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

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Supporting document 1

Risk and technical assessment report – Application A1185 Alpha-amylase 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 alpha-amylase (EC 3.2.1.1), from a genetically modified (GM) strain of *Aspergillus niger.* This production organism contains the alpha-amylase gene from *Rhizomucor pusillus.* Alpha-amylase 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. This alpha-amylase has been authorised for use in Denmark, France and Mexico since 2015; FSANZ is not aware of any reports of adverse effects in consumers.

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 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.

Alpha-amylase from GM *A. niger* was not genotoxic *in vitro*, and did not cause adverse effects in short-term toxicity studies 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 1220 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 2.86 mg/kg bw/day TOS. A comparison of these values indicates that the Margin of Exposure between the NOAEL and TMDI is more than 400.

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 and possibly wheat are used in the fermentation medium, 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.

Table of contents

1 Introduction

Novozymes Australia Pty Ltd applied to FSANZ for permission to use the enzyme alpha-amylase (EC 3.2.1.1) as a processing aid in starch processing and the production of potable alcohol. This alpha-amylase is from a genetically modified (GM) strain of *Aspergillus niger,* containing the alpha-amylase gene from the fungus *Rhizomucor pusillus.*

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*. There is already permission to use an alpha-amylase from a non-GM strain of *A. niger,* and from several other plant and microbial sources. However, the Code does not currently include a permission to use alpha-amylase produced by a GM strain of *A. niger,* containing the alpha-amylase gene from *R. pusillus.* Therefore, this enzyme needs a pre-market assessment before permission can be given for its use as a processing aid. If permitted, this alpha-amylase will provide an additional option for starch processors and manufacturers of potable alcohol.

1.1 Objectives of the assessment

The objectives of this risk and technical assessment were to:

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

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

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

2 Food technology assessment

2.1 Characterisation of the enzyme

2.1.1 Identity of the enzyme

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

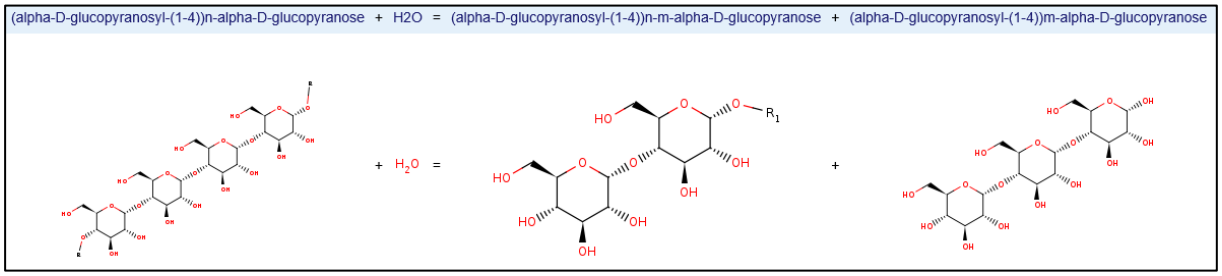
Accepted IUBMB[1] name:	α-amylase[2]
Systematic name:	4-α-D-glucan glucanohydrolase
Other names:	glycogenase; α amylase; endoamylase; Taka-amylase A; 1,4-α-D-glucan glucanohydrolase

[1] International Union of Biochemistry and Molecular Biology.
[2] Although the term that is used throughout the application, this document and the Call for Submissions is 'alpha-amylase', the term that will be used in the proposed draft variation to the Code for this enzyme is 'α-amylase', as this is the accepted IUBMB name, and will also ensure consistency with other existing permissions in Schedule 18 of the Code.

IUBMB enzyme nomenclature:	EC 3.2.1.1
CAS[3] number:	9000-90-2
Reaction:	Endohydrolysis of (1→4)-α-D-glucosidic linkages in polysaccharides containing three or more (1→4)-α-linked D-glucose units

2.1.2 Technological purpose of the enzyme

In general terms, alpha-amylases are used during the pre-saccharification of liquefied starch. They hydrolyse starch molecules randomly to release dextrins, maltose and glucose for further processing. Specifically, the enzyme catalyses the hydrolysis of 1,4-α-D-glucosidic linkages in starch polysaccharides (see Figure 1).



Source: BRENDA:EC3.2.1.1 (https://www.brenda-enzymes.org/enzyme.php?ecno=3.2.1.1)

Figure 1 *Representation of hydrolysis reaction of a polysaccharide catalysed by alpha-amylase*

The stated technological purpose of alpha-amylase in starch processing and alcohol production is consistent with the typical function of alpha-amylase and is supported by scientific literature, which indicates that this enzyme is principally responsible for rapidly reducing the average molecular weight of starch polymers (Poulson 1983; Reichelt 1983; Damodaran et al. 2008).

