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**Supporting document 1 at Approval**

Risk and technical assessment report – Application A1184

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

# Executive summary

The purpose of the application is to amend Schedule 18 – Processing Aids of the Australia New Zealand Food Standards Code (the Code) to include the enzyme glucoamylase (EC 3.2.1.3), from a genetically modified (GM) strain of *Aspergillus niger.* This production organism contains the glucoamylase gene from *Trametes cingulata.* Glucoamylase is proposed as a processing aid for starch processing and the production of potable alcohol.

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

The safety assessment of the GM production strain concluded there were no public health and safety concerns. The host *A. niger* strain is neither pathogenic nor toxigenic and has a long history of safe use as a source of enzyme processing aids, including several already permitted in the Code. Analysis of the production strain confirmed the presence and stability of the inserted DNA.

Glucoamylase from GM *A. niger* was not genotoxic *in vitro*, and did not cause adverse effects in a short-term toxicity study in rats. The no observed adverse effect level (NOAEL) in a 90-day repeated dose oral toxicity study in rats was the highest dose tested, 10 mL/kg bw/day or 1135 mg/kg bw/day on a total organic solids (TOS) basis. The applicant’s estimated theoretical maximal daily intake (TMDI) based on the proposed uses is 0.31 mg/kg bw/day TOS. A comparison of these values indicates that the Margin of Exposure between the NOAEL and TMDI is more than 3,000.

Bioinformatic analysis indicated that the enzyme has no significant homology with any known toxins or food allergens, and is unlikely to pose an allergenicity or toxicity concern. Soy may be used as an ingredient in the fermentation medium as may a glucose syrup prepared using wheat, however they are expected to be removed as a result of downstream processes.

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

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# 1 Introduction

Novozymes Australia Pty Ltd applied to FSANZ for permission to use the enzyme glucoamylase (EC 3.2.1.3) as a processing aid in starch processing and the production of potable alcohol. This glucoamylase is from a genetically modified (GM) strain of *Aspergillus niger,* containing the glucoamylase gene fromthe fungus *Trametes cingulata.*

Currently, Schedule 18 of the Australia New Zealand Food Standards Code (the Code) permits the use of over 30 different enzymes produced by *A. niger* and 6 permissions for glucoamylase. However, the Code does not currently include a permission to use glucoamylase produced by a GM strain of *A. niger* containing the glucoamylase gene from *T. cingulata.* Therefore, this enzyme needs a pre-market assessment before permission can be given for its use as a processing aid. If permitted, this glucoamylase will provide an additional option for starch processors and manufacturers of potable alcohol.

## Objectives of the assessment

The objectives of this risk and technical assessment were to:

* determine whether the proposed purpose is clearly stated and that the enzyme achieves its technological function in the quantity and form proposed to be used as a food processing aid
* evaluate potential public health and safety concerns that may arise from the use of this enzyme, produced by a GM microorganism, as a processing aid. Specifically by considering the:
* history of use of the gene donor and production microorganisms
* characterisation of the genetic modification(s), and
* safety of the enzyme.

# Food technology assessment

## 2.1 Characterisation of the enzyme

### 2.1.1 Identity of the enzyme

The applicant provided relevant information regarding the identity of the enzyme, and this has been verified using an appropriate enzyme nomenclature reference (IUBMB 2017).

Accepted IUBMB[[1]](#footnote-2) name: glucan 1,4-α-glucosidase [[2]](#footnote-3)

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

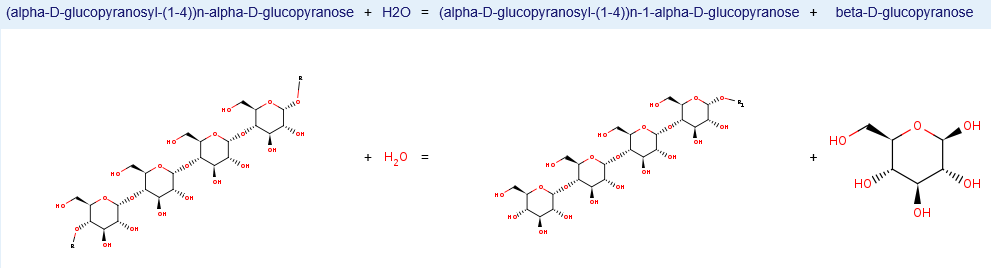
IUBMB enzyme nomenclature: EC 3.2.1.3

CAS[[3]](#footnote-4) 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. Note that most forms of the enzyme can rapidly hydrolyse 1,6-α-D-glucosidic bonds when the next bond in the sequence is 1,4.

### 2.1.2 Technological purpose of the enzyme

In general terms, glucoamylases degrade starch (and other related polysaccharides) into β-D-glucose. Specifically, the enzyme catalyses the hydrolysis of both 1,4-α and 1,6-α-D-glucosidic linkages in starch polysaccharides (Figure 1 depicts the hydrolysis of 1,4-α-D-glucosidic bonds).

Source: BRENDA:EC3.2.1.3 (<https://www.brenda-enzymes.org/enzyme.php?ecno=3.2.1.3>)

***Figure 1*** *Representation of hydrolysis reaction of a polysaccharide catalysed by glucoamylase*

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 at the mashing step to degrade gelatinised starch and dextrins to glucose and other fermentable sugars.

