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

Supporting Document 1

RISK ASSESSMENT REPORT

Summary

The *Australia New Zealand Food Standards Code* (the Code) currently permits the use of a number of microbial enzymes as processing aids in the manufacture of food. There are currently no permissions listed the Code for the enzyme, maltotetraohydrolase (EC 3.2.1.60).

Application A1033 seeks approval for the use of maltotetraohydrolase from *Bacillus licheniformis* as a processing aid. This host strain of *B*. *licheniformis* was modified using recombinant DNA techniques to contain the gene for an engineered form of maltotetraohydrolase PS4wt from *Pseudomonas stutzeri*.

The risk assessment has considered the technological suitability, the safety and identity of the donor and host microorganisms, and safety of the enzyme preparation of maltotetraohydrolase. Based on the available data, it was concluded no toxicological or hazard-related concerns with the enzyme or the donor or host microorganisms were revealed which would preclude permitting use of the enzyme as a food processing aid. The absence of any specific hazards being identified is consistent with maltotetraohydrolase undergoing normal proteolytic digestion in the gastrointestinal tract. It was further concluded that the proposed use of the enzyme, namely to retard the staling process of baked goods, was technologically justified and demonstrated to be effective.

Uncertainties in the Risk Assessment

Sufficient information was available to provide an acceptable level of confidence in the conclusions of this risk assessment.

Conclusions

- Based on the available evidence, which did not reveal any specific hazards, it is concluded that no safety concerns are associated with the proposed use of maltotetraohydrolase with *B*. *licheniformis* as the host organism.
- The absence of any specific hazards is consistent with maltotetraohydrolase undergoing normal proteolytic digestion in the gastrointestinal tract.
- There is no evidence of toxicity associated with the enzyme preparation in either the acute or 90 day toxicity studies.
- In the absence of any treatment related effects in the 90-day study, the NOAEL is 79 mg total protein/kg bw/day, which corresponds to the highest dose level tested. This is equivalent to 90.9 mg TOS/kg bw/day or 241318 BMU/kg bw/day.

- There is no evidence of genotoxicity in two in vitro studies with the enzyme preparation.

- There is no evidence of any immunologically significant amino acid similarity between maltotetraohydrolase and known allergens.

- The source organism, *Bacillus licheniformis*, is regarded as non-pathogenic and non-toxigenic and has a safe history of use in the production of food enzymes.

- The ADI (Acceptable Daily Intake) for maltotetraohydrolase produced by genetically modified *B. licheniformis* is 'not specified', indicating a food substance of very low toxicity which does not represent a hazard to health.

- Maltotetraohydrolase produced by genetically modified *B. licheniformis* has greater thermostability and baking performance over the wild type maltotetraohydrolase.

- Maltotetraohydrolase produced by genetically modified *B. licheniformis* meets international specification requirements for enzyme preparations.

- The taxonomic identity of the donor organism based on molecular techniques is *Pseudomonas stutzeri*.

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1 Introduction

Application A1033 submitted by Danisco A/S via Axiome Pty Ltd seeks approval for the use of a new processing aid (enzyme), maltotetraohydrolase, produced from *Bacillus lichenformis* containing a gene encoding for a protein engineered variant of maltotetraohydrolase PS4wt from *Pseudomonas stutzeri.* (The organism had been previously misclassified as *Pseudomonas saccharophila* in the information provided by the Applicant).

The systematic name for this enzyme is 4-α-D-glucan maltotetraohydrolase (EC 3.2.1.60, CAS No. 37288-44-1). The enzyme is a hydrolase that catalyses the hydrolysis of (1,4)-α-D-glucosidic linkages in amylaceous polysaccharides to remove successive maltotetraose residues from the non-reducing chain ends. The use of the enzyme delays the staling of bakery products and extends the acceptable eating quality period. The enzyme is expected to be largely inactivated during baking and will have no further technological function after baking.

The enzyme is not currently listed in the *Australia New Zealand Food Standards Code* (the Code) in the Table to Clause 17 (Permitted enzymes of microbial origin) of Standard 1.3.3 - *Processing Aids*. The enzyme preparation complies with international specifications for enzymes.

