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

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Supporting document

Risk and technical assessment – Application A1224

Glucose oxidase from *Penicillium rubens* as a processing aid

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Executive summary

The applicant, Shin Nihon Chemical Co., Ltd (Shin Nihon) applied to Food Standards Australia New Zealand (FSANZ) to amend the Australia New Zealand Food Standards Code (the Code) seeking to permit the use of glucose oxidase from non-genetically modified *Penicillium rubens* as a processing aid. The processing aid is for use in the manufacture of cooked products made from a dough (such as bread); pasta; noodles; and dried egg powder.

FSANZ has undertaken an assessment to determine whether the enzyme achieves its technological purpose in the quantity and form proposed to be used and to evaluate public health and safety concerns that may arise from the use of this enzyme.

FSANZ concludes that the proposed use of this glucose oxidase as an enzyme in the manufacture of cooked products made from a dough (such as bread); pasta; noodles; and dried egg powder, is consistent with its function of removing glucose (in egg processing and dough products) and facilitating the crosslinking of proteins (in dough products). Analysis of the evidence provides adequate assurance that the proposed use of the enzyme, at a level not higher than necessary to achieve the desired enzyme reaction under Good Manufacturing Practice (GMP), is technologically justified.

Glucose oxidase performs its technological purpose during the production of the specified foods and is not performing a technological purpose in the final food. It is therefore appropriately categorised as a processing aid as defined in the Code. There are relevant identity and purity specifications for the enzyme in the Code.

Penicillium rubens has a long history of safe use in food. FSANZ identified no unacceptable risk arising from potential pathogenicity or toxigenicity from use of *P. rubens* to produce the enzyme processing aid.

No public health and safety concerns were identified in the assessment of glucose oxidase under the proposed use conditions.

Toxicity testing of the enzyme showed no evidence of genotoxicity *in vitro*. The no observed adverse effect level (NOAEL) in a 90-day oral gavage study in rats was the highest dose tested,193 mg total organic solids (TOS)/kg bw/day. The theoretical maximum daily intake (TMDI) was calculated to be up to 0.38 mg TOS/kg bw/day. Comparison of the NOAEL with the TMDI gives a margin of exposure (MOE) of approximately 500.

Bioinformatics analyses indicated that the enzyme shows no significant homology with any known toxins or food allergens.

Based on the reviewed data it is concluded that, in the absence of any identifiable hazard, an acceptable daily intake (ADI) ‘not specified’ is appropriate.

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1 Introduction

The applicant, Shin Nihon Chemical Co. Ltd (Shin Nihon), applied to Food Standards Australia New Zealand (FSANZ) to amend the Australia New Zealand Food Standards Code (the Code) to permit the use of the enzyme glucose oxidase (EC 1.1.3.4), sourced from a non-genetically modified fungus, *Penicillium rubens*. The enzyme is intended to be used as a processing aid in the manufacture of cooked products made from a dough (such as bread); pasta; noodles; and dried egg powder, at the minimum level required to achieve the desired effect, in accordance with the principles of current Good Manufacturing Practice (GMP).

The Code does not currently permit glucose oxidase derived from *P. rubens* to be used as a processing aid in the manner requested by the applicant. However, the Code does permit glucose oxidase obtained from other specific microbial origins to be used as a processing aid (*Aspergillus niger,* GM *Aspergillus oryzae* and *Trichoderma reesei* - see subsections S18—4(5) and S18—9(3)) If permitted following FSANZ's pre-market assessment, the glucose oxidase that is the subject of this application would provide an additional option for manufacturers seeking to use glucose oxidase as a processing aid in the manufacture of cooked products made from a dough (such as bread); pasta; noodles; and dried egg powder.

1.1 Objectives of the assessment

The objectives of this technical and risk assessment were to:

- determine whether the proposed purpose is a solely technological purpose and that the enzyme achieves its technological purpose as a processing aid in the quantity and form proposed to be used
- evaluate potential public health and safety issues that may arise from the use of this enzyme, as a processing aid, specifically by considering the:
  - safety and history of use of the production organism
  - safety of the enzyme.

