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

16 May 2019
[80-19]

Supporting document 1

Risk and technical assessment – Application A1168

Glucoamylase from *Aspergillus niger* as a processing aid (enzyme)

Executive summary

FSANZ has assessed an application from Novozymes Australia Pty Ltd to permit the use of glucan 1,4-alpha-glucosidase (glucoamylase) as a processing aid (enzyme). The enzyme is obtained from a genetically modified strain of *Aspergillus niger*, expressing the glucoamylase gene from *Talaromyces emersonii*. The purpose of the enzyme is to convert starch into glucose in the manufacture of syrups, beverages, cereal based products, fruit products and vegetable products.

The food technological assessment concluded that the enzyme, in the form and prescribed amounts, is technologically justified and has been demonstrated to be effective in achieving its stated purpose. The enzyme performs its technological purpose during production and manufacture of foods and is therefore appropriately categorised as a processing aid and not a food additive. The enzyme preparation meets international purity specifications.

The safety assessment concluded that there are no public health and safety concerns associated with the use of glucoamylase from *A. niger* as a processing aid. The *A. niger* production strain is not toxigenic or pathogenic and is absent in the final enzyme preparation proposed to be used as a food processing aid. Glucoamylase from *A. niger* has a history of safe use in several other countries, with the earliest specified date of approval being 2008. Other glucoamylases from a large number of microbial sources have been widely used in the food industry since the 1960s.

Glucoamylase was not genotoxic in a bacterial reverse mutation assay (Ames test) or a micronucleus assay in cultured human peripheral blood lymphocytes. No adverse effects were observed in rats administered glucoamylase produced by a strain of *A. niger* of the same strain lineage as the production strain for 13 weeks.

Bioinformatic analyses did identify a high degree of homology between the recombinant glucoamylase to that of a respiratory allergen but further analysis indicated that the glucoamylase from *T. emersonii* expressed in *A. niger* is not considered to represent a food safety hazard.

In the absence of any identifiable hazard an Acceptable Daily Intake (ADI) ‘not specified’ is appropriate. A dietary exposure assessment was therefore not required.

Table of contents

1 Introduction

Glucoamylase (EC 3.2.1.3) is an enzyme which catalyses the hydrolysis of 1,4-alpha and 1,6-alpha-D-glucosidic linkages in starch polysaccharides. Produced through fermentation, its microbial source is a genetically modified (GM) strain of *Aspergillus niger*. Glucoamylase converts starch to fermentable sugars in various foods resulting in benefits including improved yield, decreased potential for microbial contamination since the enzyme can be used at higher temperatures during processing, and enhanced and more uniform colour.

The glucoamylase enzyme preparation is intended to be used as a processing aid in the starch processing industry for the production of several products, e.g. syrups, distilled beverages, beer, bread, and juices.

1.1 Objectives of the assessment

The objectives of this technical, safety and risk assessment for glucoamylase were to:

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

- evaluate any potential public health and safety issues that may arise from the use of this enzyme protein, produced by a GM organism as a processing aid. Specifically by considering the:

  - history of use of the host and gene donor organisms
  - characterisation of the genetic modification(s), and
  - safety of the enzyme protein.

2 Food technology assessment

2.1 Characterisation of the enzyme

2.1.1 Identity of the enzyme

The production microorganism of the enzyme is a GM *A. niger* (see Section 3 for information). The donor organism of the *glucoamylase gene* is the fungus, *Talaromyces emersonii*.

The glucoamylase enzyme preparation is available under the commercial name “Attenuzyme Core” as a single enzyme formulation when used for brewing processes. The preparation is standardised in glucoamylase units (AGU/g)[1] to an activity of 1600 AGU/g, being a measure of enzyme activity. The enzyme preparation is available as a liquid. The liquid product is stabilised with sucrose and glucose with water as the carrier.

A summary of the method of determining the enzyme activity is provided in the application. Glucoamylase converts maltose to D-glucose and the reaction is stopped with an alkaline solution. The glucose is subsequently phosphorylated and oxidised by other enzymes during which an amount of NAD^+ proportional to maltose is reduced to NADH. This reduction

[1] Amyloglucosidase unit (AGU), where enzyme activity is determined and analysed by an in-house method detailed in the application.

reaction can be quantified following the increase in absorbance at 340 nm. The increase is proportional to the enzyme activity.

Generic common name:	Glucoamylase
Accepted IUBMB[2] name:	glucan 1,4-α-glucosidase
Systematic name:	4-α-D-glucan glucohydrolase
Other names:	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
EC number:	3.2.1.3
CAS[3] registry number:	9032-08-0
Reaction:	Hydrolysis of terminal (1→4)-linked α-D-glucose residues successively from non-reducing ends of the chains with release of β-D-glucose

The optimum temperature for enzyme activity is 60°C, with reasonable activity between 50-70°C. The optimum pH range for enzyme activity is between 4.8 and 5.2, with the optimum being 4.8. The enzyme is deactivated during wort boiling but normal beer pasteurisation temperature treatments do not usually inactivate the enzyme.

