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Supporting document 1

Risk and technical assessment report – Application A1248

Glucoamylase from GM *Aspergillus niger* (gene donor: *Gloeophyllum trabeum*) as a processing aid

Executive summary

Novozymes Australia Pty Ltd (Novozymes) has applied to amend the Australia New Zealand Food Standards Code (the Code) to permit the use of a protein engineered variant of the glucoamylase enzyme produced from a genetically modified (GM) strain of *Aspergillus niger.* The glucoamylase enzyme preparation is proposed to be used in starch processing to produce glucose syrups and other starch hydrolysates, and in the production of potable alcohol.

FSANZ has undertaken an assessment to determine whether the enzyme achieves its technological purpose in the quantity and form proposed, and to evaluate public health and safety concerns that may arise from the use of this enzyme.

FSANZ concludes that the proposed use of glucoamylase produced by GM *A. niger* as a processing aid in starch processing and the production of potable alcohol is technologically justified. Analysis of the evidence supplied by the applicant provides adequate assurance that the enzyme achieves its technological function in the quantity and form proposed.

No public health and safety concerns were identified in the assessment of glucoamylase from GM *A. niger* under the proposed use conditions. The *A. niger* host is neither pathogenic nor toxigenic, and analysis of the modified production strain confirmed the presence and stability of the inserted DNA.

Toxicity testing of the enzyme showed no evidence of genotoxicity *in vitro* and the no observed adverse effect level (NOAEL) in a 90-day oral gavage study in rats was the highest dose tested, 1244 mg total organic solids (TOS)/kg bw/day. The theoretical maximum daily intake (TMDI) was calculated to be up to 1.8 mg TOS/kg bw/day. Comparison of the NOAEL with the TMDI gives a margin of exposure (MOE) of approximately 690.

Bioinformatics analysis indicated that the enzyme shows no significant homology with any known toxins. However, a degree of homology was identified with a respiratory allergen from *Schizophyllum commune*. Taking into account that respiratory allergens are usually not food allergens and that *S. commune* has a history of being consumed in food, the risk of food allergy from the proposed uses of the enzyme is likely to be low.

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

Novozymes Australia Pty Ltd (Novozymes) has applied to amend the Australia New Zealand Food Standards Code (the Code) to permit the use of the enzyme glucoamylase (EC 3.2.1.3) as a processing aid in distilled alcohol production and starch processing to produce glucose syrups and other starch hydrolysates. This glucoamylase is produced from a genetically modified (GM) strain of *Aspergillus niger*, containing a protein engineered variant of the glucoamylase gene from *Gloeophyllum trabeum.*

The application refers to 'distilled beverages', however the Code refers to 'potable alcohol' in its permissions, and from herein the latter term will be referenced.

There are permissions for glucoamylase from GM and non-GM microbial sources in the Code. However, this particular source is not specified as permitted. If a pre-market assessment leads to permission being granted, this glucoamylase will provide an additional option for starch processors and manufacturers of potable alcohol.

1.1 Objectives of the assessment

The objectives of this risk and technical assessment were to:

- determine whether the proposed purpose is a solely technological purpose (function) and that the enzyme achieves its technological purpose as a processing aid in the quantity and form proposed to be used
- evaluate potential public health and safety 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 enzyme is produced by a GM strain of *A. niger*, with the gene for glucoamylase provided from *G. trabeum* (see Section 3 for more information). Novozymes provided relevant information regarding the identity of the glucoamylase enzyme. FSANZ verified this information using an appropriate enzyme nomenclature reference (IUBMB 2018). Details of the identity of the enzyme are available in Table 1.

Table 1: Identity

Generic name	glucoamylase
IUBMB nomenclature	glucan 1,4-α-glucosidase
Synonyms	glucoamylase; amyloglucosidase; γ-amylase; lysosomal α-glucosidase; acid maltase; exo-1,4-α-glucosidase; glucose amylase; γ-1,4-glucan glucohydrolase; acid maltase; 1,4-α-D-glucan glucohydrolase
IUBMB No.	EC 3.2.1.3
CAS No.	9032-08-0

IUBMB: International Union of Biochemistry and Molecular Biology; CAS: Chemical Abstracts Service

The glucoamylase enzyme is available as a liquid concentrate. The concentrate is standardised in glucoamylase units (AGU(D)/g) to 1600 AGU(D)/g.