2 Food technology assessment

2.1 Characterisation of the enzyme

2.1.1 Identity of the enzyme

The production microorganism of the enzyme is a non-genetically modified strain of *P. rubens*. The applicant provided relevant information regarding the identity of the glucose oxidase enzyme. FSANZ verified this using the IUBMB[1] enzyme nomenclature database (McDonald et al 2009). Details of the identity of the enzyme are provided in Table 1.

Table 1 *Identity*

Generic common name:	Glucose oxidase
Accepted IUBMB name:	glucose oxidase
Systematic name:	β-D-glucose:oxygen 1-oxidoreductase
Other names:	glucose oxyhydrase; corylophyline; penatin; glucose aerodehydrogenase; microcid; β-D-glucose oxidase; D-glucose oxidase; D-glucose-1-oxidase; β-D-glucose:quinone oxidoreductase; glucose oxyhydrase; deoxin-1; GOD
EC number:	EC 1.1.3.4
Chemical abstracts service (CAS) number:	9001-37-0
Reaction:	β-D-glucose + O_2 = D-glucono-1,5-lactone + H_2O_2

For a graphical representation of the hydrolysis reaction catalysed by glucose oxidase, refer to its record in the enzyme database BRENDA[2] (Chang et al 2021).

2.2 Manufacturing process

2.2.1 Production of the enzyme

The applicant's glucose oxidase is produced by submerged fermentation of the *P. rubens* PGO 19-162 production strain. The fermentation steps are inoculum, seed fermentation and main fermentation. This is followed by the recovery stage involving primary and liquid separation, concentration to achieve the desired enzyme activity, polish, and germ filtration to provide a concentrated enzyme solution free of the production organism and insoluble substances. This is followed by formulation of the enzyme into an enzyme preparation. Enzymes are generally sold as enzyme preparations, which consist of the enzyme(s) and other ingredients, to facilitate their storage, sale, standardisation, dilution or dissolution.
A manufacturing flow-chart was provided as confidential commercial information (CCI) as an

[1] International Union of Biochemistry and Molecular Biology EC 1.1.3.4 (qmul.ac.uk)
[2] Information on EC 1.1.3.4 - glucose oxidase - BRENDA Enzyme Database (brenda-enzymes.org)

appendix to the application. The product is manufactured in accordance with current Good Manufacturing Practice for Food[3] and the principles of Hazard Analysis Critical Control Point (HACCP).

Details on the manufacturing process, raw materials and ingredients used in the production of the glucose oxidase enzyme preparation were provided in the application or as CCI. This includes details of the agents, reagents, and materials used in the manufacture of the enzyme and details of the filtration aids. The filtration aids are commonly used by the food industry in the purification of food ingredients. The applicant advised that the final enzyme preparation complies with the current specifications established by JECFA and the Food Chemicals Codex (FCC: see section 2.2.2 below).

The liquid enzyme concentrate is precipitated and the product dried into a powdered concentrate that exhibits 60,000 U/g of glucose oxidase activity. This concentrate is subsequently formulated with maltodextrin to produce the commercial enzyme preparation at two different enzyme activities (trade name 'Sumizyme PGO'), approximately in the ratio 35%:65% enzyme concentrate to maltodextrin, respectively.

The applicant has provided information as CCI that a raw material that is an allergen (that is required to be declared under the Code) is used during the fermentation process to produce the enzyme. However, analytical testing demonstrates that any presence in the ultra-filtered enzyme concentrate is below the limit of detection for that allergen.[4] FSANZ considers that the allergen is, therefore, highly unlikely to be present in the final commercial enzyme preparation (a mixture of the ultra-filtered enzyme concentrate and maltodextrin).

2.2.2 Specifications for identity and purity

There are international general 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) in its Compendium of Food Additive Specifications (FAO/WHO 2006) and in the Food Chemicals Codex (FCC 2008). These specifications are included in earlier publications of the primary sources listed in section S3—2 of Schedule 3 of the Code, and enzymes used as a processing aid must meet either of these specifications. The applicant states that the final enzyme preparation complies with the requirements in both specifications. Schedule 3 of the Code also includes specifications for lead, arsenic, cadmium and mercury (section S3—4) if they are not already detailed within specifications in sections S3—2 or S3—3. Limits for lead and arsenic are already included in section S3—3, under the Japan Ministry of Health standards (Japan Ministry of Health, Labour and Welfare, 2018). The applicant has advised that the enzyme preparation meets the maximum levels (1 mg/kg) for cadmium and mercury in section S3—4.