2.1.2 Technological purpose of the enzyme

The technological purpose of glucoamylase is to catalyse the hydrolysis of 1,4-alpha and 1,6-alpha-D-glucosidic linkages in starch polysaccharides.

The glucoamylase preparation is used as a processing aid during the manufacture of starch based products. Glucoamylase enzymes convert starch by removing D-glucose units in a stepwise manner from the non-reducing end of the substrate molecule to produce glucose for use in:

- the production of syrups (degrades polysaccharides into glucose)
- alcohol distilling processes (degrades gelatinised starch and dextrins into glucose and other fermentable sugars)
- beer brewing and other cereal based beverage processes utilising corn, rice or rye, (converts starch into fermentable sugars)
- baking and other cereal based processes utilising corn, rice or rye (hydrolyses starch, from milled and damaged granules into glucose to be fermented by yeast)
- processing of fruits and vegetables(to degrade starch in order to increase yield and to improve efficiencies).

[2] International Union of Biochemistry and Molecular Biology
[3] Chemical Abstracts Service

2.1.3 Technological justification of the enzyme

In the Australia New Zealand Food Standards Code (the Code), *A. niger* is an approved production source for a number of enzymes. The Code however does not permit glucoamylase from *A. niger* containing the gene for glucoamylase from *T. emersonii*.

This particular glucoamylase preparation will provide the food and beverage industry with the opportunity to improve the yield of fermentable sugars for baking, distilling, brewing and improve the yield and facilitate processing in the juice industry.

Table 1: *Benefits provided by glucoamylase*

Area of use	***Benefit***
Starch processing	• Efficient degradation of dextrins and production of glucose • Can be used at high temperatures • Less risk of microbial contamination due to enzyme being able to function at higher temperatures during processing • Stable process allowing for variations in temperature
Alcohol distilling	• Efficient degradation of dextrins and production of fermentable sugars • Reduction of raw materials used • Superior conversion of starch • Superior alcohol yields • Active at high temperature and low pH • Reduced risk of contamination because the enzyme can be used at higher temperatures and lower pH
Brewing processes	• More uniform and predictable production process • Increased brewing yield including the possibility to control the desired level of fermentable sugars
Baking processes	• Reduced baking time and yeast boosting • Uniform and slightly increased volume • Improved crust colour • More uniform and predictable production of glucose • Improved fermentation • Uniform colour
Fruit and vegetable processing	• Improved press capacity and filtration rates • Increased yield • Improved clarity final product

The applicant has provided a technical application sheet and product data sheet for brewing to show that the enzyme achieves its technological purpose in the form and quantity proposed when used as a processing aid. An assessment of this information supports the applicant's claims. The use of the enzyme for the other proposed uses and food categories use similar processing steps and purposes so can also be understood to be technically justified and appropriate. Food manufacturing companies will conduct their own production trials to ensure the enzyme performs as suggested and provides technological benefits that are economical.

2.2 Manufacturing process

2.2.1 Production of the enzyme

The enzyme manufacturing process is composed of a fermentation process, a purification process, a formulation process and finally quality control of the finished product.

Fermentation
The glucoamylase preparation is produced by submerged fed-batch pure culture fermentation of the GM strain of *A. niger*. This process is commonly used for the production of food-grade enzymes.

The fermentation process involves three steps, inoculation, seed fermentation and main fermentation.

Recovery
The recovery process is a multi-step operation designed to separate the enzyme from the microbial biomass and purify, concentrate, and stabilise the food enzyme.