2.2 Manufacturing process

2.2.1 Production of the enzyme

Novozymes' glucoamylase is produced by submerged fermentation of GM *A. niger*. The main fermentation steps are, inoculum, seed fermentation, main fermentation followed by the recovery stage involving primary and liquid separation, germ filtration, and concentration to achieve the desired enzyme activity to provide a concentrated enzyme solution free of the production strain and insoluble substances. Samples are taken from the seed fermenter and main fermenter at various stages during the process. They are examined by microscopy and plate culturing. If contamination is detected the fermentation is terminated.

The enzyme solution is manufactured in accordance with current Good Manufacturing Practices for Food and the principals of Hazard Analysis of Critical Control Point. The applicant states that their quality management system adheres to ISO 9001:2015[1]. Details of the manufacturing process, raw materials and ingredients used in the production of the glucoamylase enzyme preparation were provided in the application or as Confidential Commercial Information.

The enzyme is formulated into a final concentrated enzyme preparation. The enzyme concentrate may be used as a single enzyme preparation or blended with other food enzymes and formulated as a liquid product, depending on the characteristics of the intended food process in which it will be used.

The typical composition of the enzyme concentrate is:

Enzyme solids (TOS)	approx. 39.0 %
Sodium benzoate	approx. 0.3 %
Potassium sorbate	approx. 0.1 %

[1] ISO 9001:2015 – International Standard for Quality Management Systems

Water	approx. 60.6 %

TOS: Total organic solids

2.2.2 Allergen considerations

The applicant provided the Product Data Sheet for the enzyme preparation. This states that the following allergens are not present: celery, cereals containing gluten, crustaceans, egg, fish, lupin, milk (including lactose), molluscs, mustard, nuts, peanuts, sesame, soy, sulphur dioxide/sulphites.

2.2.3 Specifications

Internationally-recognised specifications are available for enzyme preparations used in food production. These have been established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2017) and the Food Chemicals Codex (FCC) (USPC, 2018). These specifications are included in the primary sources listed in section S3—2 of Schedule 3 of the Code and enzymes used as a processing aid must meet either of these specifications. Schedule 3 of the Code also includes specifications for arsenic and heavy metals (section S3—4) if they are not already detailed within specifications in sections S3—2 or S3—3.

Table 2: Analysis of glucoamylase compared to specifications for enzymes

			Specifications		
Analysis	**Unit**	**Novozymes analysis**	**JECFA**	**Food Chemicals Codex**	**Australia New Zealand Food Standards Code**
Lead	mg/kg	ND (LOD < 0.5)	≤ 5	≤ 5	≤ 2
Arsenic	mg/kg	ND (LOD < 0.3)	-	-	≤ 1
Cadmium	mg/kg	ND (LOD < 0.05)			≤ 1
Mercury	mg/kg	ND (LOD < 0.05)			≤ 1
Total coliforms	CFU/g	<10	≤ 30	≤ 30	-
Enteropathogenic *Escherichia coli*	CFU/25 g	ND	Absent	-	-
Salmonella spp.	CFU/25 g	ND	Absent	Negative	-
Antimicrobial activity	—	ND	Absent	-	-

ND: Not detected; LOD: Limit of detection; CFU: Colony-forming unit

Note: Analysis was performed on three batches of enzyme preparation.

2.3 Technological purpose of the enzyme

Glucoamylases break down starch polysaccharides by removing D-glucose units from the non-reducing end of the substrate molecule, in this case the starch polysaccharides amylose and amylopectin (Gudi et al, 2013). The enzymes do this by catalysing the hydrolysis of 1,4-α-D-glucosidic bonds and the 1,6-α-D-glucosidic bonds that form branches in starch molecules. The glucose molecules produced can then be used to produce potable alcohol and starch hydrolysates such as glucose syrup.

The stated technological purpose of glucoamylase in starch processing and alcohol production is supported by scientific literature (Poulson 1983; Reichelt 1983; Schuster et al. 2002). Specifically, during the production of syrups, glucoamylase degrades starch polysaccharides into glucose. During alcohol production, glucoamylase is added prior to fermentation to degrade gelatinised starch and dextrins to glucose and other fermentable sugars.

The highest level of glucoamylase used during food manufacturing is 800 AGU(D) per kg starch dry matter for both solid and liquid foods.

Use of commercial enzyme preparations should follow good manufacturing practice (GMP), where use is at a level that is not higher than that necessary to achieve the desired enzymatic reaction. The conditions of use of the enzyme in starch processing will depend on a number of factors including the nature of the application and the individual food manufacturers' production processes. The optimum use level should be assessed and adjusted using trials that reflect their particular processes.

