25 September 2020
[135–20]

Supporting document 1

Risk and technical assessment report – Application A1195

Alpha-amylase from GM *Trichoderma reesei* as a PA (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 *Trichoderma reesei.* This production organism contains the alpha-amylase gene from *Aspergillus kawachii.* Alpha-amylase is proposed as a processing aid in brewing 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 France, Denmark, as well as the USA.

The safety assessment concluded that the use of the enzyme under the proposed conditions is safe. The host is neither pathogenic nor toxigenic and analysis of the GM production strain, which has a history of safe use, confirmed presence and stability of the inserted DNA.

Alpha-amylase from *T. reesei* LOH4AkAApaA 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, 229.6 mg/kg bw/day on a total organic solids (TOS) basis. The TOS will be removed from potable alcohol by the distillation process, but dietary exposure to the enzyme may arise from use in brewing processes. The applicant's estimated theoretical maximal daily intake (TMDI) based on the proposed use in brewing is 0.39 mg/kg bw/day TOS. A comparison of these values indicates that the Margin of Exposure between the NOAEL and TMDI is more than 500.

Bioinformatic analysis indicated that the enzyme shows no significant homology with any known toxins. However a degree of homology between the recombinant alpha-amylase and several respiratory allergens was found, including an alpha-amylase from *A. oryzae* which has been implicated in three case reports of food allergy, but not in other food challenge studies with sensitised individuals. Taking into account that respiratory allergens are usually not food allergens, the very low number of case reports of food allergy to alpha-amylase from *A. oryzae* compared with its widespread use in food, and the low levels expected to be

present in final food products, the risk of food allergy from the proposed uses of the enzyme is likely to be low.

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

Danisco New Zealand Ltd applied to FSANZ for permission to use the enzyme alpha-amylase (EC 3.2.1.1) as a processing aid in brewing and the production of potable alcohol. This alpha-amylase is from a genetically modified (GM) strain of *Trichoderma reesei,* containing the alpha-amylase gene from the fungus *Aspergillus kawachii.*

There are permissions for alpha-amylase from GM and non-GM microbial sources in the Code, however, not when produced from this particular source. If permitted following a pre-market assessment, Danisco's alpha-amylase will provide an additional option for manufacturers of brewed alcohol products and 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.

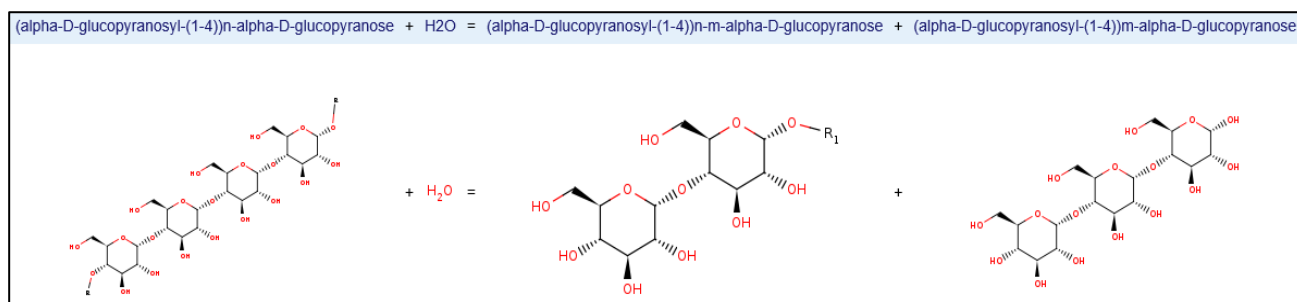# 2 Food technology assessment

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: alpha-amylase, glycogenase; α amylase; endoamylase; Taka-amylase A; 1,4-α-D-glucan glucanohydrolase

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 (Figure 1).