This risk assessment considers the safety of the donor and host bacteria, the safety of the enzyme preparation and the technological justification of the enzyme to retard the staling process of baked goods.

1.1 Objectives of the Assessment

In proposing to amend the Code to include maltotetraohydrolase produced by a genetically modified *Bacillus licheniformis* as a processing aid, a pre-market assessment is required. The objectives of this risk assessment are then to:

- Assess the risk to public health and safety; and
- Assess the technical function of the enzyme.

1.2 Key Risk Assessment Questions

The following key risk assessment questions have been developed to address the objectives of the assessment.

Question 1: What is the risk to public health and safety from the use of maltotetraohydrolase produced by a genetically modified strain of *B. licheniformis* as a processing aid?

Question 2: Is the new genetically modified strain of *B. licheniformis* safe for producing maltotetraohydrolase?

Question 3: Does the final enzyme product contain any allergenic materials?

Question 4: Does the enzyme achieve its technical function?

1.3 Production of Maltotetraohydrolase

1.3.1 Description of the genetic modification

The production organism for maltotetraohydrolase is *Bacillus licheniformis* strain GICC03279, which was derived by recombinant DNA methods from *B. licheniformis* strain Bra7. The genetic modification involved the deletion of several endogenous chromosomal genes (a sporulation gene, the native *cat* gene, and genes coding for amylase and various proteases) followed by the insertion into the chromosome of an engineered form of the wild type maltotetraohydrolase gene from *Pseudomonas stutzeri* strain IAM 1504. The engineered form of the enzyme, which was modified using site specific mutagenesis and site scanning mutagenesis, lacks the starch binding domain, which was deleted, and also contains 16 amino acid changes in the catalytic domain. The purpose of these modifications was to increase the temperature stability and baking performance of the enzyme.

The inserted gene is under the regulation of a native *B. licheniformis* promoter and terminator along with a selectable marker, the native *B. licheniformis cat* gene, encoding chloramphenicol resistance. The maltotetraohydrolase gene expression cassette was integrated into the host chromosome at the *cat* locus by recombination between direct repeated *cat* sequences. After integration, all vector sequences were deleted by recombination. The final production strain therefore contains only the integration cassette consisting of the engineered form of the maltotetraohydrolase gene, including its regulatory signals, and the re-introduced native *cat* gene. There is therefore no difference between the final production organism and the original strain in terms of the presence of the *cat* gene. The inserted genes are present in the chromosome at the site of the endogenous (deleted) *cat* gene.

1.3.2 The production process

The maltotetraohydrolase enzyme is produced by a submerged fermentation process using appropriate substrates and nutrients followed by several filtration and purification steps. The isolated extra cellular enzyme concentrate is stabilised with potassium sorbate and then dried and agglomerated using any one of the common drying methods, such as spray drying, fluid bed agglomeration or fluid bed spray drying. The process is described in detail in Confidential Commercial Information (CCI) provided by the Applicant.

2 Microbiological Assessment

2.1 Identification of the donor and host organisms

The determination of the taxonomy (genus and species) of bacteria has traditionally been established using a combination of morphological and phenotypic assessments. Examples of specific tests include size and shape of cells, the ability to grow in aerobic conditions, growth temperature ranges and the ability to metabolise carbohydrates.

More recently, molecular techniques such as ribotyping and 16s rDNA sequencing have become available. These molecular techniques are used to determine the degree of genetic similarity between a test strain and a number of characterised (type) strains of related species. The output of the taxonometric assessment is a determination of the most closely related type strain. It is not uncommon for the phenotypic and genotypic assessment of a strain to be different.

2.1.1 Host strain

The Applicant states that the host is a non-toxigenic and non-pathogenic strain of *B. licheniformis*. Non-genetically modified strains of *B*. *licheniformis* have a long history of use for the preparation of commercial enzymes for use in the food industry. Enzymes produced using *B*. *licheniformis* are currently permitted in the Code and include α-Amylase (EC 3.2.1.1) and Pullulanase (EC 3.2.1.41).

