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

21 November 2019
[102-19]

Supporting document 1

Risk and technical assessment report – Application A1182

Glucose Oxidase from a GM *Trichoderma reesei* as a Processing Aid (enzyme)

Executive summary

AB Enzymes GmbH submitted an application to Food Standards Australia New Zealand (FSANZ) seeking to permit a glucose oxidase from genetically modified (GM) *Trichoderma reesei* for use as a processing aid in baked products, cereal based products and egg processing. The *glucose oxidase gene* was derived from *Penicillium amagasakiense*.

The food technology assessment concluded that glucose oxidase, in the form and prescribed amounts, is technologically justified and has been demonstrated to be effective in achieving its stated purpose. Glucose oxidase performs its technological purpose during the processing and production of foods and is therefore appropriately categorised as a processing aid. The enzyme preparation meets international purity specifications.

The safety assessment of glucose oxidase from a GM strain of *T. reesei* concluded that there were no public health and safety concerns. The host is neither pathogenic nor toxigenic and has a history of safe use as the production strain for many food enzymes. Analysis of the GM production strain confirmed the presence and stability of the inserted DNA.

Glucose oxidase from GM *T. reesei* 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, 1000 mg/kg bw/day or 915 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 0.088 mg/kg bw/day TOS. A comparison of these values indicates that the Margin of Exposure between the NOAEL and TMDI is more than 10,000.

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. Lactose is used in the fermentation medium, however analysis of three batches of the dried enzyme concentrate indicated that lactose and casein levels were below the limit of detection (100 mg/kg and 0.25 mg/kg, respectively). Wheat flour is an ingredient in the enzyme preparation.

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.

There are no public health and safety concerns associated with the use of glucose oxidase from GM *T. reesei* when used as a food processing aid at GMP levels in baked products, cereal based products and egg processing.

Table of contents

1 Introduction

AB Enzymes GmbH submitted an application to Food Standards Australia New Zealand (FSANZ) seeking to permit a glucose oxidase (E.C. 1.1.3.4) enzyme preparation from a genetically modified (GM) *Trichoderma reesei*. This enzyme preparation will be used as a processing aid in baked products, cereal based products and egg processing.

Glucose oxidase will be used as a processing aid at a level consistent with Good Manufacturing Practice (GMP) and provides no technical function in the final food

There are two permissions for glucose oxidase as a processing aid in the Australia New Zealand Food Standards Code (the Code), however there is no permission for glucose oxidase sourced from a GM strain of *T. reesei.* Therefore, an application to amend the Code to permit the use of this enzyme as a food processing aid requires a pre-market safety assessment.

1.1 Objectives of the assessment

The objectives of this risk and technical assessment report were to:

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

- evaluate potential public health and safety risks that may arise from the use of glucose oxidase when used as a processing aid in cereal based products (baking) and egg.

2 Food technology assessment

2.1 Characterisation of the enzyme

2.1.1 Identity of the enzyme

Accepted name:	Glucose oxidaseα
Common Names:	β-D-glucose oxidase, β-D-glucose: quinone oxidoreductase, D-glucose oxidase, D-glucose-1-oxidase, glucose oxyhydrase; deoxin-1, glucose aerodehydrogenase, aero-glucose dehydrogenase, glucose oxyhydrase, Notatin , corylophyline; penatin
IUBMB[1]/EC[2] number:	1.1.3.4
CAS registry number[3]:	9001-37-0
Enzyme preparation description	A solid product, light beige in colour with an aromatic odour, comprised of wheat flour, glucose oxidase and sunflower oil

Information regarding the identity of the enzyme provided in the application has been verified using the appropriate internationally accepted reference for enzyme nomenclature, the International Union of Biology and Molecular Biology (IUBMB 2018).

2.1.2 Technological purpose of the enzyme

Baked Products: Glucose oxidase can be used in the manufacturing of cereal based products such as, but not limited to, bread, steamed bread, biscuits, cakes, pancakes, croissants, tortillas, wafers and waffles, pastas, noodles and snacks. When used as such, it generates gluconic acid which acts as a chemical leavening agent (Damodaran et al, 2008, Ramachandran et al. 2006) and strengthens the protein complexes contained in these products. This is achieved through increased formation of cross-links between proteins, thereby improving the quality of such foods. (Bonet et al. 2006, Rasiah et al. 2005). In summary, glucose oxidase has the following benefits for the manufacture of baked goods:

- Improves the handling of the dough during processing
- Reduces dough stickiness and improves dough machinability
- Improves the rheological properties of dough during shaping or moulding
- Ensures a uniform volume, an improved and more uniformed crumb structure and crust formation

A typical process for use of glucose oxidase in the baking process is shown in Figure 1.

[1] International Union of Biochemistry and Molecular Biology.
[2] Enzyme Commission, internationally recognised number that provides a unique identifier for enzymes
[3] Chemical Abstracts Service Registry Number, internationally recognised number that provides a unique identifier for organic and inorganic chemical substances