(alpha-D-glucopyranosyl-(1-4))n-alpha-D-glucopyranose + H2O = (alpha-D-glucopyranosyl-(1-4))n-m-alpha-D-glucopyranose + (alpha-D-glucopyranosyl-(1-4))m-alpha-D-glucopyranose

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 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

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[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.
[3] Chemical Abstracts Service.

of starch polymers (Poulson 1983; Reichelt 1983; Damodaran et al. 2008).

The function of alpha-amylase is to catalyse endohydrolysis of (1→4)-α-D-glucosidic linkages in polysaccharides containing three or more (1→4)-α-linked D-glucose units. Alpha-amylase is used to maximize the conversion of starchy substrates to fermentable carbohydrate. It will be used in the mashing of malted cereal and other plant sources such as barley, maize, wheat, rye, sorghum, rice, tapioca and potatoes.

In brewing, alpha-amylase is used during the mashing stage of malted cereal, cereal and other plant sources including barley, maize, wheat, rye, sorghum, rice, tapioca and potatoes. It maximises liquefaction of starchy substrates and subsequent conversion to fermentable carbohydrates, providing increased productivity, decreasing mash viscosity and hereby fouling during cereal cooker operations. The resultant worts are fermented, typically by yeast, to produce ethanol. Use of alpha-amylase also provides potential for higher alcohol yield and aids in the removal of starch related beer haze.

During potable alcohol production, alpha-amylase aids the conversion of liquefied starch into a maltose rich solution, increasing the percentage of fermentable sugars. Furthermore, it allows lower pH and temperature parameters during processing, a reduction in raw material use and increased solids concentration during mashing, all of which create efficiencies whilst providing potential for higher alcohol yield. 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	10000-13000 SSU*/g 11970 avg from 3 batch results provided
Appearance	Brown liquid
Temperature range	activity within range 60-70°C optimum 65-70°C
Temperature stability	Significant activity for 20min @ up to 70°C. 10% residual activity after approx. 20 min at 85°C
pH range	Max activity within range 2.0 – 4.0
Storage stability	approx. 90% remaining activity when stored for 24 months @ 4°C, approx. 92% @ ambient temp for 12 months

*Soluble Starch Unit/g (SSU/g). One SSU is defined as the amount of enzyme required to release 1 lmol of p-nitrophenol per minute from the non-reducing-end blocked p-nitrophenyl maltoheptaoside substrate at pH 4.5, 30°C, for 5 min.

Use of commercial enzyme preparations should follow Good Manufacturing Practice (GMP), where use is at a level that is not higher than that necessary to achieve the desired enzymatic reaction.

The conditions of use of the enzyme during alcohol production 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

Table 2 *Indicative use levels of alpha-amylase*

Application	Raw Material (RM)	Recommended usage levels (mg *TOS/kg RM)	Maximum recommended levels (mg TOS/kg RM)
Brewing	Cereal	37-371	371
Potable alcohol production	Cereal	12-116	116

*To distinguish the proportion of the enzyme preparation derived from the source material and manufacturing process from that contributed by intentionally added formulation ingredients, the content of total organic solids (TOS) is calculated as follows: % TOS = 100 - (A + W + D) where: A = % ash, W = % water and D = % diluents and/or other formulation ingredients.

2.1.3 Technological justification for the enzyme

As outlined above, the application suggests that the enzyme fulfils an important technological purpose and provides the alcoholic beverage industry with an opportunity to improve the consistency of their products, gain processing efficiencies and any cost savings associated with this. Benefits may also include increased flexibility in the choice of and a reduction in the use of raw materials used and also waste reduction.

2.2 Manufacturing process

2.2.1 Production of the enzyme

The enzyme is produced by fermentation of a GM strain of *T. reesei* via a three-part process consisting of fermentation, recovery through separation of cell mass from the enzyme followed by concentration/purification, formulation and drying, to form the stable enzyme.

The raw materials used in the fermentation and recovery process are standard ingredients used in the enzyme industry. Most raw materials conform to the specifications of the Food Chemical Codex, 6th edition (FCC 2008) and for those which do not appear in the FCC, Danisco has internal requirements in line with FCC requirements and has in place a supplier quality program. Danisco manufacture their alpha-amylase in accordance with food good manufacturing practice (GMP) with the resultant product meeting the general requirements for enzyme preparations of the Food Chemicals Codex, Sixth Edition (FCC 2008) and the General Specifications for Enzyme Preparations Used in Food.