The applicant provided the results of analysis of three different batches of the glucose oxidase concentrate. Table 2 provides a comparison of the analyses with international specifications established by JECFA and FCC. Based on these results and CCI and other information provided by the applicant, the enzyme concentrate met the relevant specifications in Schedule 3 of the Code.

[3] known as cGMP, as distinct from GMP (which refers to the level of use of the enzyme).
[4] The applicant has provided the analytical results as confidential commercial information

Table 2 *Comparison of manufacturer's glucose oxidase ultra-filtered enzyme concentrate (prior to addition of maltodextrin to produce the enzyme preparation) compared to JECFA, Food Chemicals Codex, and the Japan specification for enzymes*

Analysis	Analysis provided by manufacturer	Specifications		
		JECFA (2006)	*Food Chemicals Codex (FCC, 2020)*	*Japan Ministry of Health, 9th Edition 2018*[5]
Lead (mg/kg)	<5	≤5	≤5	≤5
Arsenic (mg/kg)	<3	-	-	≤3
Coliforms (cfu/g)	≤30	≤30	≤30	-
***Salmonella* (in 25 g)**	Not detected	Absent	Negative	-
***E. coli* (in 25 g)**	Not detected	Absent	-	-
Antibiotic activity	negative	Absent	-	-

Whilst the manufacturing processes ensure the production microorganism is removed from the final enzyme preparation, the food enzyme is a biological isolate of variable composition, containing the enzyme protein, as well as organic and inorganic material derived from the microorganism and fermentation process. Refer to section 3.3 below for the total organic solids (TOS) value.

2.3 Technological purpose of the enzyme

Under the current application, glucose oxidase is intended for use as a processing aid in the manufacture of cooked products made from a dough (such as bread); pasta; noodles; and dried egg powder. As identified by the IUBMB (section 2.1.1, above), glucose oxidase catalyses the oxidation of β-D-glucose to Dglucono-1,5-lactone in the presence of molecular oxygen, which, at the same time, converts oxygen to hydrogen peroxide. As stated in the application, the removal of glucose prevents the non-enzymatic browning of foods via the Maillard reaction. Glucose oxidase also functions to strengthen the protein complexes contained in wheat or starch-based foods. The applicant requested use of the enzyme at GMP levels.

The *Guidelines on Substances used as Processing Aids* (Codex 2010) sets out general principles for the safe use of substances used as processing aids. The guideline states that substances used as processing aids shall be used under conditions of good manufacturing practice (GMP). Therefore, use of commercial enzyme preparations should follow GMP, where use is at a level that is not higher than that necessary to achieve the desired enzymatic reaction.

The stated technological purpose of the glucose oxidase enzyme is supported by the scientific literature (Damodaran et al 2008; Rasiah et al 2005; Vemulapalli and Hoseney 1998).

The applicant provided information on the physical and chemical properties of the enzyme preparation. Table 3 summarises this information. The enzyme is heat-denatured at a temperature of 65°C. Therefore, the enzyme is inactivated in egg powder spray drying and during baking/cooking of a dough and during the drying of pasta or noodles, and would have no technological effect in these foods after they are produced. Additionally, the enzyme is

[5] Glucose oxidase Section D. monographs accessed 7 June 2022

used at GMP levels, meaning it is consumed during the production process for the nominated foods.

As stated above, the enzyme is inactivated at a temperature of 65°C. As such, it will be inactivated under food processing conditions that experience elevated temperatures, such as those that occur during baking, cooking, drying and pasteurisation. The applicant provided information to support this claim. Specifically, the applicant provided analytical results of testing done for residual glucose activity in bread-making wheat flour. The enzyme was added to the flour, which was then used to make bread dough. Following baking at typical temperatures, glucose oxidase activity was determined to be below the limit of detection. On that basis, the applicant claims, and FSANZ agrees, that glucose oxidase performs its primary technological function during food processing of the nominated foods and has no technological effect in the final food. As such, the enzyme functions as a processing aid for the purposes of the Code.