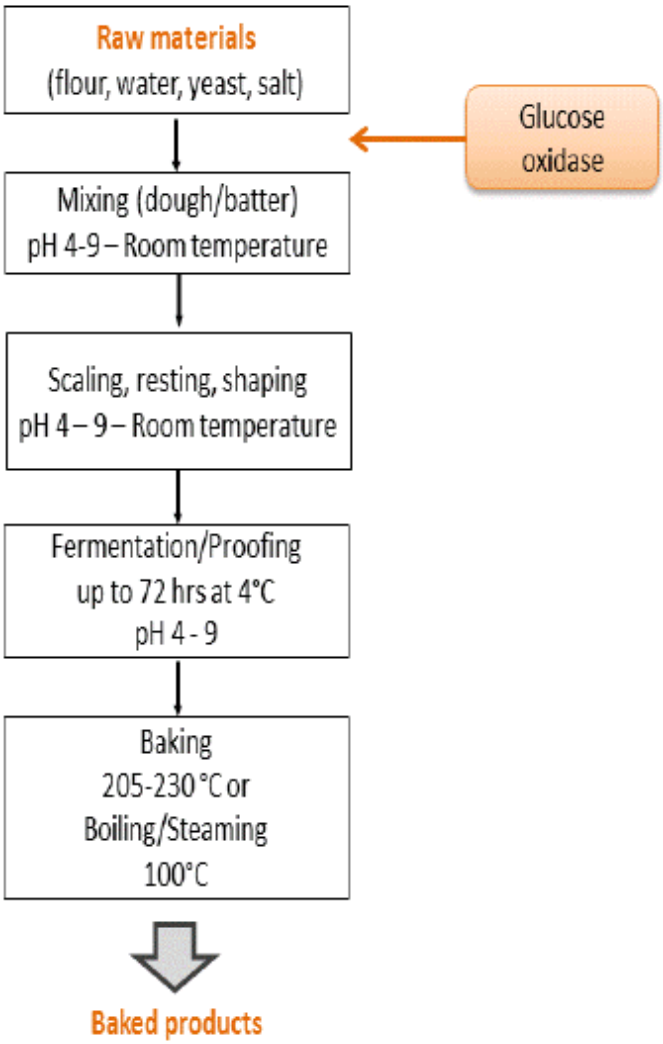


Figure 1 Typical use of glucose oxidase in baking

Pasta and Noodle making: Because gluten has a predominant role in the pasta structure, the use of glucose oxidase increases the gluten protein networks' resistance to cooking thereby decreasing surface stickiness (Kruger et al 1987; Fuad, Tina, Prabhasankar 2010).

The Applicant indicated that when utilised for the manufacture of pasta and noodles, and in snack making, glucose oxidase performs its function during dough handling and provides the following benefits:

- Facilitates the handling of the dough
- Reduces dough stickiness
- Strengthens dough structure
- Improves cooking properties of pasta and noodles
- Provides a firmer bite and better texture to pasta and noodles
- Reduces product variation, ensuring uniform/standardised quality products.

A typical process for use of glucose oxidase in pasta and noodle making is shown in Figure 2.

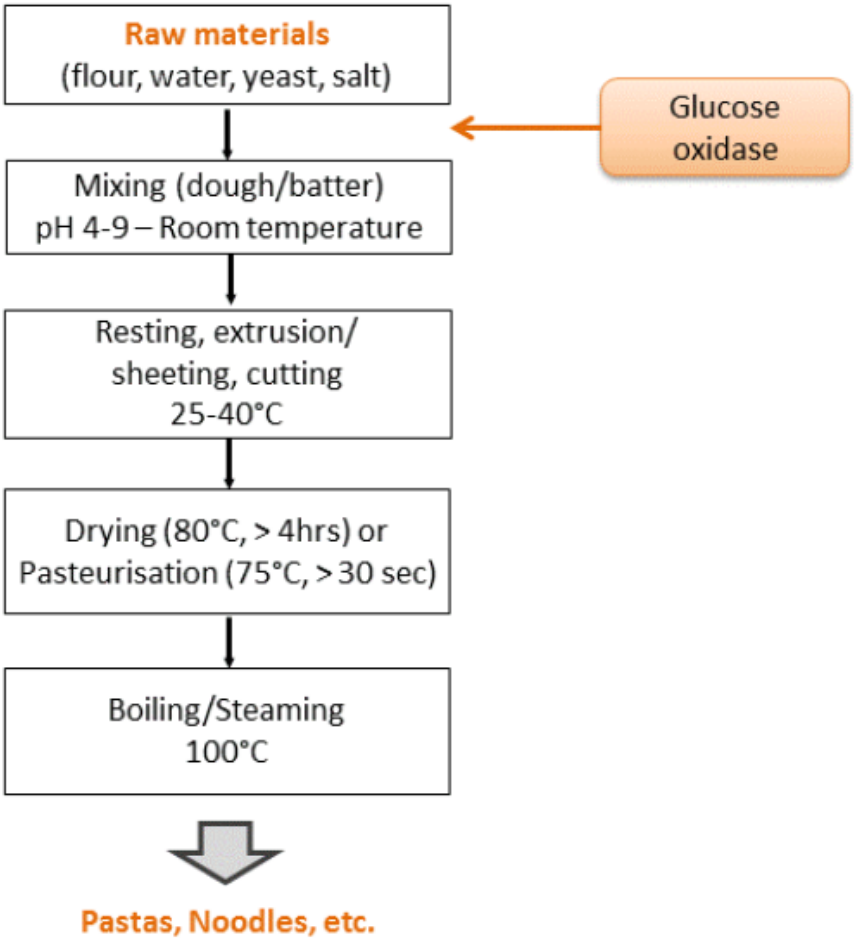


Figure 2 Typical use of glucose oxidase in pasta and noodle making

Egg Processing: Glucose oxidase is also utilised in the processing of liquid egg-white, liquid whole egg or liquid yolk, destined for spray drying. It depletes the processed liquid egg products' of glucose, thereby reducing the potential for the Maillard reaction during dehydration and storage (Damodaran et al, 2008). In the Maillard reaction, glucose and amino acids react together to give unstable compounds which react further, producing coloured, off-flavoured and insoluble products. Therefore, it is necessary for the glucose present in liquid egg to be removed before spray drying. A typical process for use of glucose oxidase in egg processing is shown in Figure 3.