Full details on the manufacturing process, raw materials and ingredients used in the production of Danisco's alpha-amylase were provided as "Confidential Commercial Information".

2.2.2 Allergen considerations

Glucose syrup is used as source material for the fermentation and to a greater extent as an excipient in the enzyme preparation. This may be sourced from wheat on occasion according to Danisco. In the unlikelihood that any wheat protein from glucose syrup was present in the enzyme preparation and considering the low usage rate of enzymes in a properly controlled potable alcohol distillation process, wheat proteins or peptides would not be carried over into the distillate (EFSA 2007, Cressey 2010).

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.

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

Analysis	Danisco analysis	Specifications		
		JECFA	*Food Chemicals Codex*	*Australia New Zealand Food Standards Code (section S3—4)*
Lead (mg/kg)	<.05, 0.01, <.05	≤ 5	≤ 5	≤2
Arsenic (mg/kg)	<0.1, 0.06, <0.1	-	-	≤1
Cadmium (mg/kg)	<0.01 (x3)	-	-	≤1
Mercury (mg/kg)	<0.01 (x3)	-	-	≤1
Coliforms (cfu/g)	<10, <5, <1	≤30	≤30	-
Salmonella (in 25 g)	Negative	Absent	Negative	-
E. coli (in 25 g)	Negative	Absent	-	-
Antimicrobial activity	Negative	Absent	-	-

2.3 Food technology conclusion

FSANZ concludes that the use of this alpha-amylase in brewing 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

T. reesei is a common, hypercellulolytic, soil fungus that was initially isolated from deteriorating canvas made from cellulosic material. The original isolate QM6a is the type strain for *T. reesei* (Olempska-Beer *et al.,* 2006) and has been registered with the American Type Culture Collection under ATCC13631. Due to the secretion of a range of cellulolytic enzymes, this fungus has been used since the 1980s for the industrial production of enzymes for a range of industries including food (Nevalainen and Peterson, 2014; Paloheimo et al., 2016).

FSANZ has previously assessed the safety of *T. reesei* as the source organism for several enzymes used as processing aids. The production strain in this application (LOH4kAApaA) is derived from *T. reesei* strain RL-P37, which is in turn derived from QM6a, and was previously confirmed as *T. reesei* in application A1174 (FSANZ 2020). This confirmation was based on 100% DNA sequence identity between RL-P37 and the *T. reesei* type strain ATCC13631 at specific conserved DNA regions.

In humans, *T. reesei* is not pathogenic and meets the requirements of a Biosafety Level 1 organism based on the *Biosafety in Microbiological and Biomedical Laboratories*[4] guidelines. Although some *T. reesei* strains can produce mycotoxins, most industrial production strains do not produce mycotoxin or antibiotics under conditions used for enzyme production (Nevalainen *et al.,* 1994; Blumenthal 2004).

3.1.2 Gene donor organisms

Aspergillus kawachii

The gene for alpha-amylase enzyme was isolated using PCR from *A. kawachii* genomic DNA. This organism is a filamentous fungus that is used for brewing shōchū, the traditional Japanese distilled spirit (Futagami et al., 2011; Nagamine et al., 2003). This organism meets the criteria for a Biosafety Level 1 agent and is not associated with disease in healthy human adults.

Regulatory and other genetic elements

Several elements were obtained from the host *T. reesei* strain. These include the promoter and terminator sequences used to drive expression of the introduced alpha-amylase gene. The orotate phosphoribosyl transferase (*pyr2*) gene from *T. reseei* was used as a selectable marker during transformation.