The application includes a description of the method used for determining enzyme activity in Appendix 3.1. In summary, glucoamylase is used to break down maltose to D-glucose units and the reaction is stopped after a period of time by adding an alkaline solution. The glucose produced is phosphorylated and oxidised by other enzymes, and an amount of nicotinamide adenine dinucleotide (NAD^+) proportional to the amount of maltose in the original reaction is

reduced to NADH. The increase in absorbance at 340 nm correlates with the amount of NADH produced, which is proportional to the enzyme activity.

2.4 Technological justification for the enzyme

As outlined above, glucoamylase assists in a more complete breakdown of large, branched polysaccharides. Increased efficiency leads to higher production of fermentable sugars for alcohol production, and higher glucose yields for manufacturing syrups. The enzyme can be used at high operating temperature and low operating pH which reduces the risk of microbial contamination, further supporting its technological justification (Bagheri et al, 2014).

2.5 Food technology conclusion

The proposed use for the enzyme is as a processing aid in the production of glucose syrups and potable alcohol products. FSANZ concludes that the evidence presented to support the proposed use provides adequate assurance that the enzyme, in the form and prescribed amounts, which must be consistent with GMP controls and processes, 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 as defined in the Code.

There are relevant identity and purity specifications for the enzyme in the Code and the applicant provided evidence that the enzyme meets these specifications.

3 Safety assessment

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

3.1 History of use

3.1.1 Host organism

A. niger is widely used as a production organism and host for the manufacture of food ingredients and enzymes. *A*. *niger* is neither pathogenic nor toxigenic and its safety as a host organism has been assessed many times by FSANZ. Schedule 18 of the Food Standards Code includes permissions for a range enzymes for which *A*. *niger* is the production or host organism. The identity of the host organism was determined using standard molecular techniques.

3.1.2 Gene donor organisms

G. trabeum is a brown rot fungus known for wood decay. The donor strain was sourced from a recognised international culture collection.

3.2 Characterisation of the genetic modification(s)

3.2.1 Description of DNA to be introduced and method of transformation

An expression cassette containing the glucoamylase gene was introduced into the *A. niger* host strain's genome, producing the production strain. The glucoamylase gene is derived from *G. trabeum* cDNA and is under the control of a hybrid *Aspergillus* promoter and hybrid *Aspergillus* and tobacco mosaic virus terminator (Figure 1). Data provided by the applicant and analysed by FSANZ confirmed the identity of the glucoamylase enzyme. The glucoamylase enzyme has been protein engineered and differs from the wild type glucoamylase enzyme by two amino acids.

A vector containing the glucoamylase expression cassette was used to transform the host strain. The expression cassette was integrated at specific integration sites in the host's genome. The final production strain was selected based on rapid growth and high glucoamylase activity.

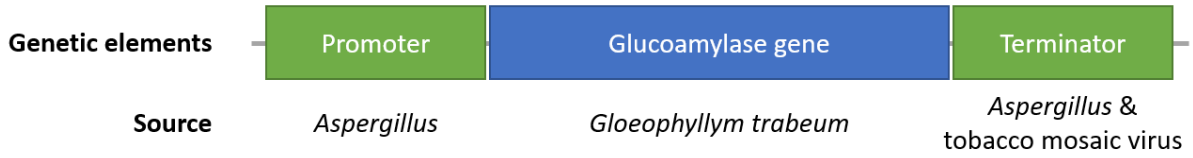

Figure 1. Representation of the glucoamylase expression cassette.

3.2.2 Characterisation of the inserted DNA

The applicant provided data to characterise the inserted DNA in the production strain. These data confirm the presence of the expression cassette in the genome of the production strain.

The applicant also provided the results of genome sequencing which confirmed the absence of antibiotic resistance genes in the production strain.

3.2.3 Stability of the production organism and inheritance of the introduced DNA

The assessment confirmed the inserted DNA is integrated into the production organism's genome and does not have the ability to replicate autonomously. The inserted gene is therefore considered to be genetically stable.

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

3.3 Safety of glucoamylase

3.3.1 History of safe use

Glucoamylases from *A. niger*, *Aspergillus oryzae*, *Rhizopus delemar*, *Rhizopus oryzae* and *Rhizopus niveusare* are currently permitted in Schedule 18 of the Code. However, glucoamylase from *A. niger* modified with a glucoamylase gene derived from *G. trabeum,* is not an approved enzyme processing aid in the Code and does not have a history of safe use in Australia or New Zealand.

There are no known reports of adverse effects in consumers arising from the use of glucoamylases in Australia or New Zealand, or any other jurisdiction where these enzymes have been approved as processing aids in food.