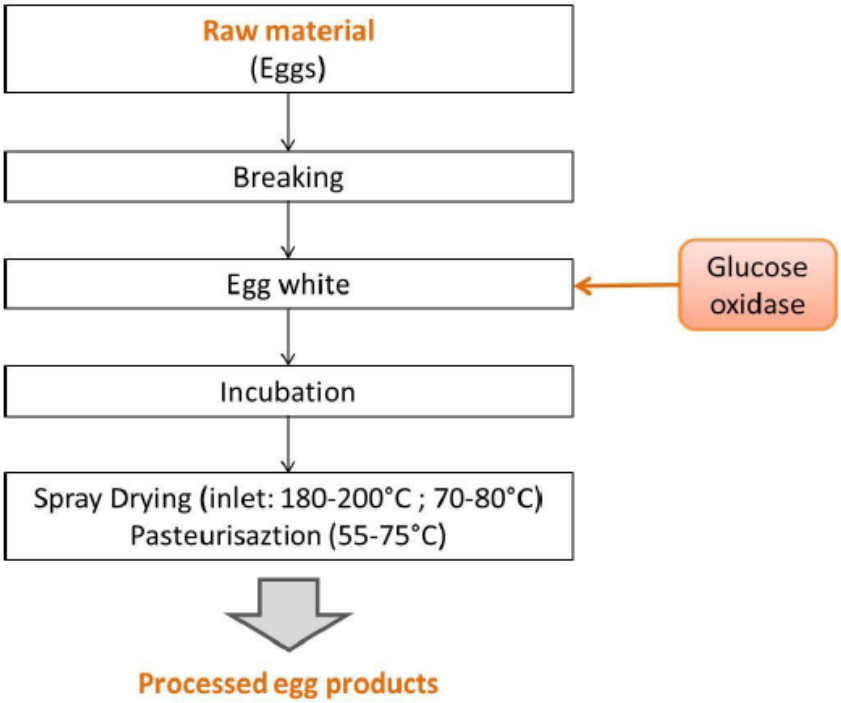


Figure 3 Typical use of glucose oxidase in egg processing

Glucose oxidase is denatured by heat during the spray drying, boiling or steaming steps and therefore does not perform a technological function in the final food.

2.1.3 Technological justification of the enzyme

There are currently permissions for α-glucosidase (EC 1.1.3.4) in S18—4(5), to be used in the manufacture of all foods. However, glucose oxidase from this particular microbial source, is not currently permitted.

Glucose oxidase is not normally present in the raw materials used in baking and therefore needs to be added to provide functionality. AB Enzymes claims that, based on market benchmarking, their glucose oxidase has superior technical characteristics, specifically tolerance to withstand mechanical shock during processing. This provides an improvement in quality for bakery products. There is also a cost benefit associated with the use of AB Enzyme's glucose oxidase which they claim results in a cost benefit that is passed on to those using the enzyme.

2.1.4 Reaction type

Glucose oxidase catalyses the oxidation of β-D-glucose to D-glucono-1, 5-lactone and the reduction of oxygen to hydrogen peroxide. In the presence of water, D-glucono-1,5 -lactone is hydrolysed to gluconic acid (*Figure 4*).

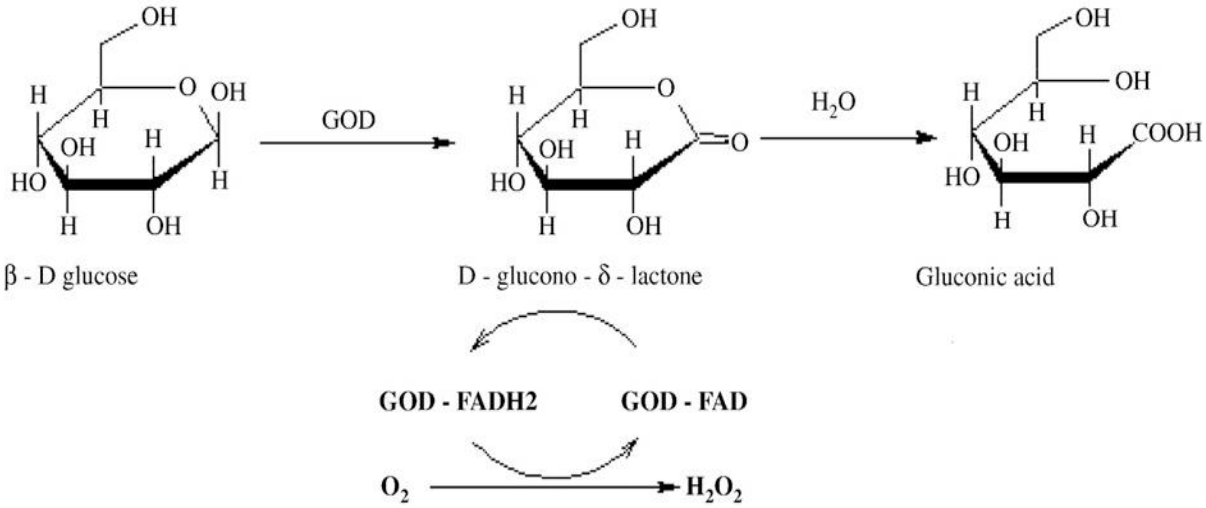


Figure 4 Representation of the Glucose oxidase reaction (GOX)

The reaction products formed as a result of the catalytic activity of glucose oxidase, are gluconolactone/ gluconic acid and hydrogen peroxide. Gluconic acid can be found in plants, fruits and other foodstuffs and is used as a food additive for several applications (Ramachandran et al. 2006). Hydrogen peroxide disappears either due to its oxidising reaction with the cysteine residues of proteins to form cystine and thus converting the sulfhydryl groups to a disulfide bond (Figure 5) or as substrate for endogenous peroxidases for phenolic crosslink formation (Rasiah et al. 2005).