The two-fold action of glucoamylase on large, branched starch polysaccharides is of technological importance in both starch processing and alcohol production. This is because, in addition to its action on terminal glucose units connected by 1,4-α-D-glucosidic bonds, it hydrolyses glucose units at branch points along the polysaccharide chain, connected in a 1,6-α-D-glucosidic conformation. These branch points could otherwise remain as non-fermentable dextrins, resulting in an incomplete, inefficient degradation of starch.

The application states that the enzyme can be used at high operating temperatures and low operating pH, as detailed in Table 1 below. Table 1 also includes a range of other physical and chemical properties of the enzyme preparation.

Table 1 Glucoamylase enzyme preparation physical/chemical properties

|  |  |
| --- | --- |
| **Physical/chemical properties of commercial enzyme preparation** | |
| Enzyme activity | 381 AGU\*/g |
| Appearance | Liquid |
| Temperature optimum | 50-70°C |
| Temperature stability | 50% residual activity after approx. 60°C |
| pH optimum | 3-5 |
| Storage stability | Stable at 0-25°C |

\*AGU – standardised glucoamylase activity units

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 applicant states that the highest use level of glucoamylase for solid foods is 400 AGU per kg starch dry matter. This corresponds to 0.07 g per kg starch dry matter, which is equivalent to 0.03 g TOS per kg starch dry matter. For liquid foods, the highest recommended use level is 900 AGU per kg starch dry matter, or 0.16 g of glucoamylase per kg starch dry matter, which is equivalent to 0.07 g TOS per kg starch dry matter. The applicant claims and FSANZ agrees that the amounts of glucoamylase and TOS in the final food are negligible.

The conditions of use of the enzyme during food 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, and also bearing in mind an acceptable economic cost.

### 2.1.3 Technological justification for the enzyme

As outlined above, the application suggests that the enzyme fulfils an important technological purpose, in that it assists in a more complete conversion of large, branched polysaccharides to glucose and other fermentable sugars during starch processing and alcohol production. Further, it is argued that the enzyme provides the food and beverage industry with an opportunity to gain these processing advantages under resource- and cost-efficient production conditions.

In particular, in starch processing, the benefits of using the glucoamylase include:

* efficient degradation of dextrins and production of glucose, leading to a higher overall glucose yield
* reduced risk of microbial contamination, because the enzyme is still functional at high operating temperatures
* thermal stability, such that variations in temperature will not affect enzyme activity.

The benefits of the action of the glucoamylase in the production of potable alcohol include:

* efficient degradation of gelatinised starch and dextrins and production of fermentable sugars
* high alcohol yields, due to a more complete conversion of starch and, as such, a reduction in the amount of raw materials needed
* reduced risk of contamination, because the enzyme is still functional at high operating temperatures and low operating pH.

## 2.2 Manufacturing process

### 2.2.1 Production of the enzyme

The enzyme is produced by fermentation of the GM strain of *A. niger.* Briefly, it comprises the processes of fermentation, purification and formulation, followed by quality control of the finished product.

The fermentation process begins with injecting the stock culture suspension into the inoculum flask. When sufficient biomass is obtained, a suspension of cells is transferred from the inoculum flask to the seed fermentation tank, and eventually to the main fermentation tank, where the desired level of biomass and enzymatic activity is achieved. During all stages of the fermentation process, samples are taken to ensure the absence of microbial contamination.

The recovery process is a multi-step operation to separate the biomass from the enzyme-containing culture medium. It involves a series of filtration, concentration and stabilisation steps, resulting in an enzyme-containing liquid concentrate that is free from any microorganisms or other impurities. For enzymatic, physical and microbial stabilisation, glycerol is added to the enzyme concentrate, and the pH is adjusted using acetic acid or sodium hydroxide.

The glucoamylase enzyme may be formulated as a single enzyme preparation or a blend with other food enzymes as a liquid or a granulate depending on the food manufacturing process for which it will be used.

The applicant has provided documentation to demonstrate that the manufacture of the enzyme follows current Good Manufacturing Practices (GMP). The company’s quality management system is ISO 9001:2015[[4]](#footnote-5) certified, and requires that the production of food enzymes complies with EC regulation 852/2004/EC[[5]](#footnote-6), including amendments, on *the hygiene of foodstuffs*.

The applicant states that the raw materials used in fermentation and recovery are food grade quality and have been analysed to ensure that they conform to the relevant specifications.

### 2.2.2 Allergen considerations

Several potential raw materials as source material for the fermentation include glucose syrup (which may be sourced from wheat and maize) and soy bean meal may be sourced from allergens (wheat and soy). The applicant has stated that product blends (which this glucoamylase is a constituent) are tested for food allergenicity risks (i.e. presence of food allergens). Data provided by the applicant of the enzyme preparation (a blend of three enzymes) has shown that downstream processes removes any traces of soy. The applicant did not provide data for wheat, however testing of product blends which include this glucoamylase are undertaken by the applicant to monitor this and such results are available to end users of the enzyme preparation to make their own allergen presence decisions which is appropriate.