The selectable marker gene for acetamidase (*amdS*) was isolated from *Aspergillus nidulans* (Kelly and Hynes, 1985). This organism meets the criteria for a Biosafety Level 1 agent not associated with disease in healthy human adults but has been associated with infections in immunocompromised individuals (Gabrielli et al, 2014). Its inclusion is regarded as 'mainstream' in filamentous fungi systems involving recombinant gene expression (Gryshyna et al., 2016) and there are no safety concerns with the protein product acetamidase.

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[4]https://www.cdc.gov/labs/BMBL.html?CDC_AA_refVal=https%3A%2F%2Fwww.cdc.gov%2Fbiosafety%2Fpublications%2Fbmbl5%2Findex.htm

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 alpha-amylase (*AkAA*) and selectable marker genes into the *T. reesei* host. The first plasmid contained an expression cassette containing the alpha-amylase gene from *A. kawachii*, regulatory sequences and the *pyr2* gene. This plasmid enabled transformation into a *pyr2*⁻ strain. The addition of the *pyr2* gene restored the enzyme function, allowing for selection of positive transformants by growth on minimal media devoid of uridine (Jørgensen et al., 2014).

The second plasmid contained the same expression cassette except the *amdS* gene replaces the *pyr2* gene. The *amdS* gene allowed for selection of positive transformants by growth on media containing acetamide (Kelly and Hynes 1985).

The two expression cassettes were introduced sequentially using protoplast-mediated transformation (Li et al. 2017). Expression cassettes were either digested from the plasmids and gel purified or amplified directly from the plasmids by PCR. The first transformation step introduced the *AkAA-pyr2* expression cassette into a *pyr2*⁻ recipient strain. After selection of a *pyr2*⁺ clone co-expressing alpha-amylase (first transformant), the *AkAA-amdS* expression cassette was then introduced. The production strain (LOH4AkAApaA) was selected based on acetamidase expression, the highest levels of alpha-amylase and a lack of bacterial DNA.

3.2.2 Characterisation of the inserted DNA

Southern blot analysis, using a probe targeting the AkAA DNA sequence, was performed on genomic DNA extracted from the first transformant, production strain and parental strain. Analysis of the DNA digested with two individual restriction enzymes showed that multiple copies of the *AkAA-pyr2* and *AkAA-amdS* expression cassettes were integrated into the genome of the LOH4AkAApaA production strain.

Hybridisation with a probe targeting the vector backbone confirmed its absence from the production strain's genome, including antibiotic resistance markers used when passaging the expression vectors in *E. coli.*

Attempts were made by the applicant using next generation sequencing to further characterise the inserted DNA, however limitations in the sequencing technique prevented the resolution of multiple inserted copies. The data provided however was sufficient to confirm that the arrangement of the DNA within one copy of the *AkAA-pyr2* and *AkAA-amdS* expression cassettes was as expected.

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

The stability of the introduced DNA in the production strain was examined by next generation sequencing over a number of generations. Genomic DNA was extracted from a stock culture and at the end of three fermentations. Two integration sites were examined, one for the AkAA-pyr2 expression cassette and the other for the AkAA-amdS expression cassette. No changes were observed at the integration sites between the stock culture and different fermentation samples. It can be concluded that the introduced DNA has been stably integrated into the host's genome.

3.3 Safety of alpha-amylase

3.3.1 History of safe use

Alpha-amylase produced by GM *T. reesei* LOH4AkAApaA containing the alpha-amylase gene from *A. kawachii* has been approved in France and Denmark.

The enzyme was self-assigned generally recognised as safe (GRAS) status as a food processing aid in manufacture of glucose and other starch syrups, and in brewing and potable alcohol/fuel ethanol manufacture processes in the USA. A GRAS notice has not been submitted to the US Food and Drug Administration (FDA).

Several alpha-amylase enzymes from other microbial sources and from malted cereals are currently permitted as processing aids in Schedule 18 of the Code.

3.3.2 Bioinformatic assessment of enzyme toxicity

A BLAST search for homology of the alpha-amylase amino acid sequence against the UniProt Protein Knowledge database was performed with a threshold E-value of 0.1. The majority of matches were alpha-amylase, with none of the top 1000 database matches being annotated as a toxin or venom.