Specifically, during the production of syrups alpha-amylase degrades starch polysaccharides into dextrins. During alcohol production, alpha-amylase converts liquefied starch into fermentable sugars. Table 1 includes a summary of the physical and chemical properties of the enzyme preparation.

Table 1 *Alpha-amylase enzyme preparation physical/chemical properties*

Physical/chemical properties of commercial enzyme preparation	
Enzyme activity	53.3 FAU(F)*/g
Appearance	Liquid
Temperature optimum	55-65°C
Temperature stability	50% residual activity after approx. 75°C
pH optimum	3

[3] Chemical Abstracts Service.

Storage stability	Stable at 0-10°C

*FAU(F) – standardised alpha-amylase 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 alpha-amylase for solid foods is 200 FAU(F) per kg starch dry matter. This corresponds to 1.33 g per kg starch dry matter, which is equivalent to 466 mg TOS per kg starch dry matter. For liquid foods, the highest recommended use level is 200 FAU(F) per kg starch dry matter, or 1.33 g of alpha-amylase per kg starch dry matter, which is equivalent to 466 mg TOS per kg starch dry matter. The applicant claims and FSANZ agrees that the amounts of alpha-amylase 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 provides the food and beverage industry with an option for improving the yield of dextrins and fermentable sugars during starch processing and alcohol production. Further, it is argued that the enzyme provides the industry with an opportunity to gain these processing advantages under resource- and cost-efficient production conditions.

The primary benefit of using alpha-amylase in starch processing relates to its efficient degradation of starch to produce dextrins for further processing and production of syrups.

The benefits of the action of the alpha-amylase in the production of potable alcohol include:

- higher ethanol yields
- fast fermentation (depending on the processing conditions, alpha-amylase can act rapidly in starch liquefaction)
- efficient production of dextrins for saccharification
- efficient production of fermentable sugars for the action of the enzymes in the yeast, producing ethanol.

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 filtration, concentration, preservation/ stabilisation and polish/germ filtration 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 alpha-amylase 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] certified, and requires that the production of food enzymes complies with EC regulation 852/2004/EC[5], 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 alpha-amylase is a constituent) are tested for food allergenicity risks. 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 alpha-amylase 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 alpha-amylase product with international specifications established by JECFA and Food Chemicals Codex, as well as those in the Code (as applicable). Based on these results, the enzyme preparation meets all relevant specifications.

[4] ISO 9001:2015 – International Standard for Quality Management Systems.
[5] Regulation (EC) No 852/2004 of the European Parliament and of the Council on the Hygiene of Foodstuffs (of 29 April 2004).

Table 2 *Analysis of enzyme alpha-amylase 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)	<0.1, 0.126, <0.1	-	-	≤1
Cadmium (mg/kg)	ND	-	-	≤1
Mercury (mg/kg)	ND	-	-	≤1
Coliforms (cfu/g)	<10	≤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 alpha-amylase in starch processing and the production of potable alcohol is clearly described in the application and is consistent with its typical function of starch hydrolysis. 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 found ubiquitously in the environment (Schuster et al. 2002; Gautam et al. 2011). 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[6], 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).

[6] https://www.cdc.gov/biosafety/publications/bmbl5/index.htm

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 non-essential proteins that would impact the yield and purity of the α-amylase 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

α-amylase gene

Rhizomucor pusillus is a thermophilic filamentous fungus, found commonly in composting and decaying plant material (Richardson 2009). Due to the thermophilic nature of this organism, several enzymes that have potential industrial application have been identified and characterised including amylases, pectinases, proteases, phytases and xylanases (Kanlayakrit et al. 1987; He et al. 2014; Robledo et al. 2016; Singh et al. 2016).

Specific strains of *R. pusillus* have been classed as Biosafety Level 2 organisms, identified as the causative agent in mucormycosis, particular in immunocompromised individuals (Binder et al. 2014; Richardson 2009). The strain used for the source of the α-amylase gene sits with the majority of strains, which are classed as Biosafety Level 1 organisms and are generally not associated with pathogenicity.

The DNA sequence encoding the mature α-amylase was amplified from a cDNA library prepared from the source organism. With subsequent standard cloning methods, there is no risk of carryover of any pathogenic factors from *R. pusillus*.

Regulatory and other genetic elements

Several regulatory elements were obtained from the host *A. niger* strain. These include the promoter and terminator sequences used to drive expression of the introduced α-amylase gene and a signal peptide, linker regions and a starch binding domain, that allow efficient expression and function of the novel enzyme.