### 2.2.3 Specifications

The JECFA Compendium of Food Additive Specifications (2017) and the Food Chemicals Codex 11th edition (2018) are international specifications for enzymes used in the production of food. These are primary sources of specifications listed in section S3—2 of Schedule 3 of the Code. Enzymes need to meet these specifications. Schedule 3 of the Code also includes specifications for heavy metals (section S3—4) if they are not already detailed within specifications in sections S3—2 or S3—3.

Table 2 provides a comparison of the analysis of different batches of the glucoamylase product with international specifications established by JECFA and Food Chemicals Codex, as well as those in the Code (as applicable). Based on these results, the enzyme preparation meets all relevant specifications.

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

| Analysis | Novozymes analysis | Specifications | | |
| --- | --- | --- | --- | --- |
| JECFA | Food Chemicals Codex | Australia New Zealand Food Standards Code  (section S3—4) |
| Lead (mg/kg) | ND | ≤ 5 | ≤ 5 | ≤2 |
| Arsenic (mg/kg) | 0.193, 0.208, 0.184 | - | - | ≤1 |
| Cadmium (mg/kg) | ND | - | - | ≤1 |
| Mercury (mg/kg) | ND | - | - | ≤1 |
| Coliforms (cfu/g) | ND | ≤30 | ≤30 | - |
| *Salmonella* (in 25 g) | ND | Absent | Negative | - |
| *E. coli* (in 25 g) | ND | Absent | - | - |
| Antimicrobial activity | ND | Absent | - | - |
| Mycotoxins | ND | No toxicologically significant levels |  |  |

ND: Not Detected

## 2.3 Food technology conclusion

FSANZ concludes that the use of this glucoamylase in starch processing and the production of potable alcohol is clearly described in the application and that the evidence presented to support its proposed use provides adequate assurance that the enzyme, in the quantity and form proposed to be used (which must be consistent with GMP), is technologically justified and effective in achieving its stated purpose. The enzyme meets international purity specifications.

# 3 Safety assessment

## 3.1 History of use

### 3.1.1 Host organism

*A. niger* is a filamentous fungus ubiquitous in the environment (Schuster et al. 2002; Gautam et al. 2015). The ability of *A. niger* to produce organic acids such as citric acid, the primary acidulant used in the food and beverage industry, has been industrially exploited since 1919 (Schuster et al. 2002; Show et al. 2015). *A. niger* has been classed as a Biosafety Level 1 organism, based on the [United States Public Health Service Guidelines](https://www.cdc.gov/biosafety/publications/bmbl5/index.htm)[[6]](#footnote-7), has a long history of safe use as a production organism for food enzymes (Gautam et al. 2011; Pariza and Johnson 2001) and is a permitted source of a number of enzymes in the Code (Schedule 18).

*A. niger* has been isolated in rare cases of aspergillosis in immunocompromised individuals (Atchade et al. 2017; Person et al. 2010) however, it is generally regarded as non-pathogenic, considering 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 mycotoxins.

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 proteins that would impact the yield and purity of the glucoamylase and to increase the ability and efficiency to transform the host. A predecessor of the host strain was characterised based on DNA sequencing of four gene regions (ribosomal internal transcribed spacer (ITS), translation elongation factor-1 (TEF-1α), calmodulin (cdl) and β-tubulin (tub2)). The sequencing data was provided by the Applicant and confirmed the host strain to be *A. niger*.

### 3.1.2 Gene donor organisms

**Glucoamylase gene**

The glucoamylase gene *amgTC* was amplified from a cDNA library prepared from the source organism *Trametes cingulata*. *T. cingulata* is a cosmopolitan mushroom from the family of white-rot fungi. These mushrooms play a major role in wood decomposition in temperate and tropical forests. Due to this role, the genome of *T. cingulata* was sequenced as part of a [Joint Genome Institute USA Community Project](https://mycocosm.jgi.doe.gov/Traci1/Traci1.home.html)[[7]](#footnote-8), to identify lignolytic enzymes that could be used for biofuel and biomass energy production (Nyanhongo et al. 2007). There is no evidence to show this organism poses any public health and safety concerns.

**Other sequences**

Regulatory sequences for expression of the glucoamylase gene and the selection marker gene aminolevulinic acid synthase (*hemA*) were sourced from the host. A second selectable marker gene acetamidase (*amdS*) were sourced from *A. nidulans* (Kelly and Hynes 1985). Inclusion of the acetamidase selection marker is regarded as ‘mainstream’ in filamentous fungi systems involving recombinant gene expression (Gryshyna et al. 2016) and there are no safety concerns with the acetamide gene product.

## 3.2 Characterisation of the genetic modification(s)

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

Two plasmids were generated to introduce the glucoamylase (*amgGT*) gene from *T. cingulata* into the *A. niger* host. The first plasmid contained an expression cassette with the *amgGT* gene flanked by promoter and terminator sequences from the host, followed by a copy of the endogenous aminolevulinic acid synthase gene (*hemA*) gene. This plasmid would be transformed into a *hemA*- strain. The addition of the *hemA* gene would restore the enzyme function, allowing for selection of positive transformants by growth on minimal media devoid of 5-aminolevulinic acid (Franken et al. 2012).

