No matches to known toxins were found.

### 3.3.3 Evaluation of toxicity studies

The *G. stearothermophilus* maltogenic alpha amylase test item used in the following toxicity studies was produced using *E. coli* BLASC and represented the commercial enzyme product.

***Animal Studies***

*Acute oral toxicity study in rats (INTOX Pvt. Ltd., 2014). Regulatory Status: GLP; conducted according to OECD Test Guideline 423.*

The maltogenic alpha amylase test item was administered by oral gavage as a single dose of 2000 mg/kg bw to two groups of female Wistar rats (3/test group). Water was used as the vehicle control. No mortality occurred during the study. No remarkable macroscopic or histopathological changes were observed at necropsy. It was concluded that the median lethal dose (LD50) of the maltogenic alpha amylase test item is greater than 5000 mg/kg bw[13].

*90-day repeated dose oral toxicity study in rats (INTOX Pvt. Ltd., 2015). Regulatory Status:*

[8] Application A1210 was gazetted on 20 September 2021.
[9] Application A1211 is running concurrently with this application.
[10] DBETH database: http://www.hpppi.iicb.res.in/btox/
[11] UniProt database: https://www.uniprot.org/
[12] 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.
[13] As outlined in the OECD TG 423, no observed mortality at the dose limit of 2000 mg/kg bw is suggestive of an LD50 being 5000 mg/kg bw or above.

*GLP; conducted according to OECD Test Guideline 408.*

The maltogenic alpha amylase test item was administered to Wistar rats (10/sex /group) at doses of 0, 250, 500 and 1000 mg/kg bw/day enzyme (0, 209, 419 and 838 mg/kg bw/day TOS, respectively) by oral gavage for 13 weeks. Additional control and high-dose animals (5/sex/group) were administered the test item for 13 weeks and allowed a further 4 week recovery period before necropsy. The vehicle control was water.

Animals were observed daily for signs of toxicity. Body weight, food consumption and detailed clinical examinations for signs of toxicity were recorded weekly. Ophthalmological examination was conducted on control and high dose test animals prior to treatment and at study termination. Functional performance and sensory reactivity tests were performed in week 13. Gross pathology 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. Following the lack of adverse finding in these groups, histopathological examinations were not extended to the low-dose and recovery 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 or motor activity parameters. No remarkable macroscopic or histopathological changes were observed at necropsy.

The no observed adverse effect level (NOAEL) was 1000 mg/kg bw/day enzyme (838 mg/kg bw/day TOS), which was the highest dose tested.

***Genotoxicity***

*Bacterial reverse mutation test (INTOX Pvt. Ltd., 2014). Regulatory Status: GLP; conducted according to OECD Test Guideline 471.*

The potential mutagenicity of maltogenic alpha amylase was evaluated in *Salmonella typhimurium* strains TA97a, TA98, TA100, TA102 and TA1535, with and without metabolic activation using rat liver homogenate (S9). Mutation tests were conducted twice independently over a dose range of 50-5000 µg protein/plate. The concentration range of the test item was based on the findings of a preliminary dose range finding study. Bacterial cells were incubated for 20 minutes with test item, before being plated in triplicate.

Positive controls in the absence metabolic activation were sodium azide (TA1535), 3-methylmethane sulphonate (TA100, TA102), ICR191 (TA97a) and 4-nitroquinoline-*N*-oxide (TA98). Positive controls in the presence of metabolic activation were 2-aminoanthracene (TA1535), 2-aminofluorene (TA97a, TA98, TA100) and 1,8-dihydroxyanthraquinone (TA102). Sterile water was used as the vehicle control.

No concentration-related increases in revertant colonies were observed in cultures treated with the test item, 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 maltogenic alpha amylase test item was not mutagenic under the conditions of this test.

*In vitro mammalian chromosomal aberration test (INTOX Pvt. Ltd., 2014). Regulatory status: GLP; conducted according to OECD test guideline 473.*

The potential of maltogenic alpha amylase to cause chromosomal aberrations in mammalian cells was tested using human lymphocytes isolated from peripheral blood, collected from a

healthy male volunteer. Treatment with the maltogenic alpha amylase test item was either a 3 hour pulse exposure with or without S9, followed by a 21 hour recovery; or 24 hours of continuous exposure without S9. Positive control assays were conducted in parallel using methyl methanesulphonate in the absence of S9 and cyclophosphamide in the short-term treatment with S9. The experiment was carried out once in duplicate.

Dose selection experiments did not show cytotoxic activity greater than 40% at a concentration of 5000 µg/mL of the maltogenic alpha amylase test item in any of the test conditions. Based on this observation, the dose range of 500-5000 µg/mL was examined for all test conditions.

There were no treatment related increases in chromosomal aberrations observed in peripheral blood lymphocytes following exposure to the maltogenic 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 aberration, validating the sensitivity of the experimental methodology. It was concluded that maltogenic alpha amylase did not cause chromosome aberrations in peripheral blood lymphocytes, under the conditions of the study.