A 5'-UTR sequence and selectable marker gene *amdS* was obtained from *Aspergillis nidulans* (Kelly and Hynes 1985). This organism meets the criteria for a Biosafety Level 1 organism and is not associated with disease in healthy human adults but has been associated with infections in immunocompromised individuals (Gabrielli et al. 2014). A further selectable marker gene and associated regulatory elements was obtained from another Biosafety Level 1 organism but the details cannot be disclosed due to CCI. This gene encodes a metabolic marker, not an antibiotic resistance marker.

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 α-amylase (*amyAM782*) and selectable marker genes into the *A. niger* host. The first plasmid contained an expression cassette containing the α-amylase gene from *R. pusillus*, regulatory sequences and a *hemA* gene. This plasmid enabled transformation into a *hemA*$^{-}$ strain. The addition of the *hemA* gene restored 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 the same expression cassette except the *hemA* gene was replaced with the *amdS* gene. Addition of the *amdS* gene allowed 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 introduced the *amyAM782-hemA* plasmid into a *hemA*$^{-}$ recipient strain. After selection of a *hemA*$^{+}$ clone co-expressing α-amylase, the *amyAM782-amdS* plasmid was then introduced. Based on the expression of acetamidase and increased α-amylase activity compared to the previous *hemA*$^{+}$*amdS* $^{-}$ strain, the production strain (667-91-15) was selected.

3.2.2 Characterisation of the inserted DNA

Southern blot analysis, using a probe targeting the *amyAM782* gene, was performed on genomic DNA extracted from 667-91-15 and the host strain. Analysis of the DNA digested with a restriction enzyme showed that the expression cassette is integrated into the genome of 667-91-15.

Real-time quantitative PCR was employed to determine gene copy number in 667-91-15 and confirmed that several copies of the *amyAM782* gene had been inserted. Attempts were made by the applicant using sequencing to confirm the arrangement of the inserted DNA however the presence of repetitive DNA regions prevented full resolution of the sequence information. The data provided however was sufficient to confirm that the *amyAM782* gene has been appropriately inserted and that the inserted DNA is genetically stable (see below).

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 667-91-15 at the end of standard fermentation runs. The results were compared to genomic DNA from a reference stock, most likely an earlier generation master stock. The results showed the same banding patterns across the different fermentation runs and the reference stock, thus indicating the stable integration of the introduced DNA into the host's genome. Morphological characteristics was also compared across the fermentation runs and to the reference stock and no differences were observed.

3.3 Safety of alpha-amylase

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.

Alpha-amylase from *A. niger* was approved for use in Denmark for starch processing, alcohol (distilling), baking and other cereal based processes 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 and Mexico since 2015, although English translations of these approvals were not supplied.

Alpha-amylases from a large number of microbial sources have been widely used in the food industry since the 1960s for the hydrolysis of starch. 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 alpha-amylases 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 alpha-amylase protein to known protein toxins identified in the UniProt[7] database. No noteworthy similarity was found between the alpha-amylase 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 alpha-amylase, PPY31016 in CD rats (Huntingdon Life Sciences Ltd., Study Number LKG0033, 2011) Regulatory status: GLP.

The test article for this study was alpha-amylase, Batch PPY31016. This batch was produced by the 667-91-15 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 11.7% w/w (dry matter content 12.4%). The vehicle and control article was water. Enzyme activity of the dose formulations and Control was determined from 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 13 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 and arena behaviour examination and bodyweight and food consumption measurement.

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, (in the absence of any observed gross differences between Controls and treated groups, quantitative measurement was not undertaken). Food consumption, bodyweight, and behaviour in an arena measurements 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

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

were subject to ophthalmologic examinations in Week 12, 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 all weeks of analysis (Week 1, 6 and 13), the enzyme activity of the 10 mL/kg bw/day formulations (i.e. formulation as supplied) were statistically significantly lower than the expected value (supplied by the sponsor in the Test Substance Data Sheet). However, as the difference from expected did not increase with time (the differences being 8%, 7% and 5% Week 1 to 13 respectively), these differences did not indicate an instability of the test substance. For each week of analysis, inter-group enzyme activity generally reflected the dose-increment, indicating the formulations were suitable for use on study. Enzyme activity was not detected in any of the Control samples.

There were no treatment-related deaths prior to scheduled termination. One female rat in the 10 mL/kg bw/day group was killed in Week 3, due to general poor clinical condition. Assessment of in-life, macroscopic and microscopic data from this rat did not reveal any findings associated with treatment. Treatment with alpha-amylase had no effects of toxicological importance on clinical observations, behaviour in an arena, sensory reactivity tests, grip strength, motor activity measured, bodyweights gains, food consumption, water consumption, ophthalmic findings, haematology, blood chemistry, organ weights or findings on gross necropsy or micropathology.