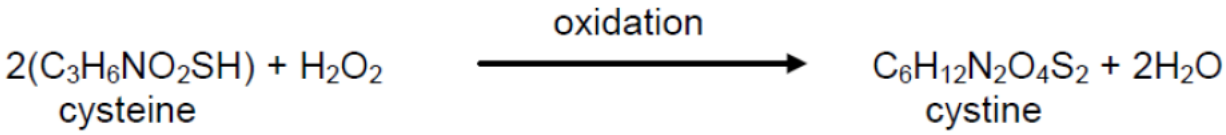


Figure 5 Hydrogen peroxide's oxidising reaction with cysteine residues

When glucose oxidase is used in egg processing, hydrogen peroxide is consumed so as to produce oxygen with the help of added catalase (Figure 6).

Figure 6 Hydrogen peroxide's catalysed reaction during egg processing

The method to analyse the activity of the enzyme is specific to AB Enzymes and is capable of quantifying glucose oxidase activity as defined by its IUBMB classification.

2.1.5 Activity and stability

The method of analysis for enzyme activity is specific to AB Enzymes and was provided as confidential information with the application. It does enable AB Enzymes to quantify glucose oxidase activity as defined by its IUBMB classification. The enzyme activity, which is the amount of enzyme oxidizing 1 µmol glucose per minute, has a specified minimum activity of 11500 GOX/g[4], set by AB Enzymes.

When stored under ambient conditions for up to a year, the activity loss of AB Enzyme's glucose oxidase will not exceed 10%.

2.1.6 Usage levels

Microbial food enzymes contain (apart from the enzyme itself), some substances derived from the production micro-organism and the fermentation medium. The presence of all organic materials is expressed as Total Organic Solids (TOS) (FAO/WHO, 2006) or more simply, dry matter minus ash. Since the usage level of a food enzyme depends on the enzyme activity present in the final enzyme preparation, the TOS is used to indicate usage levels.

Commercial enzyme preparations are typically used in accordance with GMP, whereby use is at a level that is not higher than the level necessary to achieve the desired enzymatic reaction. As such, food manufacturers adjust the usage levels depending on the food use, the type and quality of the raw materials used and based on the enzyme supplier's recommendations (Table 2). Once the enzyme has performed its function, it is inactivated by either heat, or a reduction in pH, or combination thereof.

Table 2 *Recommended use levels for AB Enzyme's Glucose oxidase*

Application	Raw material (RM)	Suggested use level (mg TOS/kg RM)
Baking and other cereal based products	Flour	10
Egg processing	Egg/egg white	10

2.2 Manufacturing process

2.2.1 Production of the enzyme

AB Enzymes glucose oxidase enzyme is produced by submerged fermentation of the GM strain of *T. reesei* using appropriate substrate and nutrients. The production process involves fermentation, recovery, formulation and packaging. (*Figure 7*).

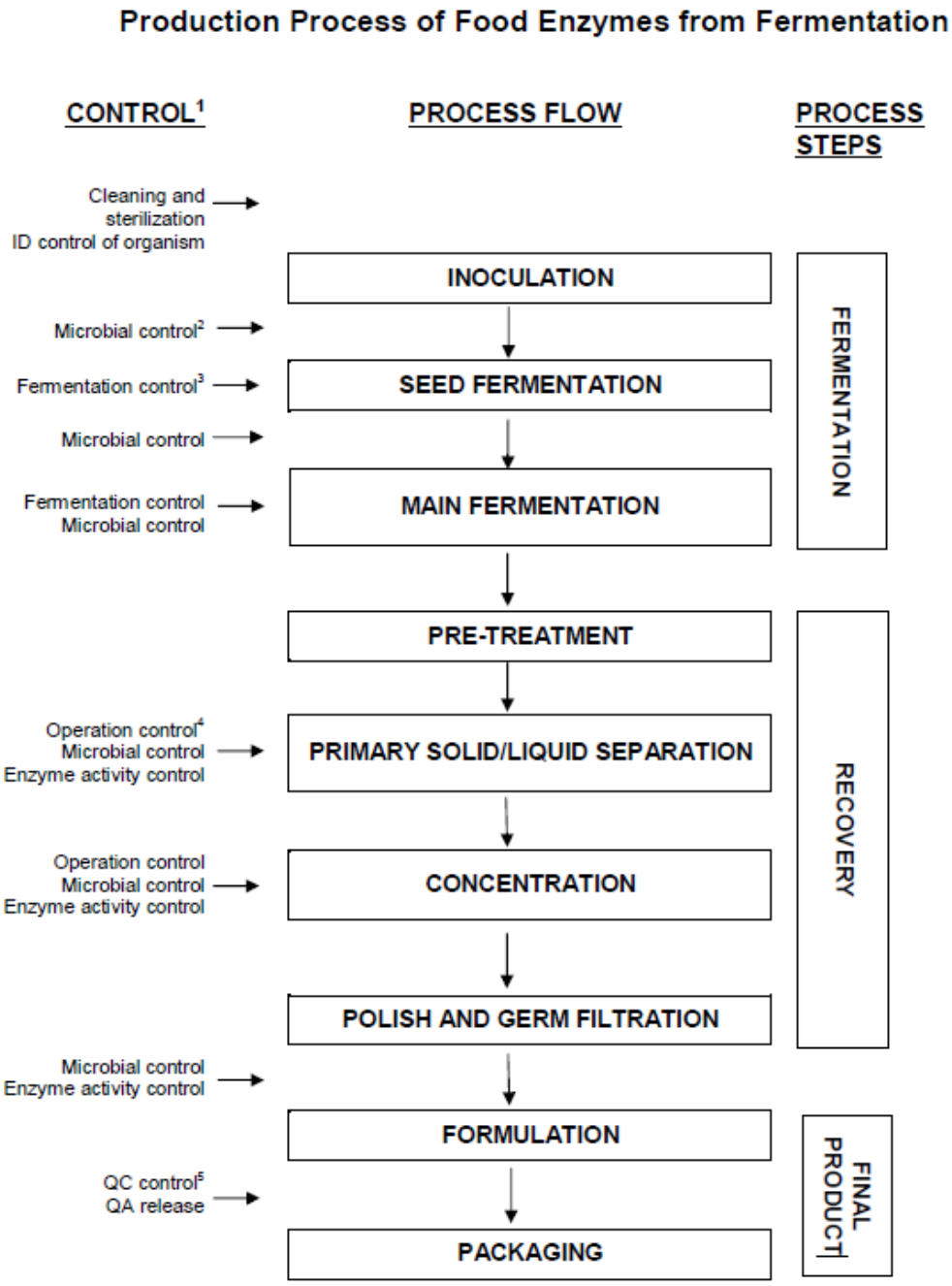
Whilst full details of the raw materials used for the fermentation were provided by AB Enzymes, this information is proprietary and as such, "Confidential Commercial Information" was granted by FSANZ. The raw materials meet predefined quality standards controlled by Quality Assurance for