### 3.3.4 Potential for toxicity arising from manufacture

Enzyme expression during fermentation is induced by the addition of IPTG, a metabolically stable analogue of beta-D-galactose. Levels of IPTG in the enzyme preparation are expected to be low as a result of ultra- and diafiltration steps applied during processing. Estimation of the likely exposures to IPTG were below the threshold of toxicological concern. The enzyme preparation was not genotoxic and no signs of toxicity were seen in a 90-day rat study. On that basis it is concluded that IPTG used in the manufacture of maltogenic alpha amylase does not raise safety concerns.

### 3.3.5 Potential for allergenicity

A FASTA search was performed using the amino acid sequence of *G. stearothermophilus* maltogenic alpha amylase using the AllergenOnline[14] database (queried November 2014) using three sequence alignments: an 80 mer sliding window (>35% homology), the full length protein (more than 50% homology), and an 8 mer sliding window (100% homology).

The applicant supplied bioinformatics search identified taka-amylase A (Asp o 21) from *Aspergillus oryzae* as a known respiratory allergen with a greater than 35% sequence identity using an 80-amino acid sliding window. Asp 0 21 is a respiratory allergen and not recognised as a food allergen[15]. The applicant also provided a recent bioinformatic search (October 2021), which identified a number of additional respiratory allergens. None were recognised food allergens.

Respiratory sensitisation of occupationally exposed individuals to some food enzyme processing aids, such as alpha-amylase and other glycoside hydrolase enzymes has 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 maltogenic alpha amylases in the scientific literature.

The applicant supplied the results of an *in vitro* pepsin digestion assay showing that maltogenic alpha amylase was liable to proteolysis within 30 seconds. Enzymes readily hydrolysed by proteolysis are considered to have a lower potential of becoming food

[14] AllergenOnline: http://www.allergenonline.org/
[15] World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee: http://www.allergen.org/

allergens (FAO/WHO 2009a).

It is concluded that the presence of maltogenic alpha amylase produced using modified *E. coli* in food is unlikely to pose an allergenicity concern to consumers.

### 3.3.6 Assessments by other regulatory agencies

An EFSA safety evaluation on maltogenic alpha amylase produced using *E. coli* BLASC was provided. EFSA concluded that, under the intended conditions of use, the evaluated food enzyme does not raise safety concerns and that the allergenic risk was likely to be low.

## 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 maltogenic alpha amylase 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.

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

- the maximum physiological requirement for solid food (including milk) is 50 g/kg body weight/day
- the maximum physiological requirement for non-milk beverages is 100 mL/kg body weight/day (the standard default level used in a budget method calculation as noted in the FAO/WHO Environmental Health Criteria (EHC) 240 Chapter 6 on dietary exposure assessment (FAO/WHO 2009b))
- 12.5% of solid food and 25% of non-milk beverages contain maltogenic alpha amylase (standard default proportions (FAO/WHO 2009b))
- the maximum maltogenic alpha amylase level in final solid food and non-milk beverages was 33.89 mg TOS/kg food (i.e. the highest use level from all uses within each group)
- all of the enzyme will remain in the final food and non-milk beverages.

Based on these assumptions, the applicant calculated the TMDI of maltogenic alpha amylase to be 1.059 mg TOS/kg body weight/day (1.06 mg TOS/kg body weight/day rounded).

FSANZ has reviewed the assumptions and accepts the calculation. The maximum physiological requirement for solid food (including milk) of 50 g/kg body weight/day 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, which for this enzyme would be from the bakery and starch processing uses.

The TMDI will be an overestimate of the dietary exposure given the conservatisms in the budget method. It was assumed that the enzyme remains in the final foods and beverages. However FSANZ notes that the enzyme will be inactivated during food processing due to process conditions such as high heat and an unconducive pH, and will have no technological function in the final food.

# 4 Discussion

No public health and safety concerns were identified in the assessment of this maltogenic alpha amylase produced from a GM strain of *E. coli* under the proposed use conditions. The *E. coli* host is neither pathogenic nor toxigenic and analysis of the GM production strain (*E. coli* BLASC) confirmed the presence and stability of the introduced DNA. Bioinformatic analysis indicated that the enzyme shows no significant homology with any known toxins.

Maltogenic alpha amylase was not genotoxic *in vitro*. The no observed adverse effect level (NOAEL) determined in a 90-day oral gavage study in rats was 1000 mg/kg bw/day total protein, equivalent to 838 mg/kg bw/day Total Organic Solids (TOS). The theoretical maximum daily intake (TMDI) was calculated to be 1.06 mg/kg bw/day TOS. A comparison of the NOAEL and the TMDI gives a Margin of Exposure (MOE) of approximately 790.

Bioinformatic analysis showed that the enzyme has a degree of homology with several known allergens. None were food allergens. No reports of sensitisation to any form of maltogenic alpha amylases was found in a search of the scientific literature and maltogenic alpha amylases from the same source organism are already permitted in the Code. On that basis, the enzyme was considered to be of low allergenic risk to consumers when used as a processing aid in food.

# 5 Conclusion

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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