Table 3 *Physical and chemical properties of glucose oxidase enzyme preparation*

Physical and chemical properties of commercial enzyme preparation	
Enzyme activity*	Formulated according to two commercial enzyme preparations – 2,000 U/g or 15,000 U/g
Appearance	The ultra-filtered enzyme concentrate is formulated with maltodextrin to produce a dry, powdered commercial product
Temperature optimum for maximum activity	37°C
Thermostability	0–45°C, denatured at 65°C (when holding for 15 minutes)
pH optimum	6.0

*Assay of glucose oxidase activity (Shin Nihon internal method). One unit of activity, expressed as U, is defined as the amount of the enzyme that oxidises 1 µmole of β-D-glucose per minute under the conditions of the assay.

2.4 Technological justification

As outlined above, the technological need of the enzymatic conversion of glucose with the help of glucose oxidase can be described as enabling a reduction in glucose content and/or functioning to strengthen protein complexes contained in wheat or starch-based foods. A summary for each food category is provided below.

The enzyme performs its function to oxidise glucose by catalysing the conversion of D-glucose in the presence of oxygen to D-glucono-1,5-lactone and hydrogen peroxide during the production of bread and bakery/dough products and in the manufacture of dried egg powder. It is, therefore, performing as a processing aid as defined by the Code.

The Code already permits glucose oxidase (from other sources) to be used in the manufacture of foods. The specific benefits of the action of glucose oxidase in the manufacture and/or processing of the applicant-nominated foods, as summarised from the application, are described below.

<u>Liquid egg processing to produce dried egg products</u>
When used in the manufacture of dried egg products, glucose oxidase reduces the content of glucose in the liquid egg, prior to drying. The removal of glucose prevents non-enzymatic

browning of the egg powder, induced by the Maillard reaction.

Bread and bakery products, pasta and noodles
Like egg processing, glucose oxidase reduces the content of glucose so, for products baked produced at elevated temperatures, non-enzymatic browning can be reduced (so the amount of browning can be controlled). In addition, glucose oxidase strengthens the protein complexes contained in starch-based dough products through increased formation of cross-links between proteins. Rasiah et al (2005) conclude that it is mainly the albumins and globulins that are cross-linked, and that this includes both disulfide and non-disulfide crosslinks. Crosslinking acts to enhance dough properties and the texture of the final baked or cooked product.

2.5 Food technology conclusion

FSANZ concludes that the proposed use of this glucose oxidase as a processing aid for use in the manufacture of cooked products made from a dough (such as bread); pasta; noodles; and dried egg powder, is consistent with its typical function as an oxidising agent. FSANZ concludes that the evidence presented to support the proposed use provides adequate assurance that the use of the enzyme in the form and requested amount (i.e., at a level consistent with GMP) is technologically justified and has been demonstrated to be effective in achieving its stated purpose.

Glucose oxidase performs its technological purpose during the production of the nominated foods, after which it is inactivated, and is not performing a technological purpose in the final food. It is, therefore, appropriately categorised as a processing aid as defined in the Code.

There are relevant identity and purity specifications for the enzyme in the Code, and the applicant provided evidence that the enzyme meets these specifications.

3 Safety assessment

3.1 Safety and history of use of the production organism

Penicillium rubens is a ubiquitous fungus which is a common cause of structural and cosmetic damage in houses (Segers et al 2016).

The applicant's production strain, *Penicillium rubens* PGO 19-162, is not genetically modified or self-cloned. It is one of several strains of *Penicillium chrysogenum* which were recently re-categorised as belonging to the species *P. rubens* based on molecular phylogenetic analysis and extrolite[6] production data (Houbraken et al 2011; 2012). The applicant provided appropriate evidence to identify the strain as *P. rubens* based on comparison of its ITS-5.8S sequence (provided as CCI) with the ITS barcodes for *P. rubens* and *P. chrysogenum* recommended by the International Commission of Penicillium and Aspergillus[7]. Taxonomic identification of the strain had also been confirmed by an independent laboratory.