The Applicant has provided additional information to establish the taxonomy of the host strain, Bra7 using two molecular methods: ribotyping and 16s rDNA sequencing. The analysis establishing the genus and species of the host strain was performed by the DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures). Three reference strains of *B*. *licheniformis*: DSM13, DSM603, a bacitracin producer and DSM8782, a pencillinase producer were used. In addition to the host strain Bra7, six other Genencor International culture collection *B*. *licheniformis* strains were evaluated.

The results of the ribotyping and 16s rDNA sequencing confirmed the host strain, Bra7, as *B*. *licheniformis*. Examination of the ribotyping and phylogenetic trees suggests this strain is closely related to the *B*. *licheniformis* type strain DSM13.

2.1.2 Donor strain

The original designation for the donor strain was *P*. *saccharophila* IAM1504. This strain was sourced from the IAM Culture Collection, located at the Institute of Molecular and Cellular Biosciences at The University of Tokyo.

A number of *Pseudomonas* sp. strains from the Genencor International culture collection were analysed against other strains of known species of *Pseudomonas* from the American Type Culture Collection, including *P*. *stutzeri* (ATCC 17588, ATCC 17685, ATCC 17686 and ATCC 17591), *P*. *putida*, *P*. *asplenii*, *P*. *mendocina*, *P*. *pseudoalcaligenes*, *P*. *saccarophila* and *P*. *pseudoflava*.

A phylogenetic tree based on the 16s rDNA sequence analysis revealed that the IAM1504 strain was most closely related to *P*. *stutzeri* ATCC 17685. The ATCC reports that the origin of strain ATCC 17685 is as a clinical isolate. On the basis of the 16s rDNA analysis it appears that the organism has been misclassified as *Pseudomonas saccharophila* and should be referred to as *Pseudomonas stutzeri*.

The source strain, *Pseudomonas stutzeri* does not have a long history of use in the food industry.

The Applicant used Medline, Chemtox and Chemical Abstracts to search for references to pathogenic, allergenic or toxicological evidence relating to both *P*. *stutzeri* and *P*. *saccharophila*. Other key words used in the search included: pathogen, allergen, allergenic allergy, toxin, toxic or toxicology for the period 1980 to 26 June 2006.

A small number of published scientific papers were identified that referred to human illness relating to *P*. *stutzeri*. In a review of 29 clinical cases of human illness associated with *P. stutzeri* it was found that many cases were probable nosocomial (hospital acquired) infections following surgical procedures and/or involved immunocompromised patients with existing conditions (Reisler and Blumberg, 1999). These clinical reports suggest that *P*. *stutzeri* is an opportunistic pathogen of low virulence and has not been associated with foodborne illness.

2.2 Conclusion

In conclusion, the host and donor microorganisms do not have significant pathogenic or toxigenic traits. Non-genetically modified strains of *B. licheniformis* are currently permitted in the Code for the production of enzymes used as processing aids. Information provided by the applicant suggests that the IAM1504 strain from the IAM Culture Collection is *P. stutzeri* and not *P. saccharophila*. Cases of human illness due to *P. stutzeri* appear to be nosocomial in nature and not associated with the consumption of food. The microbiological assessment does not raise concerns as to the safety of the host or donor microorganisms.

3 Chemical Safety Assessment

3.1 Evaluation of the safety studies

The following studies were evaluated as part of the hazard assessment:

- acute oral toxicity study in rats;
- 90 day oral (gavage) toxicity study in rats;
- bacterial reverse mutation assay (Ames test);
- *in vitro* mammalian chromosomal aberration test with human lymphocytes;
- cytotoxicity screening test for *Bacillus* sp. toxins.

In addition, the Applicant also submitted:

- a bioinformatics analysis for similarity of the maltotetraohydrolase amino acid sequence with known protein allergens; and

- an evaluation of the safety of the enzyme preparation according to the Pariza/Johnson Decision Tree (Pariza and Johnson, 2001).

3.1.1 Toxicity studies

The test material used in all the toxicity studies is described as SAS 3 amylase (Lot No. 20078126), supplied to the testing laboratories as a frozen brown liquid. The maltotetraohydrolase preparation had an enzyme activity of 241318 Betamyl unit (BMU)/g or 3000 BMU/mg enzyme, and total organic solids (TOS) of 9.09%. The total maximum protein content was 78.76 mg/ml.