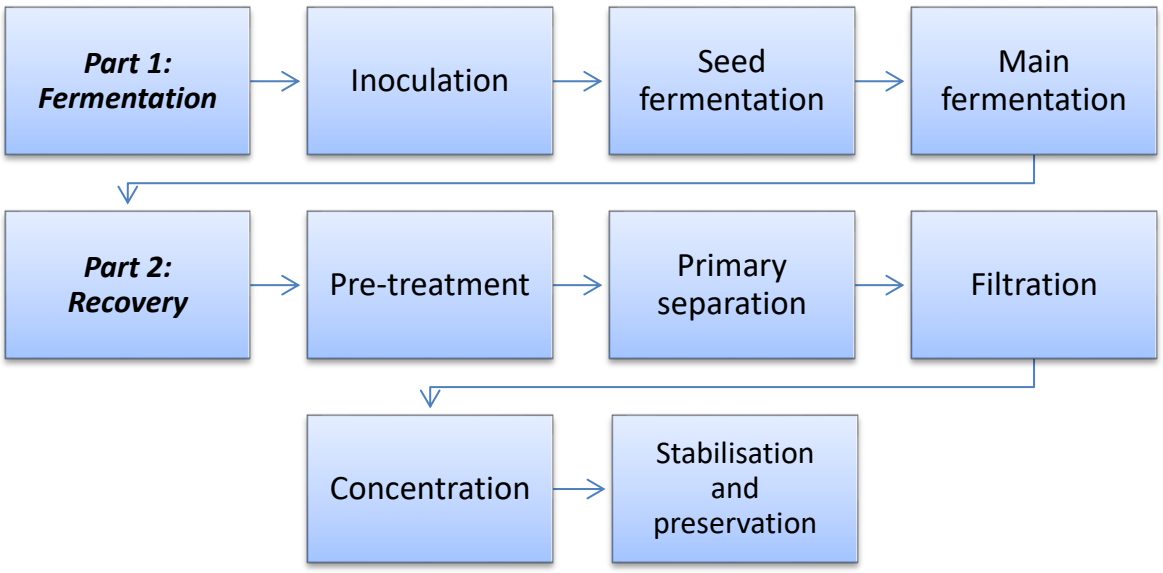

Figure 1: *Manufacturing process for glucoamylase preparation*

The enzyme preparation is manufactured in accordance with current Good Manufacturing Practices (cGMP) and the quality management system used in the manufacturing process complies with ISO 9001:2015.

The manufacturing process is such that the production microorganism, *A. niger*, is removed during processing and so is absent from the commercial enzyme preparation.

The enzyme is standardised to ensure consistent enzyme activity and produced as a liquid enzyme preparation with water as the diluent and carrier. The enzyme preparation contains 42% enzyme (as Total Organic Solids (TOS)), 1.0% of sucrose/glucose as stabilisers, sorbate and benzoate as preservatives and 57% water.

2.2.2 Specifications

There are international specifications 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) (USP, 2018). Both of these specification sources are primary sources listed in section S3—2 of the Code. Enzyme preparations must meet these specifications.

Table 2 provides a comparison of representative batch analysis of the glucoamylase enzyme preparation with the international specifications established by JECFA and Food Chemicals Codex, as well as those detailed in the Code (being section S3—4, as applicable).

Table 2: *Product specifications for commercial enzyme preparation*

Analysis	Enzyme batch analysis	Specifications		
		JECFA	Food Chemicals Codex	the Code
Lead (mg/kg)	ND[a] (DL<0.5)[b]	≤ 5	≤ 5	≤2
Arsenic (mg/kg)	0.103 (DL < 0.1)	-	-	≤1
Cadmium (mg/kg)	ND (DL < 0.05)	-	-	≤1
Mercury (mg/kg)	ND (DL < 0.03)	-	-	≤1
Total coliforms (cfu/g)	10	≤30	≤30	-
Salmonella (in 25 g)	ND	Absent	Negative	-
Enteropathic *E. coli* (in 25 g)	ND	Absent	-	-
Antimicrobial activity	ND	Absent	-	-

[a] ND = Not detected
[b] DL = Detection limit

The applicant provided additional analyses (total of heavy metals, ochratoxin A and fumonisin B2) for which no international specifications have been established to further demonstrate safety of the enzyme.

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

2.3 Food technology conclusion

FSANZ concludes that the stated purpose of this enzyme preparation; namely, for use as a processing aid in the manufacture and processing of starch based products is clearly articulated in the application. The evidence presented to support the proposed uses provides adequate assurance that the enzyme, in the form and prescribed amounts, is technologically justified and has been demonstrated to be effective in achieving its stated purpose. The stated purpose is to hydrolyse starch for the production of syrups, distilled beverages, beer, bread, and juices. The enzyme performs its technological purpose during production and manufacture of foods after which it is inactivated thereby not performing a technological function in the final food. It is therefore appropriately categorised as a processing aid and not a food additive. The enzyme preparation meets international purity specifications.

3 Safety assessment

3.1 Objectives for safety assessment

The objectives of this safety assessment for glucoamylase are to evaluate any potential public health and safety concerns that may arise from the use of this enzyme protein, produced by a GM organism, as a processing aid. Specifically by considering:

- history of use of the host and gene donor organisms
- characterisation of the genetic modification(s)
- safety of the enzyme protein.

3.2 History of use

3.2.1 Host organism

A. niger is a filamentous fungus ubiquitous in the environment. In nature it can be found in soil and litter, in compost and on decaying plant material (Schuster et al. 2002). The ability of *A. niger* to produce extracellular organic acids has been industrially exploited since 1919 (Schuster et al. 2002). Citric acid, the primary acidulant in the food and beverage industry, is mostly produced by microbial fermentation using *A. niger* (Show et al. 2015). In addition to citric acid, *A niger* is also a source of enzymes like amylase, amyloglucosidase, pectinases and many others that are currently used in food processing (Gautam et al. 2011; Pariza and Johnson 2001). The US Food and Drug Administration has accepted numerous enzymes for food use derived from *A. niger*, which is 'generally recognized as safe' (GRAS) under the condition that non-pathogenic and non-toxigenic strains are used in production. *A. niger* has a long history of safe use as a production organism for food enzymes and is a permitted source of a number of enzymes in the Code (Schedule 18).

A. niger has been reported as an opportunistic pathogen of immunocompromised individuals (Atchade et al. 2017; Person et al. 2010). However, *A. niger* is generally regarded as a non-pathogenic fungus to which humans are frequently exposed without disease becoming apparent (Schuster et al. 2002). Some strains of *A. niger* are capable of producing toxins like ochratoxins and fuminisins, which are harmful to human health (Frisvad et al. 2011). The applicant confirmed that the *A. niger* production strain belongs to a strain lineage which is non-pathogenic and does not produce any known mycotoxin.