The second plasmid contained an expression cassette with the *amgGT* gene flanked by the same promoter and terminator sequences as the first expression cassette, followed by the acetamidase gene (*amdS*). Addition of the acetamidase gene would allow for selection of positive transformants by growth on media containing acetamide (Kelly and Hynes 1985).

The two plasmids were introduced sequentially using protoplast-mediated transformation (Li et al. 2017). The first step was to introduce the *amgGT-hemA* plasmid into a *hemA-* recipient strain. After selection of a *hemA+* clone co-expressing glucoamylase, the *amgGT-amdS* plasmid was then introduced. After selection of a clone co-expressing acetamidase and glucoamylase, several rounds of chemical and ultraviolet radiation mutagenesis was performed. A final clone designated CBP5-7 showing high expression of glucoamylase was selected for production purposes.

### 3.2.2 Characterisation of the inserted DNA

Analysis of gene copy number in CBP5-7 was determined using Southern blot analysis and real-time quantitative PCR. The results showed that several copies of the *amgGT* gene was inserted into the genome of the *A. niger* host. Confirmation of the gene copy number was achieved by whole genome sequence analyses, which also showed that the gene sequence of the insert was as expected, with no rearrangements or mutations. This was important considering that several rounds of conventional mutagenesis were performed to drive higher expression levels in the production strain.

### 3.2.3 Stability of the production organisms and inheritance of the introduced DNA

Southern blot analysis was performed on genomic DNA extracted from three cultures of CBP5-7 at the end of standard fermentation runs. The results were compared to genomic DNA from a reference stock. The results showed the same banding patterns across the different fermentation runs and the reference stock. Morphological characteristics was also compared across the fermentation runs and to the reference stock and no differences were observed.

## 3.3 Safety of glucoamylase

### 3.3.1 History of safe use

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.

Glucoamylase from *A. niger* was approved for use in Denmark for starch processing, alcohol (distilling and brewery) cereal beverage and baking applications in 2015, in accordance with EFSA guidelines for the presentation of data on food enzymes. According to the applicant, the enzyme has also been approved in France, although the date of approval is not specified.

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.

### 3.3.2 Bioinformatic assessment of enzyme 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](https://www.uniprot.org/)[[8]](#footnote-9) database. No noteworthy 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.

### 3.3.3 Toxicology studies in animals

*Thirteen week oral gavage study of glucoamylase, PPY30930 in CD rats (Huntingdon Life Sciences Ltd., Study Number LKG0032, 2011) Regulatory status: GLP.*

The test article for this study was glucoamylase, Batch PPY 30930*.* This batch was produced by the CBP5-7 production strain that is the subject of this application. The test article was received as a liquid with a Total Organic Solid (TOS) content of 10.9% w/w, (dry matter content 11.4%). The vehicle and control article was water, purified by reverse osmosis. Enzyme activity of the dose formulations was determined from triplicate samples collected during Weeks 1, 6 and 13.

The test subjects were Crl:CD®(SD) rats, received at 29 to 35 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, detailed physical examination (including arena observations), bodyweight and food intake measurements.

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 appraised by visual observation on a daily basis but because visual observation did not reveal any group differences quantitative measurements were not undertaken. Bodyweight, and behaviour in an arena were recorded weekly. 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. The animal killed during the treatment period was also subject to necropsy. The following fresh organ weights were recorded for all animals killed at the end of the 13-week treatment period: 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 from all animals was preserved for histopathological examination. In the first instance histopathological examination was confined to all Control and animals receiving 10 mL/kg bw/day, however due to potential treatment-related effects being noted in the heart and kidneys of males receiving 10 mL/kg bw/day, examination of these tissues was extended to all males at 1.0 or 3.3 mL/kg bw/day surviving until the end of Week 13.

Analysis of the enzyme activity of dose formulations from Weeks 1, 6 and 13 confirmed that it did not deviate from the intended activity by more than 4%, and the formulations were therefore suitable for use on study.

There were no treatment-related deaths prior to scheduled termination. One male rat in the 1.0 mL/kg bw/day group was killed in Week 12, due to limited use of a hind limb. Assessment of in-life, macroscopic and microscopic data from this rat did not reveal any findings associated with treatment. Treatment with glucoamylase had no effects on clinical observations, behaviour in an arena, sensory reactivity tests, grip strength, motor activity measured, bodyweights gains, food consumption, water consumption, ophthalmic findings, haematology, organ weights or findings on gross necropsy. Week 13 blood chemistry investigation showed a slightly higher than control mean aspartate amino-transferase value for males at 10 mL/kg bw/day. The study director commented that the differences from control was mainly attributable to three individual males at 10 mL/kg bw/day which showed aspartate amino-transferase values above the concurrent control range. The study director further commented that all values for males at 10 mL/kg bw/day were within the background data limits, (this is an incorrect statement as the individual value for one of the males (No.4) was 118 U/L, whereas the highest background data value quoted is 98 U/L (mean 75.9 with SD 5.60)). The elevated aspartate amino-transferase levels noted for males at 10 mL/kg bw/day are considered most likely to be associated with the heart changes discussed below. Similar changes from controls, in aspartate amino-transferase levels were not evident for males at 1.0 or 3.3 mL/kg bw/day or any treated female group.