Acute toxicity

Pooles, A. (2008). SAS 3 amylase (*Bacillus licheniformis*)(GICC 03279): Acute oral toxicity in the rat – fixed dose method. (SPL Project No. 2420/0003, SafePharm Laboratories, United Kingdom).

In a GLP compliant study conducted according to OECD Test Guideline 420 (Acute Oral Toxicity – Fixed Dose Method), five fasted 8-12 week old female rats (Crl:CD® (SD) IGS BR) were given a single oral (gavage) dose of undiluted test material at a dose level of 2000 mg/kg bodyweight (dose volume 1.96 ml/kg).

Clinical observations were made at 0.5, 1, 2 and 4 hours after dosing and thereafter once daily for 14 days. Morbidity and mortality checks were made twice daily. Bodyweights were recorded on Day 0 and on Days 7 and 14. Animals were killed at the end of the observation period and subjected to gross necropsy, which involved an external examination and

opening of the abdominal and thoracic cavities.

No deaths were recorded and there were no clinical signs of any toxicity. All the animals showed normal bodyweight gain and no abnormalities were noted at necropsy.

The acute oral median lethal dose (LD50) of the test material in female rats was therefore estimated to be greater than 2000 mg/kg bodyweight.

Subchronic toxicity

Dhinsa, N.K. & Brooks, P. (2008). SAS 3 amylase (*Bacillus licheniformis*)(GICC 03279): Ninety day repeated dose oral (gavage) toxicity study in the rat (SPL Project No. 2420/0008, SafePharm Laboratories, United Kingdom).

In a GLP compliant study conducted according to OECD Test Guideline 408 (Subchronic Oral Toxicity – Rodent: 90 Day Study), the test material was administered to groups of rats (Wistar Han™:HsdRccHan™:WIST strain, 10/sex/group) by gavage at dose levels of 0 (vehicle only, 0.9% saline), 23.7 (low dose), 47.4 (intermediate dose) and 79 (high dose) mg protein/kg bw/day (corresponding to 0, 27.3, 54.5 and 90.9 mg TOS/kg bw/day or 0, 72395,144790 and 241318 BMU/kg bw/day respectively) for 90 consecutive days. Dose volume was 5 ml/kg bw/day.

Clinical signs, neurobehaviour (behaviour, motor activity, forelimb/hindlimb grip strength, sensory reactivity), bodyweight and food and water consumption were monitored throughout the study. Haematology and blood chemistry were evaluated for all surviving animals at the end of the study (Day 90); animals were not fasted prior to sampling. Ophthalmoscopic examination was performed on control group and high dose animals prior to treatment and during Week 12, prior to termination of the study. All animals underwent gross necropsy with histopathological examination of selected tissues being performed for control and high dose animals and any animals dying during the study.

Two unscheduled deaths were recorded. One low dose female was killed *in extremis* on Day 68 after showing signs of pilo-erection, lethargy, hunched posture, pallor of the extremities and staining around the snout. One intermediate dose female was found dead on Day 90 prior to study termination and had not previously exhibited any clinical signs. Necropsy was able to rule out a gavaging error. Histopathological examination of tissues from these animals did not establish the cause of death, however, both deaths appeared unrelated to treatment.

Except for isolated instances of generalised fur loss in high and intermediate dose males, no clinical signs were noted during the treatment period and there were no treatment related changes in any of the behavioural, functional performance or sensory reactivity tests. No toxicologically significant changes in bodyweight were detected during the treatment period and there were no differences in food consumption, water intake and food efficiency between control and treated groups. No treatment-related ophthalmoscopic findings were detected in any of the high dose animals prior to study termination. Haematological parameters were unchanged and statistical analyses did not reveal any significant intergroup differences. Changes in blood chemistry consisted of an increase in potassium levels in females from all treatment groups (3.881 vs. 4.526, 4.469 and 4.433 nmol/L, control vs. low, intermediate and high dose, respectively) and a reduction in chloride levels in high dose females (105.4 vs. 103.0 nmol/L). The potassium and chloride levels were however all within historical control values and in the absence of any dose-response relationship were all considered to be unrelated to treatment.