FSANZ concluded that based on the evaluation above, the highest no observed adverse effect level (NOAEL) in this study was 10 mL/kg bw/day, equivalent to 1.22 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 20108048, 2010). Regulatory status: GLP; conducted in accordance with the general recommendations of OECD Guideline No. 471.

The test article for this assay was alpha-amylase, batch number PPY31016, with a declared dry matter content of 12.4% w/w. The test article contains 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 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 concentrations tested in both 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 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 in plate preparation in the second experiment, toxicity for TA98 with S-9 was determined by examination of background lawn. In the absence of any toxicity, as determined by a standard viability count, in the first experiment with this strain and any evidence of toxicity from examination of the background lawn for the second experiment, this deviation from protocol is not considered to 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 8228052, 2010). Regulatory status: GLP; in accordance with the 2009 draft proposal OECD Guideline 487

This assay was performed using Batch PPY31016, which is alpha-amylase 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 obtained from two healthy non-smoking male donors for each experiment phase (Range-Finder or Micronucleus), were pooled. In all 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 0-7%) of cytotoxicity was observed. On this basis the doses for both the three and 24-hour exposure Micronucleus assays were 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 0-12%). 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 (in June 2018) by comparing the alpha-amylase 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 the alpha-amylase from *A. niger* showed significant similarity, to the alpha-amylases from *Aspergillus oryzae* and the glucoamylase from *Schizophyllum commune* (splitgill mushroom). The similarities were up to 60.3% and 55.2% respectively with E values $<1x10^{-7}$. The preceding values provide some evidence that the *A. niger* alpha-amylase has the potential to be an allergen. However, a search for exact matches of 8 amino acid sequences identified no matches and a full length sequence alignment search revealed protein homology below 35%.

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 considered unlikely that this alpha-amylase will act as an allergen. In addition, the glucoamylase from *S. commune* and the alpha-amylase from *A. oryzae* have been identified as respiratory allergens (Toyotome et al. 2014; Tsuchiya et al. 1993). 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), nor *A. oryzae,* (which has a long safe history of use in food, including in the fermentation process for some traditional Japanese foods including soy sauce, bean paste and sake). In a double blind, placebo-controlled food challenge described in the Poulsen (2004), 18 patients with known respiratory allergy to alpha-amylase from *A. Oryzae* did not show evidence of food allergy to the enzyme.

Respiratory allergens are usually not digestive allergens (Bindslev-Jensen et al. 2006; Poulsen 2004), 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 alpha-amylase 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. The homology therefore between the recombinant alpha-amylase to that of *A. oryzae* or the glucoamylase from *S. commune* is not considered to represent a food allergy concern.

In summary, the alpha-amylase 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 alpha-amylase and the glucoamylase of *S. commune* or the alpha-amylase of *A. oryzae* is not considered to represent a food allergy

concern.

The applicant has indicated that soy and possibly wheat (as a source of starch) are possible ingredients in the fermentation medium. The applicant has stated that the final food will not contain significant residual amounts of these raw materials due to downstream processes such as washing and filtration, which are expected to remove remaining amounts. Testing of product blends which include this alpha-amylase 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 provided (in original languages) copies of “positive lists” entries for the alpha-amylase enzyme for processing aids, from France and Mexico, both dated 2016.

4 Discussion

No public health and safety concerns were identified with the use of alpha-amylase 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 genetically modified production strain 667-91-15 confirmed both presence and stable inheritance of the inserted alpha-amylase gene.

Alpha-amylase from *A. niger* strain 667-91-15 showed no evidence of genotoxicity in a bacterial reverse mutation assay or a micronucleus assay in human lymphocytes. Alpha-amylase 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 1220* mg/kg bw/day on a TOS basis. The applicant’s estimated theoretical maximal daily intake (TMDI) of alpha-amylase is 2.86 mg/kg bw/day TOS, resulting in a Margin of Exposure (MoE) of 427 between the NOAEL and TMDI.

Bioinformatic analyses did not identify any homology with any known toxins but did show a degree of homology between the recombinant alpha-amylase to that of two respiratory allergens. However, further analysis indicated that the alpha-amylase from *R. pusillus* expressed in *A. niger* is unlikely to be a food allergy concern.

Soy and possibly wheat are used in the fermentation medium, however due to washing and filtration processed they are not expected to be present in the final product.

> * This value was calculated based on the nominal enzyme activity of 53.3 FAU(F)/g, however the in-study dose formulation check revealed FAU activity for the high level to be up to 8% lower than expected. When considering this slightly lower than expected enzyme activity, the NOAEL would be 1122 mg/kg bw/day on a TOS basis. Which in turn would give a MoE of 392. As the MoE value is still considered to provide a large margin of safety, the slightly lower enzyme activity does not adversely affect the outcome of the study.

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.

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