The host strain had previously been modified using a range of conventional mutagenesis and genetic modification steps that are routinely used to optimise organisms for industrial use. These changes have been characterised and include silencing of unnecessary proteins that would impact the yield and purity of glucoamylase and to increase the ability and efficiency to transform the host. An intermediate strain was deposited and taxonomically characterized by the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ). The characterization was based on morphological and molecular methods including DNA sequencing, and confirmed the taxonomy as *A. niger*.

3.2.2 Gene donor organism(s)

Talaromyces emersonii

The gene sequence for the glucoamylase processing aid (*amgGT*) was isolated from *T. emersonii* (Nielsen et al, 2002). This organism is a thermophilic fungi that was initially

isolated from compost. It has not been listed in the NIH Guidelines[4] in risk groups 2-4 and is thus considered a risk group 1 agent, which is not associated with disease in healthy adult humans.

Other organisms

The endogenous acid stable amylase (*asaA*) gene, including the native promoter and terminator sequences, were isolated from the host *A*. *niger* strain. Other regulatory elements (promoters and terminator sequences) were also isolated from the *A. niger* host to drive expression of the glucoamylase gene. The gene for acetamidase (*amdS*), a nutritional selection marker, including the native promoter and terminator sequences, was isolated from *A. nidulans* (Kelly and Hynes, 1985). An additional regulatory element (5'UTR) was also used from *A. nidulans* for expression of the glucoamylase gene. A second selectable marker system was generated using the orotidine-5'-phosphate (OMP) decarboxylase gene (*pyrG*) from *A. oryzae.*

The *Aspergillus* strains used to source the DNA are considered risk group 1 agents according to the NIH Guidelines[4].

3.3 Characterisation of the genetic modification(s)

3.3.1 Description of DNA to be introduced and method of transformation

Two vectors designated A and B were generated, each containing unique glucoamylase (*amgGT*) expression cassettes (Figure 2). The expression cassette from vector A contained the *amgGT* gene followed by a copy of the endogenous acid stable amylase (*asaA)* gene and a copy of the selectable marker gene for acetamidase (*amdS)*. The presence of the acetamidase allows for selection of positive transformants by growth on media containing acetamide. The expression cassette from vector B contained the *amgGT* gene followed by the selectable marker decarboxylase gene (*pyrG*). When transformed into a *pyrG*$^{-}$ strain, the decarboxylase allows transformed cells to produce the essential nutrient uridine, thus allowing survival on minimal media without uridine.

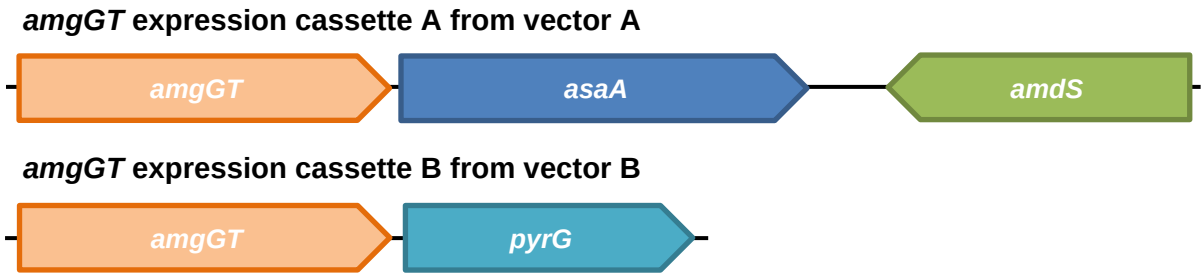

Figure 2*: Map of the amgGT expression cassettes from vectors A and B.*

In the backbone sequence of vector A there is an ampicillin resistance gene for passage in *E. coli*. Vector B makes use of the *Saccharomyces cerevisiae* OMP decarboxylase gene (*URA3*) for passage in *E. coli* (Rose et al, 1984). The backbone sequences for both vectors are removed prior to transforming the *Aspergillus* host.

A further vector designated C was generated for disrupting the endogenous oxalic acid gene. The aim of this gene disruption was to eliminate the production of oxalic acid during fermentation.

[4] https://osp.od.nih.gov/wp-content/uploads/NIH_Guidelines.html#_Toc446948380

The three expression cassettes were introduced into the *Aspergillus* host by sequential transformation, using standard methodology for transformation of protoplasts (Ruiz-Diez, 2002). Before transformation, each vector was digested with restriction enzymes to isolate the expression cassette away from the vector backbone, thereby ensuring the absence of any vector backbone sequence. After the sequential transformation, which generated a clone that was designated pre-41SaM2-54, several rounds of conventional mutagenesis were performed to generate the final production strain, 41SaM2-54 (Figure 3).