Reductions in absolute and relative ovary weights were detected for females from all treatment groups. The control values were however higher than the historical range, and in the absence of a convincing dose response or any associated histopathological changes, these reductions were not considered to be treatment related. No other organ weight changes were noted. No treatment-related macroscopic abnormalities or histopathological changes were detected.

The 'No Observed Adverse Effect Level' (NOAEL) was considered to be 79 mg total protein/kg bw/day (corresponding to 90.9 mg TOS/kg bw/day or 241318 BMU/kg bw/day) based on the absence of any treatment-related effects.

Genotoxicity

The results of two *in vitro* genotoxicity studies with maltotetraohydrolase are summarised in Table 1 below. Both studies were GLP compliant and conducted in accordance with OECD Test Guidelines 471 (Bacterial Reverse Mutation Test) and 473 (*In vitro* Mammalian Chromosome Aberration Test). Neither test revealed any genotoxic potential associated with maltotetraohydrolase.

Table 1: Genotoxicity of maltotetraohydrolase

Assay	Test System	Concentration	Results
Reverse mutation assay "Ames Test" SafePharm Laboratories, UK (SPL Project No. 2420/0006). Bowles (2008)	*Salmonella typhimurium* TA1535, TA1537, TA98 and TA100; *Escherichia coli* WP2uvrA¯ ± metabolic activation using liver microsomal preparation from Sprague-Dawley rats (S9-mix)	Dose ranging assay: TA100 and WP2uvrA¯ tested at 0.15 to 5000 µg total protein/plate ± S9. Mutation assay: All strains tested at 50 to 5000 µg total protein ± S9.	**Negative** – no significant increases in frequency of revertant colonies, with or without metabolic activation.
Chromosome aberration SafePharm Laboratories, UK (SPL Project No. 2420/0007). Morris (2008)	Human peripheral blood lymphocytes ± metabolic activation using liver microsomal preparation from Sprague-Dawley rats (S9-mix) Positive controls: mitomycin C (- S9) and cyclophosphamide (+ S9)	Dose ranging assay: 19.5 to 5000 µg/ml ± S9. Test 1: 156.25, 312.50 and 625 µg/ml ± S9 (4 hours exposure to test material). Test 2: 156.25, 312.50 and 625 µg/ml ± S9 (24 hours exposure to test material without S9 – 312.5 µg/ml max. dose, 4 hours exposure to test material with S9)	**Negative** – the test material did not induce a statistically significant increase in the frequency of cells with aberrations or numbers of polyploid cells, with or without metabolic activation.

3.1.2 Production strain analyses

Analysis for toxin production

Wilkins, S., Hubbard, J.O. & Douglas, J. (2002). CHO-MTT cytotoxicity screening test for *Bacillus* toxins (GNC 001B/021251, Huntingdon Life Sciences Ltd, United Kingdom).

Bacterial broth supernatants from 11 industrial *Bacillus* spp. strains (2 *B. subtilis* strains, 7 *B. licheniformis* strains and 2 *B. amyloliquifaciens* strains) were screened for the production of toxins using the MTT assay, which detects toxin-induced damage to Chinese Hamster Ovary (CHO) cells (CHO-K1 epithelial cell line). The toxicity of each supernatant was compared to the background toxicity of broth alone. Broths from reference *Bacillus* spp. strains, negative (*B. licheniformis* NCTC 6346) and positive (*B. cereus* NCTC 11145 and *B. cereus* NCTC 11143) for toxin production, were included as controls.

Doubling dilutions (50% to 0.781% broth in culture medium) of blank broth or test broth were applied to duplicate cultures of CHO cells. After 24 hours, viability and mitochondrial activity of the CHO cells was measured using the MTT assay[1].

The results for the reference strains were negative for the non-toxin producing *Bacillus* spp. strains and positive for the toxin producing *Bacillus* spp. strains. The results for the 11 test *Bacillus* spp. strains were all negative for toxin production.

Pariza-Johnson decision tree analysis

The Applicant has submitted their assessment of the maltotetraohydrolase enzyme preparation according to the Pariza-Johnson Decision Tree (Pariza and Johnson, 2001). This decision tree has been constructed based on experience in the production, use and safety of enzyme preparations and is based upon an evaluation of the toxigenic potential of the production organism. Specifically, this analysis relies on determining whether the production strain is derived from a safe strain lineage, as well as consideration of the safety of all new DNA that has been introduced into the host organism.