[4] 1 glucose oxidase unit is the amount of enzyme oxidizing 1 µmol glucose per minute. Unit of activity is GOX/g.

ROAL Oy[5]. They also conform to either specifications set out in the Food Chemical Codex, 11th edition, 2018 or The Council Regulation 93/315/EEC, setting the basic principles of EU legislation on contaminants and food, and Commission Regulation (EC) No 1881/2006 setting maximum limits for certain contaminants in food.

The production of AB Enzyme's glucose oxidase is monitored and controlled by analytical and quality assurance procedures that ensure that the finished preparation complies with these specifications and is of the appropriate quality for use as a processing aid in food applications.

[5] Roal Oy Ltd is one of the world´s largest enzyme companies producing enzymes for different industrial applications, e.g. baking, food, technical and feed industries.



[1] The controls shown on the flow chart may vary depending on the production set-up. Controls are conducted at various steps throughout the production process as relevant.
[2] Microbial control: Absence of significant microbial contamination is analyzed by microscope or plate counts
[3] During fermentation parameters like e.g. pH, temperature, oxygen, CO_2, sterile air overflow are monitored / controlled.
[4] Operation control in downstream processes cover monitoring and control of parameters like e.g. pH, temperature
[5] Final QC control will check that product does live up to specifications like e.g. enzyme activity as well as chemical and microbial specification.

Figure 7 *Manufacturing process for AB Enzyme's Glucose oxidase*

2.2.2 Specifications

There are international specifications for enzyme preparations used in the production of food. These have been established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) Compendium of Food Additive Specifications (JECFA 2017) and the United States Pharmacopeial Convention (USPC) Food Chemicals Codex 11th edition (USPC 2018). Both of these specification sources are primary sources listed in section S3—2 of the Code. Enzyme preparations must meet these specifications.

Table 3 provides a comparison of three representative batch analyses of Glucose oxidase with the international specifications established by JECFA and USPC, as well as those detailed in the Code (as applicable).

Table 3 *Product specifications for commercial α-glucosidase enzyme preparation*

Analysis	Average Enzyme analysis from 3 batches	Specifications		
		JECFA	USPC	the Code
Lead (mg/kg)	< 0.05	≤5.0	≤5.0	≤2.0
Arsenic (mg/kg)	< 0.5	-	-	≤1.0
Cadmium (mg/kg)	*< 0.5	-	-	≤1.0
Mercury (mg/kg)	*< 0.5	-	-	≤1.0
Total coliforms (cfu/g)	< 1	≤ 30	≤ 30	-
Salmonella (in 25 g)	Negative	Absent	Absent	-
Enteropathic *E. coli* (in 25 g)	Negative	Absent	-	-
Antibiotic activity	Negative	Absent	-	-
Enzyme Activity GOX/g	12467	N/A	N/A	N/A

[a] ND = Not detected

[b] DL = Detection limit

*Cadmium and Mercury based on two independent batches

Based on the above results for the enzyme preparation, where there is no specification under section S3—2 or S3—3, or if the monographs referred to in those sections do not contain a specification for cadmium or mercury, the enzyme preparation *must* meet the conditions in S3—4 i.e. arsenic, cadmium and mercury (all ≤1.0 mg/kg).

2.3 Food technology conclusion

FSANZ concludes that the stated purpose of this glucose oxidase enzyme preparation, namely for use as a processing aid in the production is clearly articulated in the application. AB Enzymes has provided adequate assurance that the enzyme preparation, in the form and prescribed amounts, is technologically justified and has been demonstrated to be effective in achieving its stated purpose. Glucose oxidase performs its technological function during the production and manufacture of foods, after which it is inactivated, thereby performing no technological function in the final food. It is therefore appropriately categorised as a processing aid. The glucose oxidase enzyme preparation needs to meet international purity specifications, or those set out in the Code to be sold in Australia and New Zealand.

3 Risk assessment

3.1 Objective for risk assessment

The objective of this risk assessment for glucose oxidase from *T. reesei* is to evaluate any potential public health and safety concerns that may arise from the use of this enzyme as a processing aid. Consideration is given to the history of use of the host and gene donor organisms, characterisation of the genetic modification(s), and safety of the enzyme.