3.3.2 Bioinformatic assessment of enzyme toxicity

A ClustalW[2] alignment was performed using the mature glucoamylase protein sequence against all proteins in the UniProt[3] database annotated as a toxin (Feb 2021). No toxic proteins shared notable sequence similarity with the glucoamylase protein sequence (17.2% identity or lower). Based on these bioinformatics results, glucoamylase does not demonstrate toxicity potential.

3.3.3 Evaluation of toxicity studies

The *G. trabeum* glucoamylase test item used in the following toxicity studies was produced using modified *A. niger* and represented the commercial enzyme product.

Animal Studies

90-day repeated dose oral toxicity study in rats (Huntington Life Sciences, 2014). Regulatory Status: GLP; conducted according to OECD Test Guideline (TG) 408 (1998).

The glucoamylase test item was administered by oral gavage to Sprague-Dawley (Crl:CD(SD)) rats (10/sex/group) for 13 weeks at dosages of 0, 1, 3.3 and 10 mL/kg body weight (bw)/day (equivalent to 124, 410 or 1244 mg/kg bw/day of total organic solids (TOS)). The vehicle control was water. Animals were housed in groups of 5 by sex, with *ad libitum* access to food and water.

Animals were observed daily for signs of toxicity. Body weight, food and water consumption, and detailed clinical examinations for signs of toxicity were recorded weekly. Functional performance tests were conducted on all animals in week 12. Ophthalmological examinations were conducted on all animals prior to first treatment, and in week 13 on the control and high-dose groups only. Haematology, clinical biochemistry, urinalysis, gross necropsy and measurement of organ weights were conducted on all animals at study termination, and a histopathological examination was conducted on organs and tissues from the control and high-dose group animals.

No mortality nor treatment related adverse clinical observations occurred during the study. There were no treatment-related differences compared to controls with respect to feed consumption, body weights, haematology, clinical chemistry, ophthalmology, functional observations or motor activity parameters. No remarkable macroscopic or histopathological changes were observed at necropsy.

The no observed adverse effect level (NOAEL) was 1244 mg TOS/kg bw/day, which was the highest dose tested.

3.3.4 Genotoxicity assays

Bacterial reverse mutation test (Novozymes, 2014). Regulatory Status: GLP; conducted according to OECD TG 471 (1997).

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[2] Thompson et al. (1994)

[3] UniProt toxins database: https://www.uniprot.org/program/Toxins

The potential mutagenicity of glucoamylase was evaluated in *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537, and *Escherichia coli* strain WP2*uvrA*pKM101, with and without metabolic activation using rat liver homogenate (S9). Mutation tests were conducted twice independently over a dose range of 156–5000 µg/mL dry matter. Bacterial cells were incubated for 3 hours with test item. Positive controls in the absence metabolic activation were 2-nitroflurene (TA98), ICR-191 (TA1537) and 1-methyl-3-nitro-*N*-nitrosoguanidine (TA100, TA1535, WP2*uvrA*pKM101). Positive controls in the presence of metabolic activation were 2-aminoanthracene (all strains). Sterile water was used as the vehicle control.

No concentration-related increases relative to vehicle controls 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 cell micronucleus test (Covance Laboratories Ltd., 2014). Regulatory status: GLP; conducted according to OECD TG 487 (2010).

The potential of glucoamylase to cause chromosomal aberrations in mammalian cells was tested using human lymphocytes isolated from peripheral blood, collected from two healthy male volunteers. Treatment with the glucoamylase test item was either a 3 hour pulse exposure with or without S9, followed by a 21 hour recovery; or 24 hours exposure without S9, followed by 24 hours recovery. Positive controls assays were conducted in parallel using mitomycin C or vinblastine for the short- or long-term exposure respectively in the absence of S9, and cyclophosphamide in the short-term treatment with S9. The vehicle control was purified water. The experiment was carried out once in duplicate.

Dose selection experiments did not show cytotoxic activity 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 3000–5000 µg/mL was examined for all test conditions.

There were no treatment-related increases in micronucleated or binucleated cells observed in peripheral blood lymphocytes following exposure to the glucoamylase test item, relative to the vehicle controls, under any of the conditions tested. The positive controls demonstrated a statistically significant increase in micronucleated/binucleated cells, validating the sensitivity of the experimental methodology. It was concluded that glucoamylase did not demonstrate clastogenic or aneugenic properties, in peripheral blood lymphocytes under the conditions of the study.

3.3.5 Potential for allergenicity

Alignments were performed using the amino acid sequence of the glucoamylase against known allergen sequences from the AllergenOnline[4] database (February 2021). The Needleman-Wunsch algorithm was used for full length protein alignments (>35% identity cut-off) and the Smith–Waterman algorithm was used for 80 mer sliding window (>35% identity cut-off), scaled 80 mer sliding window (>35% identity cut-off) and 8 mer sliding window (100% identity cut-off) alignments.