The Applicant states that the enzyme product encoded by the introduced DNA in *B. licheniformis* has a history of safe use in food based on the following:

- three previous generations of the enzyme, produced in *B. subtilis*, have been determined to be GRAS (data not provided);
- amylases with the designation 3.2.1.1., which are produced from fungal, bacterial and cereal sources, are widely used in the food industry;
- maltotetraohydrolases (EC 3.2.1.60) from *P. stutzeri* strains, which are closely related to the three generations of the Amylase SAS3 preparation, have been used for producing maltotetraose and maltotetraose syrups for use in foods since 1980;
- maltotetraose-forming amylase from *P. stutzeri* is listed as a natural additive in the Korean Food Additives Code.

The only inserted DNA is the endogenous *B. licheniformis cat* gene and the engineered form of the maltotetraohydrolase gene, neither of which code for any known toxins. The DNA is integrated into the bacterial chromosome at a known locus (the *cat* locus). Given this targeted integration, the Applicant states there is no concern regarding any unintended pleiotropic effects. The Applicant states that the *B. licheniformis* strain used as the production organism is derived from a safe lineage of *B. licheniformis*, meaning it is a thoroughly characterised non-pathogenic, non-toxigenic strain with a history of safe use in food enzyme manufacture. The Bra7 strain is a classical industrial strain that has been used for α-amylase production by the Applicant since 1989. The Applicant states that toxicology testing on 5 products from this strain (data not provided) confirms the safety of the lineage. The Applicant concluded that the maltotetraohydrolase enzyme preparation is safe for its intended use.

3.1.3 Potential allergenicity of maltotetraohydrolase

The amino acid sequence similarity of maltotetraohydrolase to known allergens was determined using bioinformatic analyses. The amino acid sequence of the engineered form of the maltotetraohydrolase enzyme was compared to that of known allergens in the SDAP

[1] The MTT assay depends on the ability of viable cells to metabolise a water-soluble dye (MTT or 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) into an insoluble salt. The quantity of insoluble salt produced is proportional to the number and/or metabolic activity of viable cells present in each culture.

and Allermatch databases, containing 737 and 792 allergens, respectively.

The FASTA algorithm was used to determine the degree of similarity between the maltotetraohydrolase enzyme and proteins contained in the above databases. The degree of structural similarity was determined by examining the alignment of the sequences, percent identity and *E*-score. The latter is a statistical measure of the likelihood that the similarity between two sequences could have occurred by chance alone. The FASTA alignment threshold for potential allergenicity was 35% homology over 80 amino acids, which is consistent with the recommendations from the Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology (FAO, 2001). The potential allergenicity of the enzyme was further evaluated using a sliding window search for exact matches of short stretches (6 amino acids) of sequence that could serve as potential IgE binding sites.

No immunologically significant sequence similarity was identified. A low degree of similarity (approximately 20%, E score 10^{-8}) to α–amylase from *Aspergillus oryzae* (Asp o 21, *A. oryzae* TAKA amylase a) was detected. The fungal amylases have been recognised as inhaled allergens associated with baker's asthma (Blanco Carmona *et al*., 1991) although they do not cause food allergy. Furthermore, the degree of similarity with the engineered form of the maltotetraohydrolase is low and therefore not considered to be immunologically meaningful.

3.1.4 JECFA consideration of maltotetraohydrolase

To date, there has been no evaluation of maltotetraohydrolase from *B. licheniformis* by the Joint FAO/WHO Expert Committee on Food Additives and Contaminants (JECFA). The Applicant notes that amylase from *B. licheniformis* was reviewed by JECFA in 1986 with an ADI 'not specified' being established (WHO, 1986).

An ADI 'not specified' is applicable to a food substance of very low toxicity which, on the basis of the available data (chemical, biochemical, toxicological, and other), the total dietary intake of the substance arising from its use at the levels necessary to achieve the desired effect and from its acceptable background in food, does not represent a hazard to health.