3.3.3 Toxicology studies in animals

90-day repeated dose oral toxicity study in rats (LAB Research [Scantox] 2010) Regulatory status: GLP; Conducted in accordance with OECD Test Guideline (TG) 408 (1998)

The alpha-amylase test item in this study was an ultra-filtered concentrate. Sprague Dawley rats 10/sex/group, approximately 6 weeks old) were administered alpha-amylase at doses of 0, 46, 92 or 184 mg/kg bw/day total protein by oral gavage for 13 weeks. Doses were equivalent to 0, 57.4, 114.8 or 229.6 mg/kg bw/day total organic solids (TOS). The vehicle and negative control was 0.9% saline. Clinical signs were monitored daily with more detailed observations performed weekly. A functional observation battery was performed at termination. Body weight, food and water consumption were monitored at regular intervals. Ophthalmoscopy was performed before the start of treatment and before termination. Blood and urine were collected from all animals during week 13 for evaluation of clinical chemistry and haematology parameters. At necropsy a macroscopic evaluation was performed on all animals, organ weights were recorded and selected organs and tissues were fixed and examined.

No test item-related clinical signs of toxicity were observed during the study. No treatment-related adverse effects were observed in the functional observation battery. No treatment-related effects on body weight and body weight gain, food consumption, water consumption, ophthalmoscopy, haematology, clinical chemistry, urinalysis or organ weights were observed. No treatment-related macroscopic or histopathological changes were reported. It was concluded that the no observed adverse effect level (NOAEL) in this study was 184 mg/kg bw/day total protein, the highest dose tested. The NOAEL corresponded to 229.6 mg/kg bw/day on a TOS basis.

3.3.4 Genotoxicity assays

Two *in vitro* genotoxicity studies were submitted, a bacterial reverse mutation assay (Ames test) and a chromosomal aberration test in human lymphocytes. The test item used in these studies was the same as that used in the 90-day oral toxicity study.

Bacterial reverse mutation assay (LAB Research [Scantox] 2010) Regulatory status: GLP; conducted in accordance with OECD TG 471 (1997)

Test systems for the assay were *Salmonella typhimurium* strains TA102, TA100, TA98, TA1537 and TA1535. Following a preliminary toxicity assay with strain TA98, concentrations of 50, 160, 500, 1600 and 5000 µg total protein/plate were used in two independent main tests, both conducted in the presence and absence of metabolic activation (S9 mix). The treat and plate method was used in each test to minimise the possibility that histidine in the test item might compromise the test. The vehicle and negative control was sterile saline solution. The positive control in the presence of metabolic activation was 2-aminoanthracene for all strains. In the absence of metabolic activation, positive controls were cumene hydroperoxide for TA102, sodium azide for TA100 and TA1535, 2-nitrofluorene for TA98 and 9-aminoacridine for TA1537. All assays were conducted in triplicate.

No cytotoxicity was observed following treatment with the test item. No biologically significant increases in the number of revertant colonies were observed following treatment with the test item compared with vehicle controls, in the presence or absence of metabolic activation. Substantial increases in revertant colonies were observed following treatment with positive controls, confirming the validity of the assays.

It was concluded that alpha-amylase was not mutagenic in the Ames test under the conditions of the study.

Chromosome aberration assay in cultured human lymphocytes (LAB Research [Scantox] 2010) Regulatory status: GLP; conducted in accordance with OECD TG 473 (1997)

The test system was cultured human peripheral lymphocytes. Two experiments were conducted. In experiment I cells were exposed to the test substance in the presence or absence of metabolic activation (S9 mix) for 3 hours. In experiment II cells were exposed to the test substance for 18 hours without S9 and 3 hours in the presence of S9. Concentrations up to 5000 µg total protein/mL were tested in all experiments. All cultures were harvested 18 hours after the start of treatment. Cells were cultured in duplicate. Two hours prior to harvest, demecolcin was added to the cultures. Positive controls were cyclophosphamide and daunomycin in the presence and absence of metabolic activation, respectively. At harvest cells were fixed on slides, stained and scored for chromosomal aberrations. Approximately 100 metaphases per culture (i.e. 200 per concentration) were evaluated for the presence of chromosomal aberrations, with 1000 cells per culture counted for determination of the mitotic index. The numbers of polyploidy and endoreduplicated metaphases in 200 metaphases were also counted for each culture. The Test Guideline for this study has subsequently been updated to require scoring of at least 300 metaphases per concentration for aberrations, but the study was compliant with the previous version of the Guideline.