The allergenicity search identified a single sequence match to Sch c 1, a glucoamylase respiratory allergen from *Schizophyllum commune*. *S. commune* allergic reactions include

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

rhinitis, sinusitis and allergic bronchopulmonary mycosis (Chowdhary et al., 2014; Toyotome et al., 2014). However, the *S. commune* fungus is eaten in Africa, Asia, the Indian subcontinent and central America and does not have a documented association with oral allergenicity.

Glucoamylases used as enzyme processing aids are a known source of allergic respiratory sensitisation in commercial settings (i.e. baker's asthma) (Cartier, 2010). However, there are no reports in the scientific literature of allergic reactions to oral exposure and considerable evidence exists to show that people with respiratory allergens can consume the peptide allergens safely (Cullinan et al. 1997; Brisman 2002; Poulsen 2004; Armentia et al. 2009).

It is concluded that the presence of glucoamylase produced using modified *A. niger* in food is unlikely to pose an allergenicity concern to consumers.

3.3.6 Assessments by other regulatory agencies

Documents were provided by the applicant to show that *G. trabeum* glucoamylase produced using modified *A. niger* was approved for use in Denmark (in 2000) and France (in 2016). These approvals were not accompanied by written assessments.

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 glucoamylase enzyme from GM *A. niger* remained in the food.

The budget method is a valid screening tool for estimating the theoretical maximum daily intake (TMDI) of a food additive (Douglass *et al.,* 1997). The calculation is based on physiological food and liquid requirements, the food additive concentration in foods and beverages, and the proportion of foods and beverages that may contain the food additive. The TMDI can then be compared to an acceptable daily intake (ADI) or a NOAEL to estimate a margin of exposure for risk characterisation purposes.

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

- the maximum physiological requirement of solid foods (including milk) is 25 g/kg body weight/day
- the maximum physiological requirement for non-milk beverages is 100 mL/kg body weight/day. This is the standard level used in a budget method calculation (FAO/WHO, 2009)
- 50% of solid foods and 25% of non-milk beverages are processed foods
- 25% of processed solid foods and 12% of processed non-milk beverages contain glucoamylase
- the maximum glucoamylase level in both final solid foods and non-milk beverages was 195 mg TOS/kg food (i.e. the highest use level from all uses within each group)
- liquid density ~ 1 g/mL.

Based on these assumptions, the applicant calculated the TMDI of glucoamylase to be 1.2 mg TOS/kg body weight/day.

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

- the maximum physiological requirement for solid food (including milk) is 50 g/kg body weight/day. This is the standard level used in a budget method calculation where there is potential for the enzyme to be in baby foods or general purpose foods that would be consumed by infants (FAO/WHO, 2009), which for this enzyme would be from the glucose syrups and other starch hydrolysates.

All other inputs and assumptions used by FSANZ remained as per those used by the applicant. The TMDI based on FSANZ's calculations for solid food and non-milk beverages were 1.22 mg TOS/kg body weight/day and 0.59 mg TOS/kg body weight/day respectively, resulting in a total of 1.80 mg TOS/kg bw/day.

Both the FSANZ and applicant's estimates of the TMDI will be overestimates of the dietary exposure given the conservatisms in the budget method. This includes that it was assumed that the enzyme remains in the final foods and beverages. The applicant has stated that the enzyme is denatured by heat during processing or removed by distillation and does not have a function in the final food.

4 Discussion

No public health and safety concerns were identified in the assessment of protein engineered glucoamylase from modified *A. niger* under the proposed use conditions. The *A. niger* host is neither pathogenic nor toxigenic, and analysis of the modified production strain confirmed presence and stability of the inserted DNA.

Toxicity testing of the enzyme showed no evidence of genotoxicity *in vitro* and the NOAEL in a 90-day oral gavage study in rats was the highest dose tested, 1244 mg TOS/kg bw/day. The TMDI was calculated by the applicant to be 1.2 mg TOS /kg bw/day, while FSANZ calculated the TMDI to be 1.8 mg/kg bw/day. Comparison of the NOAEL with the TMDI calculated by the applicant or FSANZ gives a Margin of Exposure (MOE) of approximately 1040 and 690, respectively.

Bioinformatics analysis indicated that the enzyme shows no significant homology with any known toxins. However, a degree of homology was identified with a respiratory allergen from *S. commune*. Taking into account that respiratory allergens are usually not food allergens and that *S. commune* has a history of being consumed in food, the risk of food allergy from the proposed uses of the enzyme is likely to be low.

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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