3.2 Conclusion

Following the safety assessment of maltotetraohydrolase from *B. licheniformis*, it is concluded that:

- there is no evidence of toxicity in either the acute or 90 day toxicity studies;
- in the absence of any treatment related effects in the 90-day study, the NOAEL is 79 mg total protein/kg bw/day, which corresponds to the highest dose level tested. This is equivalent to 90.9 mg TOS/kg bw/day or 241318 BMU/kg bw/day.
- there is no evidence of genotoxicity in two *in vitro* studies with the enzyme preparation;
- there is no evidence of any immunologically significant amino acid similarity between maltotetraohydrolase and known allergens.

Based on the available evidence, which did not reveal any specific hazards, it is concluded that there are no safety concerns associated with the proposed use of maltotetraohydrolase from *B. licheniformis*. The absence of any specific hazards is consistent with maltotetraohydrolase undergoing normal proteolytic digestion in the gastrointestinal tract.

4 Food Technology Assessment

4.1 Chemistry

Systematic name:	4-α-D-glucan maltotetraohydrolase
IUBMB Enzyme Nomenclature:	EC 3.2.1.60
CAS number:	37288-44-1
Common name:	Maltotetraohydrolase
Other names:	1,4-α-D-glucan maltotetraohydrolase, exo-maltotetraohydrolase, maltotetraose-forming amylase, Amylase SAS 3.

Maltotetraohydrolase is a hydrolase enzyme that catalyses the hydrolysis of (1,4)–α–D-glucosidic linkages in amylaceous polysaccharides to remove successive maltotetraose residues from the non-reducing chain ends.

4.2 Technological function of the enzyme

The enzyme preparation will be used in bakery products such as bread, bread buns, whole wheat toast bread, soft rolls and tortillas to delay staling and thereby extend the acceptable eating quality period.

Anti-staling enzymes have to be sufficiently heat-stable to be active during the baking step to modify starch after initial gelatinization of starch granules. The Applicant claims that this maltotetraohydrolase, a G4-amylase enzyme, has improved thermostability and baking performance over the wild type maltotetraohydrolase.

The temperature optimum is found by measuring release of reducing sugars from 0.4% boiled waxy maize amylopectin for 15 minutes in 50 mM sodium-citrate, 5 mM calcium chloride, pH 6.5. The temperature optimum for this maltotetraohydrolase was determined to be 60-65 °C. Thermal stability, measured in half-lives at 75, 80 and 85 °C, were found to be 78.0, 45.3 and 18.2 minutes respectively. In comparison, the thermal stability of another maltotetraose-forming amylase at 85 °C was less than 5 minutes.

Anti-staling exo-amylases, such as maltotetraohydrolase, shorten the amylopectin side chains and release maltooligosaccharides. This reduces staling by lowering the rate of amylopectin retrogradation without disadvantageous side effects caused by excessive weakening of the amylose network. One way to measure staling, a highly complex phenomenon, is to determine firming.

In a comparative analysis provided by the Applicant, maltotetraohydrolase effectively showed lower firmness and higher resilience on US sponge and dough pan bread over 14 days compared with a standard market amylase.

Recommended use levels range from 2 to 30 mg enzyme protein per kilogram of flour. The enzyme is expected to be largely inactivated during baking and have no further technical effect after baking.

4.3 Analysis and Specifications

4.3.1 Methods of analysis

The method of analysis used for production quality control and in the final food to measure the activity of this maltotetraohydrolase is based on the enzyme's ability to break down blocked p-nitrophenyl maltoheptoside and is measured colorimetrically. The rate of *p*-nitrophenyl release is proportional to amylase activity and is monitored at 410 nm.

The specific activity, measured in BMK, is 3.0 BMK/mg enzyme protein using this assay (Appendix A3 in A1033).

4.3.2 Specifications

The Applicant states specifications written for this maltotetraohydrolase comply with the international specifications relevant for enzymes prepared by the FAO/WHO Expert Committee on Food Additives at its sixty-seventh meeting for publication in FAO JECFA Monographs 3 (JECFA, 2006). These specifications are primary reference sources listed in Clause 2 of Standard 1.3.4: Identity and Purity, of the Code.