Treatment related microscopic changes were confined to males at 10 mL/kg bw/day and consisted of changes in the heart and kidneys. Multifocal myocardial inflammation/

degeneration in the heart was recorded for four males (minimal degree in three animals and slight in one) at 10 mL/kg bw/day, with a further male at this dosage showing the same change but focal (minimal). The same heart finding was noted in three male controls but limited to focal and minimal. The study director commented that the incidence for males at 10 mL/kg bw/day was within the background data range, and that the multifocal nature may have arisen due to an exacerbation of a background finding. The background data presented were not a direct match to the study findings, i.e. in the study the finding was reported as combined myocardial inflammation/degeneration, whereas the background data reports as separate entries “myocardial inflammation” and “cardiomyopathy”. In addition the background data does not provide any grading information e.g. focal and multifocal. The study director makes no acknowledgment/clarification of these discrepancies. The differences in data presentation between the study results section and the background data makes it difficult to judge whether the heart findings are consistent with background findings, which in-turn impacts on the assessment of toxicological importance of the heart changes seen in males at 10 mL/kg bw/day. In the report Discussion, the study director comments they are considered to be of “little toxicological significance”, which indicates they are adverse, albeit minor in degree. However the study director later concludes “Since none of the findings in this study was considered of any toxicological importance, the no observed adverse effect level (NOAEL) in this study is considered to be 10 mL/kg/day (equivalent to 1.135 g/kg bw/day TOS).” Of the three aforementioned males at 10 mL/kg bw/day which had elevated aspartate amino-transferase levels two had histopathological heart changes, multifocal in extent, indicating a possible correlation. Control males with heart lesions did not show any corresponding effects on aspartate amino-transferase levels. There was also some evidence of a correlation between individual heart weights and the presence of histopathological heart changes. The three males at 10 mL/kg bw/day and the three controls with focal heart alterations had the highest heart weights within their respective group. As the values for the affected males at 10 mL/kg bw/day were higher than the highest concurrent control heart weight (in the region of 6% higher) this provides support that the heart effects at 10 mL/kg bw/day are greater than expected from normal biological variation.

The lack of similar treatment-related heart findings in females on this study is consistent with naturally occurring spontaneous progressive cardiomyopathy in rats, which is reported to have a higher incidence in males than females (Berridge et al.2016; Herman and Eldridge, 2019; Jokinen et al. 2011). In addition, these authors reported onset of these lesions as early as three months of age. Herman and Eldridge state the most common sites of these alterations are the left ventricle, right ventricle and septum, again this is consistent with the findings in this study. Of the five males at 10 mL/kg bw/day and three Controls with reported heart changes, the change was seen in the ventricles. The absence of any reports of fibrosis in the hearts of affected males indicates that the heart changes are at an early stage of the pattern of spontaneous progressive cardiomyopathy. The typical pattern of change begins with focal and multifocal myocyte degeneration and necrosis, with varying degrees of inflammation and interstitial cell infiltration, and ultimately fibrosis (Herman and Eldridge 2011).

The scientific literature cited above supports the conclusion that the heart changes in males at 10 mL/kg bw/day are most likely to be attributable to a spontaneous background change which treatment has slightly exacerbated. There was no evidence that the heart changes were adversely affecting the general health of the rats and although treatment-related were not considered to be of a severity to class as adverse.

Males receiving 1.0 or 3.3 mL/kg bw/day also showed a slightly higher than control incidence of multifocal myocardial inflammation/degeneration, however there was no dose response, between these groups, in either incidence or degree and no corresponding effects on aspartate amino-transferase or heart weights, therefore the heart lesions in these treated groups were considered to be attributable to normal biological variation.

The kidney changes were confined to males and consisted of higher, than control, dosage-related incidences of hyaline droplets at 3.3 or 10 mL/kg bw/day. The incidence of this finding for males at 1 mL/kg bw/day was slightly lower than controls. The study director commented that the severity of the finding for males at 3.3 or 10 mL/kg bw/day was similar to that seen for the five (50%) male controls, the finding is common in male rats, (related to high levels of α-2 globulin), it was not apparent in female rats on this study, and is not relevant to humans as extensively documented in the literature (Gopinath et al 1987; Greave 1990; Hascheck and Rousseaux 1991; Swenberg et al. 1989). Therefore, the finding was considered to be of no toxicological significance.

FSANZ concluded that based on the evaluation above, the no observed adverse effect level (NOAEL) in this study was 10 mL/kg bw/day, (equivalent to 1.135 g TOS/kg bw/day). This is consistent with the NOAEL assigned by the Study Director.