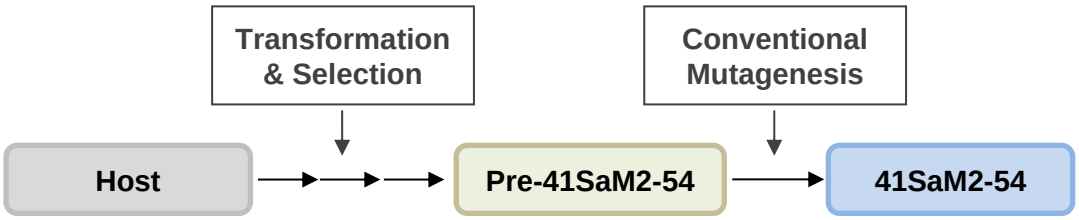

***Figure 3**: Outline of the steps used to generate the final production strain 41SaM2-54.*

3.3.2 Characterisation of inserted DNA

Southern blotting was performed on genomic DNA extracted from the 41SaM2-54 and host strains that had been digested with various restriction enzymes. Hybridisation with a probe targeting the *amgGT* gene showed multiple full-length inserts had been integrated into 41SaM2-54. The results also showed that some of the insertions had undergone rearrangement, leading to some tandem repeats of the expression cassette.

Hybridisation with a probe targeting the *asaA* gene produced two different banding patterns that allowed differentiation of the endogenous and introduced genes. The endogenous gene was shown to be present in both the host and 41SaM2-54, whereas the introduced gene was only present in 41Sam2-54. The banding size for the introduced gene fragment also confirmed that the full-length product had been inserted.

Southern blotting was also performed on a range of intermediate strains to examine the disruption of the oxalic acid gene. Each clone generated a distinct banding pattern that confirmed the gene was disrupted.

A quantitative PCR method was used to determine how many copies of the insert were present in the genome of 41AaM2-54. Using a primer-probe combination that targeted the terminator region of the *amgGT* gene, the data showed multiple copies of the gene were present. Using a primer-probe combination specific for the *asaA* gene, multiple inserts were also identified.

Next generation sequencing was performed on 41SaM2-54 to determine if the post-transformation exposure to conventional mutagenesis resulted in changes to the *amgGT* gene. The results showed that the *amgGT* gene present in 41SaM2-54 had the same sequence as the gene that was present in vectors A and B thus confirming that there were no changes to the inserted gene arising from the mutagenesis process.

3.3.3 Genetic stability of the inserted gene

Southern blotting was performed on genomic DNA extracted from cells obtained from the end of three distinct fermentation runs. As a control, a comparison was made to an early generation master stock of the 41SaM2-54 strain. After digestion of the DNA, hybridisation

with a probe targeting the *amgGT* gene showed consistent presence across the four generations analysed, indicating the glucoamylase gene was stably incorporated in the production strain.

3.4 Safety of glucoamylase

In considering the safety of novel proteins it is important to consider that a large and diverse range of proteins are ingested as part of the normal human diet without any adverse effects. Only a small number of dietary proteins have the potential to cause adverse health effects, because they have anti-nutrient properties or they can cause allergies in some consumers (Delaney et al. 2008). Furthermore, proteins perform a wide range of functions in humans. To encompass this range of type and function, the safety assessment of the novel protein must consider if there is a history of safe use, and whether there are any potential toxic or allergenic effects.

3.4.1 History of safe use of the enzyme

Glucoamylase from *A. niger* has been approved for use in Brazil since 2009, and China since 2015. Denmark approved the use of the enzyme for production of glucose syrup, alcohol and beer in 2008 and for baking applications in 2011, and wrote that their evaluations had been made in accordance with EFSA guidelines for the presentation of data on food enzymes. According to the applicant, the enzyme has also been approved in Canada, France and Mexico, although the dates of approval are not specified. The applicant also states that the enzyme is used in unspecified countries in which there are no restrictions on the use of enzymes as processing aids, or where the enzyme is covered by a positive list.

By volume, glucoamylase is one of the most used commercial biocatalysts in the food industry (Kumar and Satyanarayana, 2009). Glucoamylases from a large number of microbial sources have been widely used in the food industry since the 1960s for the hydrolysis of starch (Kumar and Satyanarayana, 2009), particularly in the starch, brewing, distilling and baking industries. FSANZ and a number of other regulatory authorities, including those of Brazil, Canada, China, Denmark, France, Japan and Mexico have authorized the use of various glucoamylases as processing aids.

3.4.2 Toxicology studies in animals

Thirteen week oral gavage study of glucoamylase in CD rats (Huntingdon Life Sciences Ltd., Study Number NVZ0028/053878, 2006) Regulatory status: GLP.

The test article for this study was glucoamylase from *A. niger*, Batch PPY24900. This batch was produced by a strain that is of the same strain lineage as the production strain used to produce the glucoamylase that is the subject of this application. The test article was received as a liquid with a Total Organic Solid (TOS) content of 13.9% w/w. The vehicle and control article was water, purified by reverse osmosis. Enzyme activity of the dose formulations was determined from samples collected during Weeks 1, 6 and 13.

The test subjects were Crl:CD®(SD)BR rats, received at 33 to 37 days of age. Rats were acclimatized to the study room and standard laboratory environmental conditions for 12 days prior to the start of the study. Rats were group-housed by sex, 5/cage, in polycarbonate cages and provided with water *ad libitum*, and standard rat diet *ad libitum* except during scheduled fasting prior to blood collection. All rats were subject to prestudy ophthalmologic examination.