Specifications for the commercial product, as provided by the Applicant, are described in the following table.

Table 2: Commercial maltotetraohydrolase specifications (Appendix A4 in A1033)

	Specification
Amylase, betamyl units	150,000 BMU/g (liquid) 1000 – 1500 BMK/g (powder for blends)
Heavy metals (as Pb)	Less than 30 mg/kg
Arsenic (as As)	Less than 3 mg/kg
Lead	Less than 5 mg/kg
Viable bacteria count	Less than 5×10^4 cfu/ml
Coliforms	Less than 30 cfu/ml
Salmonella	Absent in 25 g
E. coli	Absent in 25 g
***Antibiotic activity**	Negative
Description	Off-white powder

* FAO Food and Nutrition Paper: 25th Session of the Joint FAO/WHO Expert Committee on Food Additives; Geneva 1981; p317-318; Appendix A

4.3.3 Allergenicity

The Applicant states that sorbitol and glucose (derived from gluten containing cereals), soy flour and lactose are used as fermentation nutrients during the fermentation process.

If these products are present in the final enzyme preparation they must be labelled in accordance with the requirements of Standard 1.2.3 – *Mandatory Warning and Advisory Statements and Declarations*.

4.4 Conclusion

Maltotetraohydrolase produced by genetically modified *Bacillus licheniformis* meets the international specification requirements for the enzyme. According to the Applicant, the enzyme provides superior anti-staling properties for bakery products due to its greater thermostability and baking performance over the wild type maltotetraohydrolase.

The half-life and crumb firmness and resilience data presented by the Applicant provides adequate assurance that the stated purpose for this maltotetraohydrolase, namely to reduce staling, is technologically justified and has been demonstrated to be effective in achieving this purpose.

5 Response to Risk Assessment Questions

Question 1: What is the risk to public health and safety from the use of maltotetraohydrolase produced by a genetically modified strain of B. licheniformis as a processing aid?

The Safety Assessment (Section 3) reviewed evidence for toxicity (acute and 90 day animal studies) or genotoxicity associated with the enzyme preparation. Based on suitable data, it was concluded that no toxicological or hazard-related concerns with the enzyme were revealed which would preclude permitting use of the enzyme as a food processing aid. The absence of any specific hazards being identified is consistent with maltotetraohydrolase undergoing normal proteolytic digestion in the gastrointestinal tract.

Question 2: Is the new genetically modified strain of B. licheniformis safe for producing maltotetraohydrolase?

The microbiological evidence assessed to establish the safety of the new genetically modified strain of *B*. *licheniformis* included a review of the donor and host microorganisms (Secton 2) and cytotoxicity screening tests (Section 3). The host strain of *B. licheniformis* has a long history of safe industrial use and is currently listed in the Code for the production of enzymes used as processing aids. Cytotoxicity tests using broth supernatant solutions were negative for all of the industrial strains of *Bacillus* spp. tested.

Question 3: Does the final enzyme product contain any allergenic materials?

No evidence of any immunologically significant amino acid similarity between maltotetraohydrolase and known allergens were identified. The growth media used during the fermentation process includes sorbitol and glucose (derived from gluten containing cereals), soy flour and lactose. If these products are present in the final enzyme preparation they must be labelled in accordance with the requirements of Standard 1.2.3 – *Mandatory Warning and Advisory Statements and Declarations*.

Question 4: Does the enzyme achieve its technical function?

The proposed use of maltotetraohydrolase as an enzyme to retard the staling process of baked goods is technologically justified and it has been demonstrated to be effective.

6 Conclusion

The risk assessment has considered the technological suitability, the safety and identity of the donor and host microorganisms and safety of the enzyme preparation of maltotetraohydrolase.

Based on the available data, it was concluded no toxicological or hazard-related concerns with the enzyme or the donor or host microorganisms were revealed which would preclude permitting use of the enzyme as a food processing aid. The absence of any specific hazards being identified is consistent with maltotetraohydrolase undergoing normal proteolytic digestion in the gastrointestinal tract. It was further concluded that the proposed use of the enzyme, namely to retard the staling process of baked goods, was technologically justified and demonstrated to be effective.

7 References

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