### 3.3.4 Genotoxicity assays

*Bacterial reverse mutation assay (Novozymes A/S, Study Number 20118002, 2011). Regulatory status: GLP; conducted in accordance with the general recommendations of OECD Guideline No. 471.*

The test article for this assay was glucoamylase, batch number PPY 30930, with a declared dry matter content of 11.4% w/w, which is glucoamylase from the production strain that is the subject of the application. The test article contains histidine and tryptophan, the amino acids that are the growth-limiting factors for the test strains of *Salmonella typhimurium* and *Escherichia coli* respectively. A standard bacterial reverse mutation assay conducted by the plate incorporation method 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 using the “treat and plate” method. This method includes a rinsing step which removes the test article.

The test systems for this assay were *S. typhimurium* strains TA98, TA100, TA1535 and TA1537 and *E. coli* WP2 *uvr*ApKM101. The test article was diluted to the required concentrations using the vehicle/negative control article, deionised water. Positive control articles for assays without addition of S9 mix were 2-nitrofluorene for TA98, ICR-191 for TA1537, and N-methyl-N-nitro-N-nitrosoguanidine for TA100, TA1535, and WP2 *uvr*ApKM101. For assays with addition of S9 mix for metabolic activation, the positive control article was 2-aminoanthracene for all bacterial strains.

The dose levels tested, in the initial and confirmatory run were 156, 313, 625, 1250, 2500, 5000 μg/mL. S9 mix or sham mix, tester strain, vehicle, and test article or positive control article were mixed in a test tube mixed by vortex and incubated for 3 h at 37°C with shaking. The contents of the tube were then centrifuged and the supernatant was removed. The tester strain was resuspended in phosphate buffer and an aliquot was added to molten selective top agar. This mixture was overlaid onto the surface of minimal bottom agar. After the overlay had solidified, the plates were inverted and incubated for 64 hours at 37±2°C. Revertant bacterial colonies were counted.

No precipitation of the test article was observed in any strain in either experiment with or without metabolic activation, and no cytotoxicity was detected. There was no biologically relevant increase in revertant colonies, with or without S9 mix. Positive controls induced mutagenesis at expected levels, based on background data, confirming the validity of the assay.

Due to a failure to add bacteria suspension there were no confirmatory data for strain TA100, 2500 μg/mL with S9 mix. Considering there was clearly no effect of treatment noted for this group on the initial run and in the absence of any treatment-related effects for this strain at other treatment levels, including at 5000 μg/mL, the lack of data is not considered to adversely affect the outcome/validity of the study.

It was concluded that under the conditions of this study, the test article did not cause a positive mutagenic response with any of the tester strains in either the presence or absence of metabolic activation.

*Micronucleus assay in cultured human peripheral blood lymphocytes (Covance Laboratories, Study Number 8228638, 2010). Regulatory status: GLP; in accordance with the 2009 draft proposal OECD Guideline 487.*

This assay was performed using Batch PPY 30930, with a declared dry matter content of 11.4% w/w (TOS 10.9%) 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. Positive control articles were mitomycin C, cyclophosphamide and vinblastine. Lymphocytes were obtained from healthy non-smoking male donors, for each experiment phase (Range-Finder or Micronucleus), samples from two donors were pooled. In both phases, lymphocytes were pre-treated with phytohaemagglutinin to stimulate division. In both the Range-Finder and Micronucleus phases, two time regimes were investigated: three-hours incubation with test material followed, after washing, by a 21-hour incubation period (with or without S9 mix), and a 24-hour incubation followed, after washing, by a 24-hour incubation period (without S9). Positive controls were only used in the Micronucleus phase of the study, with and without S9 mix for the three hour incubation regime and without S9 mix for the 24-hour incubation regime. Independent of the treatment time, each Micronucleus test with test article and positive control was conducted in duplicate whereas assays with the negative control were conducted in fourfold. Incubation was at 37ºC. At the end of the incubation cells were harvested, processed to slides and examined. Osmolality and pH measurements on post-treatment incubation medium were undertaken. At 5000 µg/mL (highest concentration tested) osmolality and pH were similar to the concurrent vehicle control.

The Range-Finder experiment was carried out to determine whether there was evidence of cytotoxicity and assist in selection of dose levels for the Micronucleus test. The Range-Finder investigated the following concentrations of test article 0, 18.1, 30.2, 50.4, 84, 140, 233, 389, 648, 1080, 1800, 3000 and 5000 µg/mL. Under all conditions investigated on the Range-Finder experiment only a low level (range 0-19%) of cytotoxicity was observed. On this basis the doses for both the three and 24-hour exposure Micronucleus assays were 500, 1000, 2000, 3000, 4000 and 5000 µg/mL. As expected from the Range-Finder study the level of cytotoxicity in the Micronucleus phase was low, (range 0-7%). 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. No significant differences were observed between treated and negative control cells.

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

### 3.3.5 Potential for allergenicity

A sequence homology assessment to known allergens was conducted by the applicant and FSANZ 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>). The applicant’s search was carried out using FASTA version 34 in January 2018, whilst FSANZ used the most recent version (at the time of this assessment), FASTA 36.