The test item did not cause marked cytotoxicity at any concentration tested. A statistically significant increase in the number of cells with chromosomal aberrations compared with negative controls was seen in the first run of experiment I with S9 at 5000 µg/mL, and in the first run of experiment II without S9 at 1250 µg/mL. However, both of these increases only occurred in one of the two replicate cultures. In addition, the first run of experiment II without S9 was not valid because three of the four positive control treatments did not produce adequate increases in the frequency of aberrant metaphases. Both experiments were therefore repeated.

The repeated experiments I and II were considered valid as all controls performed as expected. No significant increases in the frequency of metaphases with chromosomal aberrations were observed in cultures treated with alpha-amylase in the presence or

absence of S9 in either of the repeat tests.

It was concluded that alpha-amylase did not cause chromosomal aberrations in cultured human lymphocytes under the conditions of this study.

3.3.5 Potential for allergenicity

The applicant performed several searches for homology of the amino acid sequence of the alpha-amylase enzyme to known allergens using the [Allergen Online database](#) of the University of Nebraska's Food Allergy Research and Resource Program (FARRP). The following searches were conducted:

- A search for full-length sequence alignment for matches of > 35% identity
- A search using a sliding window of 80 amino acid stretches for identities > 35%
- A search for exact matches of 8 contiguous amino acids

The search for full-length sequence alignment found two matches with more than 35% identity to the alpha-amylase sequence (both 67.5% identity): a fungal alpha-amylase A type 1/2 precursor and a Taka-amylase A precursor, both from *Aspergillus oryzae*, also known as Asp o 21 and Asp o 21.0101, respectively. Matches to these allergens were also identified in the searches for 80 amino acid stretches with > 35% identity to known allergens and for exact matches of 8 contiguous amino acids.

Both of these matches are listed as airway allergens in the FARRP database and the WHO/IUIS Allergen Nomenclature Database. Alpha-amylase from *A. oryzae* is widely used as a processing aid in bakeries and is also used in the pharmaceutical industry. It is known to cause respiratory allergies such as Baker's asthma in occupationally exposed workers (Brisman and Belin 1991, Quirce *et al.* 1992). Workers with Bakers asthma can usually tolerate bread consumption, however (Armentia *et al.* 2009).

A small number of case reports were identified which suggest a link between alpha-amylase from *A. oryzae* and food allergy. Three individuals have been reported to have allergic reactions to bread containing alpha-amylase from *A. oryzae* (Asp o 2) (Baur *et al.* 1995, Kanny and Moneret-Vautrin 1995, Moreno-Ancillo *et al.* 2004). Symptoms reported included sneezing, cough and oral angioedema, wheezing, urticaria, rhinitis and conjunctivitis. An allergic reaction was also reported in a fourth individual following an oral challenge with the enzyme, but this person was able to ingest bread and other cereal products without any symptoms (Losada *et al.* 1992).

Sensitisation reactions to alpha-amylase from another fungal source used in food production (*Bacillus subtillus*) were tested in two of these patients and found to be absent, suggesting that the reaction was specific to alpha-amylase from *A. oryzae* (Kanny and Moneret-Vautin 1995; Moreno-Ancillo et al 2004). Similarly, Losada *et al.* reported that subjects occupationally sensitised to alpha-amylase from *A. oryzae* showed no evidence of sensitisation to alpha-amylase from pig pancreas.