In Japan, *P. rubens* PGO 19-162 has been used for the production of glucose oxidase for over two decades. The production organism is, therefore, considered to have a history of safe use in production of this enzyme food processing aid. *P. chrysogenum* also has a history of safe use in Europe as a starter culture for the production of dry sausages and in the fermentation and bio-preservation of meat (Sunesen and Stahnke 2003; Mogensen et al 2002)[8]. The Ministry of Health, Labour and Welfare (MHLW) in Japan approved the use of *Penicillium* species for the production of food additives in 2018. China has permitted the use of the organism, under its former name of *Penicillium chrysogenum*, in food processing since 2014, and South Korea has permitted the use of *P. chrysogenum* for production of food additives since 2020.

Infections caused by *P. rubens* or *P. chrysogenum* are extremely rare, opportunistic, and usually identified only in individuals with compromised immune systems or after injury or surgery. Only two cases of infection in non-immunocompromised individuals have been reported (López-Martínez et al., 1999; Kantarcioğlu et al., 2004). The applicant provided data on the effective removal of the production organism during production of the enzyme preparation, and the risk of infection due to its use is considered negligible.

Both *P. rubens* and *P. chrysogenum* are known to produce a wide variety of extrolites, including antibiotics (such as penicillins, meleagrin and xanthocillin X) and mycotoxins such as roquefortine C (Houbraken et al 2012). The applicant provided results of batch testing of the enzyme preparation for a standard range of mycotoxins—ochratoxin A; aflatoxins B1, B2, G1 and G2; zearalenone; sterigmatocystin; and T-2 toxin—and for *Penicillium*-specific extrolites such as roquefortine C and the pigment chrysogine. No detectable levels were reported. Evidence was also provided by the applicant to show that no residual antibacterial activity is present in the food enzyme preparation. These results imply that the production organism does not produce toxicologically significant amounts of mycotoxins or other extrolites of concern under industrial fermentation conditions.

[6] Extrolites, also called secondary metabolites, are chemical compounds that are excreted or accumulate in cell walls or membranes. They tend to be of limited taxonomic distribution, and are of assistance in differentiating between similar species.
[7] www.aspergilluspenicillium.org
[8] Note: given the relatively recent reclassification of the production organism from *P. chrysogenum* to *P. rubens*, it is necessary to consider some information regarding *P. chrysogenum*.

3.2 Safety of the enzyme

3.2.1 History of safe use

In 2014, the US FDA responded with a “No Questions” letter to a GRAS Notification filed for the enzyme that is the subject of this application.

3.2.2 Bioinformatics concerning potential for toxicity

A recent (2021) search was conducted using the NCBI’s blastp program to conduct a sequence alignment query of the amino acid sequence of glucose oxidase against downloaded protein sequences obtained from a curated database of 7,262 animal venom proteins and toxins maintained by UniProt[9]. The default blastp parameters were used (i.e., Expect threshold = 0.05; BLOSUM62 matrix). No significant similarities between the enzyme and any known toxins or venoms were identified.

3.2.3 Toxicity studies

All toxicology tests were performed on a batch representative of the food enzyme before the addition of other components of the commercial food enzyme preparation.

3.2.3.1 Animal Studies

90-day repeat-dose oral gavage study of glucose oxidase in Sprague Dawley rats (Konishi et al 2013). Regulatory status: GLP; in accordance with OECD test guideline 408

The vehicle and control article for this study was distilled water. Doses of glucose oxidase, selected on the basis of a preceding dose range-finding study, were 0, 1.93, 19.3 and 193 mg TOS/kg bw/day. Rats were received at 4 weeks of age, acclimatized for eight days and subjected to ophthalmological examinations before being assigned to groups, 10/sex/group. Rats were individually housed under standard laboratory conditions of environment and husbandry. Food and water were provided *ad libitum,* except when study activities precluded this.