Using the 80-mer sliding window both searches found significant similarity, up to 84% from FASTA 34 and 82.5% using FASTA 36, (E values <1x10-7) to the glucoamylase from *Schizophyllum commune* (splitgill mushroom). A full length sequence alignment search revealed a high degree of protein homology (70%) which was not confined to a distinct region; a search for exact matches of 8 amino acid sequences identified 43 matches. These results of high similarity are not unexpected as the donor organism *T. cingulata* is also a mushroom/fungus. *T. cingulata* is also known as “white rot fungus” and is commonly found on debarked trees and rotting wood. No reports of *T. cingulata* as a food were found, nor reports of any adverse effects associated with this mushroom.

According to Aalberse et al. (2000) 70% homology over the full length with an allergen has a moderate likelihood of cross-reactivity. On this basis it is possible that this glucoamylase will act as an allergen. However, as mentioned in the assessment for A1168, the glucoamylase from *S. commune,* which this glucoamylaseis showing similarity with, has been identified as a respiratory allergen, associated with fungal rhinitis, sinusitis and allergic bronchopulmonary mycosis (Toyotome et al. 2014), rather than a food allergen. 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. This is consistent with the finding that respiratory allergens are usually not digestive allergens, which in turn is considered to be due to insufficient amounts of the allergen being ingested to trigger a clinical reaction. The use pattern of the glucoamylase in this application is such that it will be used at the minimal level to achieve its technical function and if present in the final food, the amount present would be expected to be very low and insufficient to elicit a clinically evident allergic reaction. In addition, one of the enzymes investigated by Bindslev-Jensen et al. (2006) was a glucoamylase that had a sequence identity of up to 68% (*cf* 70% in this application) to glucoamylase from *S. commune;* none of the tested patients on the study showed clinical symptoms of an allergenic reaction after oral ingestion of the glucoamylase.

In summary, the glucoamylase in this application has no identified association with causing food allergy and is not expected to be at a high enough level in food to cause concern. The homology therefore between the recombinant glucoamylase and glucoamylase from *S. commune* is not considered to represent a food safety concern.

The FASTA sequence homology assessment conducted by the applicant also revealed a 35.2% identity over an 80 amino acid window for beta-xylosidase from *A. niger.* This beta-xylosidase is classified as an occupational allergen (especially in bakers), that is not a food allergen. On this basis and considering a) the very low % of homology identity (35%) and b) in the more recent FASTA 36 search this enzyme was not identified as a homology match, the homology between the recombinant glucoamylase and beta-xylosidase is not considered to represent a food safety concern.

The applicant has indicated that soy may be used as an ingredient in the fermentation medium as may a glucose syrup prepared using wheat. The applicant has stated that the final food will not contain significant residual amounts of these raw materials due to down-stream processes such as washing and filtration, which are expected to remove remaining amounts. Testing of product blends which include this glucoamylase are undertaken by the applicant to monitor this.

### 3.3.6 Approvals by other regulatory agencies

The applicant provided a letter of approval from Danish authorities for the use of the enzyme for starch processing and production of alcohol (distilled and brewed products), cereal based beverages and for baking applications, dated 2015. The evaluations had been made in accordance with EFSA guidelines for the presentation of data on food enzymes. In addition, the applicant has stated the enzyme is included in the French positive list for processing aids, including food enzymes (The French order of October 19, 2006 on use of processing aids in the manufacture of certain foodstuff), as amended.

# 4 Discussion

No public health and safety concerns were identified with the use of glucoamylase from *A. niger* when used as a food processing aid at GMP levels for starch processing and the production of potable alcohol.

The host *A. niger* strain has a long history of safe use as a source of enzyme processing aids, including several already permitted in the Code and is neither toxigenic or pathogenic. Molecular characterisation of the production strain CBP5-7 confirmed that the gene insert was as expected and would encode the glucoamylase as specified. The insert was also shown to be genetically stable and heritable.

Glucoamylase from CPB5-7 showed no evidence of genotoxicity in a bacterial reverse mutation assay or a micronucleus assay in human lymphocytes. Glucoamylase did not cause any adverse effects in a sub-chronic toxicity study in rats. The NOAEL was the highest dose tested, 10 mL/kg bw/day or 1135 mg/kg bw/day on a TOS basis. The applicant’s estimated theoretical maximal daily intake (TMDI) of glucoamylase is 0.31 mg kg bw/day TOS, resulting in a Margin of Exposure (MoE) of more than 3000 between the NOAEL and TMDI.

Bioinformatic analyses did not identify any homology with any known toxins but did show a high degree of homology between the recombinant glucoamylase to that of a respiratory allergen. However, further analysis indicated that the glucoamylase from *T. cingulata* expressed in *A. niger* is unlikely to be a food safety concern. Soy may be used as an ingredient in the fermentation medium as may a glucose syrup prepared using wheat, however due to washing and filtration processes they are not expected to be present in the final product.

# 5 Conclusion

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

Aalberse RC (2000). Structural biology of allergens. J Allergy Clin Immunol 106:206-238

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. Med Mycol Case Rep 17:4-7

Bindslev-Jensen C, Skov PS, Roggen EL, Hvass P, Brinch DS (2006) Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry. Food and Chemical Toxicology 44: 1909-1915

Berridge BR, Mowat V, Naga H, Nyska A, Okazaki Y, Clements PJ, Rinke M, Snyder PW, Boyle MC, Wells MY (2016) Non-proliferative and Proliferative Lesions of the Cardiovascular System of the Rat and Mouse. Journal of Toxicological Pathology 29 (3 suppl): 1S-47S

Bucci T.J (1991) Evaluation of altered morphology In: (Hascheck W and Rousseaux (eds) Handbook of Toxicologic Pathology. 1st ed. Elsevier p23-35.