Rats were assigned, 10/sex/group, to groups gavaged once daily at a dose volume of 10 mL/kg bw, to deliver a dose of 0, 1, 3.3 or 10 mL/kg bw/day of the test article as supplied to

the laboratory. The volume delivered was based on the most recently recorded body weight. During the in-life phase, rats were subject to cageside observations twice daily. Detailed clinical observations were recorded daily through the first week, twice weekly during Weeks 2 to 4, and once weekly thereafter. Water consumption was measured for all rats in a cage was recorded every 2 to 3 days, and food consumption for all rats in a cage was recorded weekly. Bodyweight, and behaviour in an arena were recorded weekly from prior to initiation of dosing. Sensory reactivity and grip strength were assessed during Week 12 prior to administration of the daily dose. Sensory reactivity was assessed by response to approaching the face with a probe, auditory startle response, tail pinch response and touch response. Motor activity was also measured electronically during Week 12. Rats in the control and 10 mL/kg bw/day groups were subject to ophthalmologic examinations in Week 13 but because no differences in the prevalence of ocular abnormalities were observed between those two groups, examination was not extended to the 1 or 3.3 mL/kg bw/day groups.

Blood was collected from all surviving rats in Week 13 for haematology (including assessment of coagulation factors) and clinical chemistry. All surviving animals were killed at the end of 13 weeks of treatment, and subject to necropsy. Fresh organ weights were recorded for adrenals, brain, heart, kidneys, liver, spleen, thymus, and either testes and epididymides or ovaries and uterus as sex-appropriate. A comprehensive list of organs and tissues was preserved for histopathological examination.

Analysis of the enzyme activity of dose formulations in Weeks 1, 6 and 13 confirmed that it did not deviate from the intended activity by more than 2%, and the formulations were therefore suitable for use on study.

There were no treatment-related mortalities prior to scheduled termination. One male rat in the 3.3 mL/kg bw/day group was killed in moribund condition in Week 3. The rat was found to have meningitis. Treatment with glucoamylase had no effects on clinical observations, behavior in an arena, sensory reactivity tests, grip strength, motor activity in Week 12, bodyweights, bodyweight gains, food consumption, food conversion efficiency, water consumption, ophthalmic findings, haematology, clinical chemistry, organ weights or findings on gross necropsy. Findings that showed a dose-response relationship were limited to an increase in the frequency or severity of vacuolation of adrenal cortical cells in male rats dosed with ≥3.3 mL/kg bw/day. Some vacuolation of these cells was observed in control and 1 mL/kg bw/day males, but the frequency and severity increased in the 3.3 and 10 mL/kg bw/day males in a dose-related manner. The study director commented that since glucocorticoids are involved in mediating protein catabolism, this could be an adaptive response to the administration of glucoamylase. It was noted that the frequency and severity of the vacuolation remained within the historical control range for male rats of this strain, similar changes were not observed in females, and there was no associated necrosis or inflammatory response. Consequently, the finding was not considered to be adverse.

The study director concluded that the No Observed Adverse Effect Level (NOAEL) in this study was 10 mL/kg bw/day, which when expressed as TOS is 1.47 g/kg bw/day.

Although the test article is from a different strain of *A. niger* than the production strain for the enzyme that is the subject of this application, the two strains are of the same lineage and the study is considered to be informative of the safety of glucoamylase from *A. niger* when administered orally.

3.4.3 Genotoxicity assays

Bacterial reverse mutation assay (Novozymes A/S, Study Number 20118069, 2011). Regulatory status: GLP; conducted in accordance with the general recommendations of OECD Guideline No. 471.

This assay was performed using Batch PPY 32789, which is glucoamylase from the production strain that is the subject of the application. The test article was received as a brown liquid with a declared dry matter content of 10.2% w/w. The enzyme was prepared as a solution of 5% w/v in sterile deionised water, and serial dilutions made with water so that the test concentrations were 156, 313, 625, 1250, 2500 and 5000 µg/mL. The negative control article was sterile deionised water. The test bacterial strains were *Escherichia coli* WP2uvrApKM101 and four strains of *Salmonella typhimurium*; TA1535, TA100, TA1537 and TA98. The positive control article for all strains in the presence of S9 mix for metabolic activation was 2-aminoanthracene. In the absence of S9 mix, the positive control article was 1-methyl-3-nitro-N-nitrosoguanidine for TA1535, TA100 and *E. coli* WP2uvrApKM101, 2-nitrofluorene for TA98, and acridine mutagen for TA 1537.