3.2 History of use

3.2.1 Host organism

T. reesei is a common 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). In humans *T. reesei* is not pathogenic and meets the requirements of a biosafety level 1 organism based on the *Biosafety in Microbiological and Bioemedical Laboratories*[6] 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).

FSANZ has previously assessed the safety of *T. reesei* as the source organism for several enzymes used as processing aids. The applicant provided data to confirm that the optimised host strain derived from QM6a was *T. reesei* using sequence analysis of the internal transcribed spacer 1 and 2 and the 5.8s (*ITS*) and the elongation factor 1-alpha (*EF1α*) genes.

3.2.2 Gene donor organism(s)

Penicillium amagasakiense

The gene for glucose oxidase was chemically synthesised based on the gene sequence from *Penicillium amagasakiense*, an anamorphic fungus commonly found in soil. This organism meets the criteria for a biosafety level 1 agent not associated with disease in healthy human adults.

Aspergillus nidulans

The selectable marker gene for acetamidase (*amdS*) was isolated from *Apsergillis 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 (Gabirelli 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 gene product acetamidase.

3.3 Characterisation of the genetic modification(s)

3.3.1 Description of DNA to be introduced and method of transformation

The host *T. reesei* strain was derived from the parental QM6a strain that had been optimised for industrial use via classical mutagenesis.

The glucose oxidase gene from *P. amagasakiense* was chemically synthesised using the

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

preferred codon usage for *T. reesei* and cloned into a commercially available vector. Standard molecular biology methods were used to create an expression cassette containing;

i) a *T. reesei* promoter that has been modified to improve the expression of glucose oxidase
ii) the glucose oxidase gene from *P. amagasakiense*
iii) a terminator sequence from *T. reesei*
iv) an acetamidase gene (*amdS)*, including the native promoter and terminator sequences were from *A. nidulans* (Kelly and Hynes, 1985).

The expression cassette was excised via restriction digest and introduced into *T. reesei* protoplasts using heat shock and polyethylene glycol-mediated transformation (Penttila *et al.,* 1987; Karhunen *et al.,* 1993). The DNA used to transform the *T. reesei* protoplasts only contained the expression cassette sequences (i.e. free from any vector-derived sequences). The resulting production strain was identified as RF11400.

3.3.2 Characterisation of inserted DNA

Southern blot analysis, using a probe targeting the entire expression cassette, was performed on genomic DNA extracted from the production (RF11400) and host strains. Analysis of the DNA digested with three individual restriction enzymes showed that at least one intact copy of the expression cassette is integrated into the genome of the 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 vector in *E. coli*.

3.3.3 Genetic stability of the inserted gene

The stability of the inserted gene in the production strain was examined by Southern blot analysis over a number of generations. Genomic DNA was extracted from a stock culture and at the end of three fermentations. Hybridisation was performed using a probe targeting the entire expression cassette. The results showed there was no change in band pattern in the DNA samples taken from the stock culture and different fermentations. It can be concluded that the DNA has been stably integrated into the host's genome.

3.4 Safety of glucose oxidase

3.4.1 History of safe use of the enzyme

The enzyme preparation has been authorised for use in food in France and Denmark since 2017, and in Mexico as of 2019. The enzyme is also approved for food use in Canada as well as being considered Generally Recognized as Safe (GRAS) in the USA since 2017.

A number of glucose oxidase enzymes from other microbial sources are currently permitted as processing aids in Schedule 18 of the Code.

3.4.2 Bioinformatics concerning potential for toxicity

A search for homology of the amino acid sequence of glucose oxidase from *T. reesei* with proteins identified as toxins was conducted using the BLAST-P (protein – protein BLAST) program. Glucose oxidase did not show significant homology to any known toxins.

3.4.3 Toxicology studies in animals

90-day repeated dose oral toxicity study in rats (Eurofins 2016) Regulatory status: GLP; Conducted in accordance with OECD TG 408 (1998) and Commission Regulation (EC) No. 440/2008, L142, Annex Part B

The test item in this study was glucose oxidase from *T. reesei* RF11400 (Batch No. P 140032 J, 91.5% total organic solids [TOS]). The test item was an ultra-filtrated concentrate representative of the glucose oxidase that is the subject of the application. This is the most concentrated form of glucose oxidase before its formulation into a food enzyme preparation.

Groups of Wistar Crl: WI(Han) rats (10/sex/group) were administered 0, 100, 300 or 1000 mg/kg bw/day glucose oxidase by oral gavage for 90 days. The vehicle and negative control was water. Animals were observed daily for clinical signs of toxicity and body weight and food consumption were measured weekly. Ophthalmological examinations were performed before the first administration and in the last week of the treatment period. Detailed behavioural observations using a functional observational battery of tests were made once before the first exposure and once in the last week of treatment. Blood was collected from the animals at the end of the treatment periods for assessment of haematology, blood coagulation and clinical biochemistry parameters. Urine samples were also collected at the end of the treatment period for urinalysis. On study day 91 all surviving animals were killed and subjected to gross necropsy. Organ weights were recorded. Histopathological evaluation of tissues was performed on all animals in the control and high dose groups and any animal found dead or euthanised before the end of the study.

Two animals were found dead during the treatment period: one female from the low dose group (study day 27) and one female from the high dose group (study day 82). Based on a histopathological assessment, the cause of these deaths was considered an accidental influx of the dosing solution into the respiratory tract or an accidental tissue injury relating to dosing in the respiratory tract. Neither death was related to exposure to the test item. No clinical signs of toxicity were observed during the course of the study, and all other animals survived to the end of the treatment period. No significant differences in body weight, body weight gain or food consumption were observed. No adverse effects on neurobehavioral parameters were found in the functional observational battery. There were no treatment-related effects on haematology, blood coagulation, clinical biochemistry, urinalysis, absolute or relative organ weights, gross findings on necropsy, or microscopic findings.