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

Franken ACW, Lokman BC, Ram AFJ, van den Hondel CAMJJ, de Weert S, Punt PJ (2012) Analysis of the role of the *Aspergillus niger* aminolevulinic acid synthase (*hemA*) gene illustrates the difference between regulation of yeast and fungal haem- and sirohaem-dependent pathways. FEMS Microbiol Lett 335:104-112

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. Res J Microbiol 6(3):270-280

Greave P (1990) Cardiovascular system. In Greave P (ed) Histopathology of Pre-clinical toxicity studies : interpretation and relevance in drug safety evaluation. 4th ed, Elsevier, Canada, p 349-371

Gopinath C, Prentice D, Lewis (1987) The cardiovascular system In: Prentice D (ed) Atlas of experimental toxicology pathology. 1st ed. Springer, Netherlands, p11-21

Gryshyna A, Kautto L, Peterson R, Nevalainen KMH (2016) On the safety of filamentous fungi with special emphasis on *Trichoderma reesei* and products made by recombinant means. In Schmoll M, Dattenböck C (eds) Gene expression systems in fungi: advancements and applications. Fungal Biology. Springer, Cham

Herman E and Eldridge S (2019) Spontaneously occurring cardiovascular lesions in commonly used laboratory animals. Cardio-Oncology Vol 5, p3

<https://link.springer.com/article/10.1186/s40959-019-0040-y>

IUBMB (2018) EC 3.2.1.3. <https://www.qmul.ac.uk/sbcs/iubmb/enzyme/EC3/2/1/3.html>

JECFA (2017) Combined compendium of food additive specifications (FAO JECFA Monograph 1) <http://www.fao.org/docrep/009/a0691e/A0691E03.htm>

Jokinen MP, Lieuallen WG, Boyle MC, Johnson CL, Malarkey DE, Nyska A (2011) Morphologic Aspects of Rodent Cardiotoxicity in a Retrospective Evaluation of National Toxicology Program Studies. Toxicologic Pathology. Vol. 39 p 850-860 [https://journals.sagepub.com/doi/full/10.1177/0192623311413788#](https://journals.sagepub.com/doi/full/10.1177/0192623311413788)

Kelly JM, Hynes MJ (1985) Transformation of *Aspergillus niger* by the *amdS* gene of *Aspergillus nidulans.* EMBO J 4(2):475–479

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

Li D, Tang Y, Lin J, Cai W (2017) Methods for genetic transformation of filamentous fungi. Microb Cell Fact 16:e168

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

Nyanhongo GS, Gubitz G, Sukyai P, Leitnwe C, Haltrich D, Ludwig R (2007) Oxidoreductases from *Trametes* spp. in biotechnology: a wealth of catalytic activity. Food Technol Biotechnol 45(3):250-268

OECD (2009) “Genetic Toxicology: OECD Guideline for the testing of chemicals”. Draft proposal for a new Guideline 487; *In vitro* micronucleus test

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

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

Poulson PB (1983) Alcohol - Potable. In Godfrey T and Reichelt J (eds) Industrial Enzymology. The application of enzymes in industry. The Nature Press, New York 170-178

Reichelt JR (1983) Starch. In Godfrey T and Reichelt J (eds) Industrial Enzymology. The application of enzymes in industry. The Nature Press, New York 375-396

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. Appl Microbiol Biotechnol 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*. Front Life Sci 8(3):271-283

Swenberg JA, Short B, Borghoff S, Strasser J and Charbonneau M (1989) The comparative pathobiology of α2µ-globulin nephropathy. Toxicology and Applied Pharmacology Vol 97 p35-46

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

USPC (2018) Food Chemicals Codex 11th Edition, United States Pharmacopeial Convention, Rockville, MD. <http://publications.usp.org/>

1. International Union of Biochemistry and Molecular Biology. [↑](#footnote-ref-2)
2. Although the accepted IUBMB name is ‘glucan 1,4-α-glucosidase’, the name used in the application, this document, the Call for Submissions and the Approval Report is ‘glucoamylase’. This is the name that will be used in the proposed draft variation to the Code for this enzyme. [↑](#footnote-ref-3)
3. Chemical Abstracts Service. [↑](#footnote-ref-4)
4. ISO 9001:2015 – International Standard for Quality Management Systems. [↑](#footnote-ref-5)
5. Regulation (EC) No 852/2004 of the European Parliament and of the Council on the Hygiene of Foodstuffs (of 29 April 2004). [↑](#footnote-ref-6)
6. <https://www.cdc.gov/biosafety/publications/bmbl5/index.htm> [↑](#footnote-ref-7)
7. <https://mycocosm.jgi.doe.gov/Traci1/Traci1.home.html> [↑](#footnote-ref-8)
8. <https://www.uniprot.org/> [↑](#footnote-ref-9)