Alpha-amylase from *A. oryzae* and other microbial sources have a long history of use in food, but no further case reports of food allergy have been identified since that of Moreno-Ancillo et al. in 2004. Other studies have found no food allergy reactions to alpha-amylase from *A. oryzae* and other microbial sources. Double blind placebo controlled food challenges have found no evidence of food allergy to bread containing alpha-amylase from *A. oryzae* in a group of 18 patients with respiratory allergy to the enzyme (Poulsen 2004) or in persons sensitised to *A. fumigatus* (Cullinan *et al.* 1997). No food allergy responses to 19 microbially-derived food enzymes, including alpha-amylases from *A. oryzae* and four other sources, were found in a study of 400 individuals with allergy to inhalation, food or other allergens

(Bindslev-Jensen et al. 2006).

Overall, the evidence indicates that food allergy to alpha-amylase from *A. oryzae* is very rare and these allergens are not listed as food allergens in the WHO/IUIS Allergen Nomenclature Database. Alpha-amylase from *T. reesei* will be removed from potable alcohol during the distillation process. For brewing uses, the enzyme is expected to be denatured by heat during wort boiling. The risk of food allergy from consumption of alpha-amylase from *T. reesei* is therefore considered to be low.

The search for 80 amino acid stretches within the sequence with greater than 35% identity to known allergens found two additional matches, one of which was also found in the 8 amino acid search. Neither of these two additional matches are listed as food allergens in the FARRP Allergen Online database or the WHO/IUIS Allergen Nomenclature database: both are listed as airway allergens. Respiratory allergens are not usually food allergens (Poulsen 2004, Bindslev-Jensen et al. 2006), and no reports of food allergy responses to these allergens were identified.

3.3.6 Approvals by other regulatory agencies

The applicant has provided documentation indicating that the enzyme preparation is approved in France and Denmark.

The safety of alpha-amylase from the same production strain was evaluated by the European Food Safety Authority (EFSA) in 2018. EFSA concluded that no safety issues were identified with the use of the enzyme in distilled alcohol production and brewing processes. EFSA noted that the risk of allergic sensitisation and elicitation reactions upon dietary exposure to the enzyme cannot be excluded, but the likelihood was considered low (EFSA 2019).

4 Discussion

The safety assessment concluded that the use of alpha-amylase from *T. reesei* LOH4AkAApaA as a food processing aid at GMP levels in brewing and potable alcohol production is safe.

T. reesei has a long history of safe use as a source of enzyme processing aids, including several that are already permitted in the Code. This fungus is neither toxigenic nor pathogenic. Characterisation of the GM production strain (LOH4AkAApaA) confirmed both presence and stable inheritance of the inserted alpha-amylase gene.

Alpha-amylase from *T. reesei* LOH4AkAApaA showed no evidence of genotoxicity in a bacterial reverse mutation assay or a chromosomal aberration assay in human lymphocytes. The enzyme did not cause adverse effects in short-term toxicity studies in rats. The NOAEL in a 90-day repeated dose oral toxicity study in rats was the highest dose tested, 229.6 mg/kg bw/day on a TOS basis. The TOS will be removed from potable alcohol by the distillation process, but dietary exposure to the enzyme may arise from use in brewing processes. The applicant's estimated theoretical maximal daily intake (TMDI) based on the proposed use in brewing is 0.39 mg/kg bw/day TOS. A comparison of these values indicates that the Margin of Exposure between the NOAEL and TMDI is more than 500.

Bioinformatic analysis indicated that the enzyme shows no significant homology with any known toxins. However a degree of homology between the recombinant alpha-amylase and several respiratory allergens was found, including an alpha-amylase from *A. oryzae* which has been implicated in three case reports of food allergy, but not in other food challenge studies with sensitised individuals. Taking into account that respiratory allergens are usually

not food allergens, the very low number of case reports of food allergy to alpha-amylase from *A. oryzae* compared with its widespread use in food, and the low levels expected to be present in final food products, the risk of food allergy from the proposed uses of the enzyme is likely to be low.

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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