It was concluded that the no observed adverse effect level (NOAEL) in this study was 1000 mg/kg bw/day, the highest dose tested. On the basis that the authors report the test item as containing 91.5% TOS, FSANZ has concluded that the high dose corresponds to 915 mg/kg bw/day TOS.

3.4.4 Genotoxicity assays

Two in vitro genotoxicity studies were submitted, a bacterial reverse mutation assay (Ames test) and a micronucleus 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 (Eurofins 2015a) Regulatory status: GLP; conducted in compliance with OECD TG 471 (1997), US EPA test guideline OPPTS 870.5100 (1998) and Commission Regulation (EC) No. 440/2008 B.13/14 (1998)

The test item in this study was glucose oxidase from T. reesei RF11400 (Batch No. P 140032 J, 91.5% TOS). The solvent and negative control was water. Because glucose oxidase in the

presence of glucose produces hydrogen peroxide, a well-known cytotoxic and mutagenic compound in vitro, the enzyme was inactivated by pH shift through the preparation of a 10% w/v solution of the enzyme concentrate in 0.5 M sodium hydroxide solution, with the pH adjusted to 7.0.The TOS concentration was therefore reduced 10-fold, to 9.2%.

Test systems for this assay were Salmonella typhimurium strains TA100, TA1535, TA102, TA1537 and TA98. For assays conducted without S9 mix for metabolic activation, positive control articles were sodium azide in water for TA1535 and TA100, 4-nitro-o-phenylene-diamine in DMSO for TA1537 and TA98 and methyl methanesulfonate in DI water for TA102. The positive control article for use with all bacterial strains employing a metabolic activation system was 2-aminoanthracene in DMSO.

Following a dose range-finding study with strains TA 98 and TA 100, two experiments were performed in triplicate, one using the plate incorporation method and one using the preincubation methods. Concentrations of test article used in both experiments were 31.6, 100, 316. 100, 2500 and 5000 µg/plate, calculated based on the TOS content. For the plate incorporation test, test solution (100 µL), 500 µL S9 mix or substitution buffer, 100 µL bacterial solution and 2000 µL overlay agar were mixed in a test tube and poured over the surface of a minimal agar plate. For the pre-incubation method the test item preparation was mixed in a test tube with the tester strains and the S9-mix or substitution buffer for 60 minutes at 37ºC before adding the overlay agar and pouring on minimal agar plates. After the overlay had solidified, the plates were inverted and incubated for 48 hours at 37 °C.

No precipitation of the test item 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. In the plate incorporation test with strain TA1537 a 3.2-fold increase in the number of revertant colonies compared with the negative controls, but the revertant colony number was within the range of historical negative control data, no dose-response relationship was observed and the effect was not reproduced in the pre-incubation test. A substantial increase in revertant colonies was observed in the presence of positive control articles, confirming the validity of the assays.

It was concluded that glucose oxidase did not cause base pair changes or frameshift mutations in the experimental strains used in either the presence or absence of metabolic activation.

Micronucleus assay in cultured human lymphocytes (Eurofins 2015b) Regulatory status: GLP; conducted in accordance with OECD TG 487 (2014)

The test item in this study was glucose oxidase from T. reesei RF11400 (Batch No. P 140032 J, 91.5% TOS). RPMI cell culture medium was used as the solvent and negative control. Lymphocytes were obtained from a single healthy non-smoking donor with no known recent exposure to genotoxic chemicals. Two micronucleus experiments were conducted following a range-finding study. In both studies proliferation of lymphocytes was initiated by the addition of phytohaemagglutinin to the culture medium for 48 hours prior to exposure to the test item. In experiment I cells were exposed to the test item for 4 hours in the presence or absence of S9 metabolic activation, washed and then cultured in the presence of cytochalasin B for 40 hours. In experiment II, cells were exposed to the test item for 44 hours without metabolic activation, with cytochalasin B added to the culture medium 1 hour after addition of the test item.

In experiment I without metabolic activation concentrations of 5, 10 and 15 µg/mL glucose oxidase were evaluated for micronuclei frequency, concentrations of 50, 100, 200 and 275 µg/mL were evaluated in experiment I with metabolic activation, while concentrations of 0.1, 0.25, 0.5, 1.0 and 2.5 µg/mL were assessed in experiment II without metabolic activation. Concentrations were calculated based on the TOS content of the test item. Duplicate cultures were included and

for each dose group at least 2000 binucleated cells (cytotoxicity permitting) were analysed for micronuclei. Positive clastogenic controls were ethylmethanesulfonate or cyclophosphamide in the absence and presence of S9, respectively. The positive aneugenic control, used in the absence of metabolic activation, was colcemid.

In experiment I without metabolic activation no increase of cytostasis above 30% relative to the negative controls was noted up to a concentration of 10 µg/mL. At 15 µg/mL relative cytostasis was 32%. In experiment I with metabolic activation relative cytotasis was greater than 30% at concentrations ≥ 100 µg/mL: 33%, 48% and 60% at 100, 200 and 275 µg/mL respectively. In experiment II cytostasis was below 30% up to a concentration of 0.25 µg/mL, at higher concentrations, relative cytostasis was 31%, 50% and 69% at concentrations of 0.5, 1 and 2.5 µg/mL, respectively. In experiment I without metabolic activation, a significant increase was observed at 10 µg/mL ($p = 0.0191$) but not at 5 or 15 µg/mL, however the frequency of micronucleated cells was within the historical negative control limits so was not considered to be biologically relevant. In experiment I with metabolic action a significant increase in the frequency of micronucleated cells compared with controls was observed at the highest concentration (275 µg/mL; $p = 0.0011$)), and a significant concentration-related trend was also found using the chi-squared test ($p < 0.05$). However, the frequencies of micronucleated cells at all concentrations were within the historical negative control ranges, and substantial cytotoxicity was observed at 275 µg/mL (60% relative cytostasis), so this increase was not considered biologically relevant. No statistically significant increases in the frequency of micronucleated cells compared with the negative controls were observed in experiment II without metabolic activation. The positive controls produced the expected significant increases in frequency of micronuclei, demonstrating the validity of the assay.