The test article provides a rich medium for bacterial growth, and a source of histidine and tryptophan, the amino acids that are the growth-limiting factors for the test strains of *S. typhimurium* and *E.coli* respectively. A standard bacterial reverse mutation assay in agar plates is likely to be confounded by a “feeding effect” in which the bacterial lawn is increased and the number of spontaneous mutations are increased as a result of more nutrient. In order to overcome this confounding, all strains were exposed to the test article in liquid culture using the “treat and plate” method. For each assay bacterial culture, test or control article, S9 mix or an equal volume of buffer, and nutrient broth were combined in a test tube and incubated with shaking at 37ºC for 3 h. Bacterial cells were then washed twice by centrifugation and poured into agar plates, and soft top agar was added. After the top agar had set, plates were inverted and incubated at 37ºC for 72 h. Each assay was conducted in triplicate for assays in which the bacteria were exposed to test article or positive control, while the negative (solvent) control was conducted five-fold. The entire experiment was conducted twice.

Colonies were counted automatically, unless there were fewer than 20 colonies in which case they were counted manually.

No toxicity of the test article on bacteria was observed. On the contrary, bacterial growth was stimulated by the test article, relative to the solvent control. There was a weak associated increase in revertant colonies, which was predictable and expected. However no concentration of the test article, with or without metabolic activation by S9 mix, resulted in an increase in revertant colonies that met the criteria for a response to a mutagen. The positive controls all induced the expected response to a mutagen, confirming the validity of the assay.

Micronucleus assay in cultured human peripheral blood lymphocytes (Covance Laboratories, Study Number 8259272, 2012). Regulatory status: GLP; in accordance with OECD Guideline 487

This assay was performed using Batch PPY 32789, which is glucoamylase from the production strain that is the subject of the application. The test article was received as a liquid and diluted with purified water to obtain the range of concentrations tested. Purified water was used as the negative control article. Lymphocytes were obtained and pooled from two healthy non-smoking male donors. Positive control articles were mitomycin C (MMC), cyclophosphamide (CPA) and vinblastine (VIN).

A Range-Finder experiment was conducted with concentrations of test article of 0, 18.1, 30.2, 50.4, 84, 140, 233.3, 389, 648, 1080, 1800, 3000 and 5000 µg/mL, in order to determine whether there was any evidence of cytotoxicity. Lymphocytes that had previously been stimulated to divide using phytohaemagglutinin were incubated with test article or negative control article, and with or without S9 mix for metabolic activation, for three hours and then washed and incubated for a further 21 hours. Incubation was at 37ºC. Additionally lymphocytes or negative control article were incubated without S9 mix for 24 hours, then washed and incubated for a further 24 hours. At the end of the incubation cells were harvested, processed to slides and examined. No significant differences were observed between treated and negative control cells.

As a result of the Range-Finder experiment, the doses for the Micronucleus experiment were 0, 1000, 2000, 3000, 4000, and 5000 µg/mL for a three-hour exposure followed by 21 hours continued incubation without exposure to the test article, while doses of 500, 1000, 2000, 3000, 4000 and 5000 µg/mL were used for a 24-hour exposure followed by 24 hours continued incubation without exposure. The three-hour exposure experiment was conducted both with and without S9 mix whereas the 24 hour exposure experiment was conducted without S9 mix. Assays with test article were conducted in duplicate whereas assays with the negative control were conducted in fourfold. Concurrent assays with the positive control articles were also conducted. The expected significant increase in binucleate cells with micronuclei was observed with all positive control articles. However no treatment-related increase in binucleate cells with micronuclei, relative to negative control assays, was observed in assays containing the test article, with or without S9 mix.

It was concluded that the test article, with or without metabolic activation, did not induce micronuclei in human peripheral blood lymphocytes at concentrations up to 5000 µg/mL

3.4.4 Bioinformatics concerning potential for toxicity

The applicant provided results from *in silico* analyses comparing the amino acid sequence for the glucoamylase protein to known protein toxins identified in the UniProt[5] database. No significant similarity was found between the glucoamylase protein to any known protein toxins, thus it can be concluded that the toxigenic potential of this protein is low.

3.4.5 Bioinformatics concerning potential for allergenicity

Sequence homology assessment to known allergens was conducted by the applicant by comparing the glucoamylase to the databases of FARRP (http://www.allergenonline.org) and the World Health Organisation and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee (http://www.allergen.org). Using the 80-mer sliding window search, significant similarity (E value <1 x 10-7) was found to the glucoamylase from *Schizophyllum commune* (splitgill mushroom). The glucoamylase from *S. commune* has been identified as a respiratory allergen, associated with fungal rhinitis, sinusitis and allergic bronchopulmonary mycosis (Toyotome et al, 2014). There have been no reports of allergic reactions associated with the consumption of *S. commune*, which is eaten in Africa, Asia, the Indian subcontinent, and central America. Further analysis showed that the high degree of homology occurred in a distinct region, whereas homology of the full length proteins is only 49%. The glucoamylase from *T. emersonii* shows greater similarity to the same protein in *Penicillium camemberti* (63%), which is used in the production of camembert and brie, common foods in Australia and New Zealand. The homology therefore between the recombinant glucoamylase to that of *S. commune* is not considered to represent a food safety hazard.

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

In addition to the bioinformatic searches, the applicant provided the Product Data Sheet for the commercial enzyme. 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.

3.4.6 Approvals by other regulatory agencies

The applicant provided letters of approval from Danish authorities for the use of the enzyme for production of glucose syrup, alcohol and beer, dated 2008, and for baking applications, dated 2011. The evaluations had been made in accordance with EFSA guidelines for the presentation of data on food enzymes.

4 Discussion

No public health and safety issues were identified with the production strain. *A. niger* is not a pathogenic organism and is absent in the final enzyme preparation proposed to be used as a food processing aid. *A. niger* has a history of safe use as the production organism for a number of enzyme processing aids that are already permitted in the Code. Molecular characterisation of the production strain has confirmed the insertion and genetic stability of the novel DNA.

No public health and safety concerns associated with the use of glucoamylase from GM *A. niger* were identified as a result of this hazard assessment. This specific glucoamylase has a history of safe use in several other countries, with the earliest specified date of approval being 2008. Other glucoamylases from a large number of microbial sources have been widely used in the food industry since the 1960s.

Glucoamylase, from a strain of *A. niger* of the same strain lineage as the production strain of the glucoamylase that is the subject of this application, was the test article in a 13-week oral gavage study in CD rats. The NOAEL was the highest dose tested, 10 mL/kg bw/day, which when expressed as TOS is 1.47 g/kg bw/day. The Theoretical Maximum Daily Intake (TMDI) of glucoamylase is 6.16 mg TOS/kg bw/day resulting in a Margin of Exposure (MoE) of 240 between the NOAEL and TMDI.

Genotoxicity assays of the glucoamylase that is the subject of this application, comprising a bacterial reverse mutation assay (Ames test) and a micronucleus assay in cultured human peripheral blood lymphocytes, did not show any evidence of genotoxicity.

Bioinformatic analyses did identify a high degree of homology between the recombinant glucoamylase to that of a respiratory allergen but further analysis indicated that the glucoamylase from *T. emersonii* expressed in *A. niger* is not considered to represent a food safety hazard.

5 Conclusions

There are are no public health and safety concerns associated with the use of glucoamylase from *A. niger* as a processing aid.

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

6 References

Atchade E, Jean-Baptiste S, Houzé S, Chabut C, Massias L, Castier Y, Brugière O, Mal H, Montravers P (2017) Fatal invasive aspergillosis caused by *Aspergillus niger* after bilateral lung transplantation. Medical mycology case reports 17:4-7

Delaney B, Astwood JD, Cunny H, Conn RE, Herouet-Guicheney C, MacIntosh S, Meyer LS, Privalle L, Gao Y, Mattsson J, Levine M, ILSI Food Biotechnology Committee Task Force on Protein Safety (2008) Evaluation of protein safety in the context of agricultural biotechnology. Food Chem Toxicol 46: S71-S97

Frisvad JC, Larsen TO, Thrane U, Meijer M, Varga J, Samson RA, Nielsen KF (2011) Fumonisin and ochratoxin production in industrial *Aspergillus niger* strains. PLoS One 6(8):e23496

Gautam AK, Sahrma S, Avasthi S, Bhadauria R (2011) Diversity, pathogenicity and toxicology of *A. niger*: An important spoilage fungi. Research Journal of Microbiology 6(3):270-280

Kelly JM, Hynes MJ (1985) Transformation of *Aspergillus niger* by the *amdS* gene of *Aspergillus nidulans*. EMBO J 4:475-479

Kumar P and Satyanarayana T (2009) Microbial glucoamylases: characteristics and applications. Crit Rev Biotechnol 29(3): 225–255

Nielsen BR, Lehmbeck J, Frandsen TP (2002) Cloning, heterologous expression, and enzymatic characterization of a thermostable glucoamylase from *Talaromyces emersonii*. Protein Expr Purif 26(1):1-8

Pariza MW, Johnson EA (2001) Evaluating the safety of microbial enzyme preparations used in food processing: update for a new century. Regulatory Toxicology and Pharmacology 33:173-186

Perrone G, Stea G, Epifani F, Varga J, Frisvad JC, Samson RA (2011) *Aspergillus niger* contains the cryptic phylogenetic species *A. awamori*. Fungal biology, 115(11):1138-1150

Person AK, Chudgar SM, Norton BL, Tong BC, Stout, JE (2010) *Aspergillus niger*: an unusual cause of invasive pulmonary aspergillosis. Journal of Medical Microbiology 59(7):834-838

Rose M, Grisafi P, Botstein D (1984) Structure and function of the yeast *URA3* gene: expression in *Escherichia coli*. Gene 29(1-2):113-124

Ruiz-Diez B (2002) Strategies for the transformation of filamentous fungi. J Appl Microbiol 92:189-195

Schuster E, Dunn-Coleman N, Frisvad JC, van Dijck PWM (2002) On the safety of *Aspergillus niger* - a review. Applied Microbiology and Biotechnology 59:426-435

Show PL, Oladele KO, Siew QY, Aziz Zakry FA, Lan JC, Ling TC (2015) Overview of citric acid production from *Aspergillus niger*. Frontiers in Life Science 8(3):271-283

Toyotome T, Satoh M, Yahiro M, watanabe A, Nomura F, Kamei K (2014) Glucoamylase is a major allergen of Schizophyllum commune. Clin Exp Allergy 44:450-457