It was concluded that glucose oxidase did not induce structural and/or numerical chromosomal aberrations in human lymphocytes under the conditions of this study.

3.4.5 Potential for allergenicity

A full length sequence alignment search was conducted using the Food Allergy Research and Resource Program (FARRP) AllergenOnline database, with identify matches greater than 50% indicating possible cross-reactivity (Aalberse 2000). No matches with greater than 50% identity were identified. An identity of 30.7% was found with Mala s 12 allergen precursors produced by the fungal species *Malassezia sympodialis*.

The same database was used to conduct an 80 amino acid sliding window search with a cut-off of ≥35% identity to known allergens. One identity match of 41.2% was identified, with Mala s 12 allergen precursor produced by *M. sympodialis*. *M. sympodialis* is a yeast present among normal human skin microflora and can induce IgE- and T-cell-mediated allergic reactions in atopic eczema patients (Zargari *et al.* 2007).

The European Food Safety Authority (EFSA) recently considered amino acid sequence similarity between Mala s 12 and a glucose oxidase enzyme from another microbial source, *Aspergillus niger* strain ZGL (EFSA CEP Panel 2019). EFSA noted that given oral allergic reactions are mediated by IgE, elicitation reactions upon dietary exposure to the food enzyme cannot be excluded but as the yeast that expresses Mala s 12 is an ubiquitous component of the skin microflora, the likelihood of such elicitation reactions to occur after oral exposure through food was considered to be low.

Mala s 12 is listed as a contact allergen in the AllergenOnline database and the World Health Organization (WHO)/International Union of Immunological Societies (IUIS) Allergen Nomenclature database, but not as a food allergen. A literature search identified references to Mala s 12 as an allergen in patients with atopic eczema, but no references to it as a food

allergen. Studies on the association between sensitisation to *M. sympodialis* and atopic dermatitis were also identified, but none demonstrating a link between *M. sympodialis* and cases of food allergy. Furthermore, the risk of food allergy associated with cross-reactivity between a food enzyme processing aid and respiratory or dermal allergens is considered to be very low (Dauvrin *et al.,* 1998; Bindslev-Jensen *et al.,* 2006). Therefore the homology with Mala s allergen precursor is not considered to represent a food safety hazard.

A search for exact matches of 8 amino acid sequences did not identify any matches.

The applicant has indicated that lactose and glucose are included in the fermentation medium. The glucose is sourced from maize. The applicant submitted results of analysis for lactose content of three batches of the dried ultra-filtrated enzyme concentrate, which found that lactose levels were below the limit of detection (100 mg/kg). Analysis of three batches of the enzyme concentrate indicated that levels of casein in the enzyme concentrate were below the limit of detection (0.25 mg/kg).

The formulation of the enzyme preparation contains wheat flour.

3.4.6 Approvals by other regulatory agencies

The applicant has provided documentation indicating that the enzyme preparation was authorised for use in food in France and Denmark in 2017, and in Mexico in 2019. In Denmark and Mexico, approvals were for use in baking processes, other cereal processes and egg processing with a recommended maximum dose of 10 mg TOS/kg raw material.

In Canada, the enzyme preparation is approved for use in bread, flour, whole wheat flour, liquid egg white, pasta and unstandardized bakery products[7]. The US Food and Drug Administration (FDA) responded with a 'No Questions' letter to a GRAS Notice (GRN 707[8]) for the enzyme preparation in 2017.

4 Discussion

There are no public health and safety concerns associated with the use of glucose oxidase from *T. reesei* when used as a food processing aid at GMP levels in baked products, cereal based products and egg processing.

T. reesei has a long history of safe use in the source of enzyme processing aids, including several that are already permitted in the Code. This fungus is neither toxigenic nor pathogenic. Characterisation of the genetically modified production strain confirmed both presence and stable inheritance of the inserted glucose oxidase gene.

Glucose oxidase from genetically modified *T. reesei* showed no evidence of genotoxicity in a bacterial reverse mutation assay or a micronucleus assay in human lymphocytes. Glucose oxidase 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, 1000 mg/kg bw/day or 915 mg/kg bw/day on a TOS basis. The applicant's estimated theoretical maximal daily intake (TMDI) based on the proposed uses is 0.088 mg/kg bw/day TOS. A comparison of these values indicates that the Margin of Exposure between the NOAEL and TMDI is more than 10,000.

[7] https://www.canada.ca/en/health-canada/services/food-nutrition/food-safety/food-additives/lists-permitted/5-enzymes.html

[8] https://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&id=707&sort=GRN_No&order=DESC&startrow=1&type=basic&search=glucose%20oxidase

Bioinformatic analysis indicated that the enzyme glucose oxidase from genetically modified *T. reesei* shows no significant homology with any known toxins or food allergens, and is unlikely to pose an allergenicity concern. Lactose is used in the fermentation medium, however analysis of three batches of the dried ultra-filtrated enzyme concentrate indicated that lactose and casein levels were below the limit of detection (100 mg/kg and 0.25 mg/kg, respectively). Wheat flour is an ingredient in the enzyme preparation.

5 Conclusions

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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