Parameters determined during the study included survival, clinical observations, bodyweight changes and food consumption. In week 12, all rats were subjected to ophthalmological examination. Urine was collected for analysis from all animals in the final week of the in-life phase, and blood was collected from the abdominal aorta, immediately prior to killing, for haematology, clinical chemistry and measurement of coagulation times. Detailed necropsy was performed. Fresh organ weights, as sex-appropriate, were recorded for organs specified in the OECD guideline. A comprehensive list of organs and tissues was preserved for histopathology.

All rats survived to the scheduled end of the in-life phase, and there were no treatment-related adverse effects on any of the parameters measured. It was concluded that the no observed adverse effect level (NOAEL) was the highest dose tested, 193 mg TOS/kg bw/day.

3.2.3.2 Genotoxicity

Bacterial reverse mutation assay of glucose oxidase (Konishi et al 2013). Regulatory status: GLP; in accordance with OECD test guideline 471

[9] https://www.uniprot.org/program/Toxins

For the purpose of this assay, the enzyme was inactivated by heating to 60°C and adjusting the pH to 2. The pH was then re-adjusted to the original pH of the test article (approximately 4). The reason for the inactivation was that the reaction catalysed by glucose oxidase produces hydrogen peroxide (H_2O_2) as a byproduct, and H_2O_2 may cause cellular and sub-cellular oxidative damage, including damage to DNA.

Bacterial test strains used in the assay were *Salmonella enterica* var. Typhimurium strains TA1535, TA100, TA1537 and TA98 and *Escherichia coli* WP2uvrA. The solvent and negative control article was sterile water. Appropriate positive control articles were used to confirm the validity of the assay. A preliminary concentration-range finding test and a definitive test were performed. Both tests were conducted in triplicate at final test article concentrations of 0.00611, 0.0193, 0.0611, 0.193, 0.611 and 1.93 mg TOS/plate in the presence and absence of S9 metabolic activation.

In both the range finding test and the main test, no positive mutagenic responses were observed in any bacterial strain at any concentration tested in either the presence or absence of S9 mix for metabolic activation. Positive control substances displayed the expected mutagenic activity, confirming the validity of the assay. Based on the results of this study, it was concluded that the glucose oxidase was non-mutagenic in the bacterial reverse mutation test.

In vitro *chromosomal aberration assay of glucose oxidase in human peripheral lymphocytes (Konishi et al 2013). Regulatory status: GLP; in accordance with OECD test guideline 473*

The enzyme used for this assay was inactivated as described for the bacterial reverse mutation assay. Lymphocytes were collected from two healthy non-smoking volunteers, and treated with phytohaemagglutinin for 48 h prior to use. The solvent and negative control was distilled water. Positive controls were mitomycin C in the absence of S9 mix and cyclophosphamide in the presence of S9 mix for metabolic activation. In the short-term exposure assay, cells were exposed to the enzyme at concentrations of 0, 0.0151, 0.0303, 0.0605, 0.121, 0.242 and 0.484 mg TOS/mL for 3 h in the absence and presence of S9 mix. Under the continuous exposure used for this assay, cells were exposed to the enzyme at concentrations of 0, 0.000118, 0.000236, 0.000472, 0.000945, 0.00188 and 0.00378 TOS/mL, respectively, for 24 h in the absence of S9 mix. All control and enzyme treatments were conducted in duplicate.

The incidence of cells with structural chromosome aberrations was not significantly different in any enzyme treatment group compared to the negative control. In addition, the incidence of polyploid cells was low (0.0 or 0.5%) at all enzyme concentrations and in the negative control (0.0%), and no significant differences were observed between the negative control and enzyme treatments. In all assays, a dose-dependent decrease in relative Mitotic Index was observed with exposure to the glucose oxidase. Treatment with positive control agents produced a significant increase in the percentage of cells with structural chromosome aberrations compared to the negative control. No precipitation of the test article was observed either at the start or end of treatment at any concentration. It was concluded that glucose oxidase was not clastogenic under the conditions of this study.

Combined in vivo *mammalian erythrocyte micronucleus test and comet assay (Konishi et al 2013). Regulatory status: GLP. The mammalian erythrocyte micronucleus test was performed according to OECD test guideline 474.*

Male Crl:CD (SD) rats were received at 7 weeks of age and acclimatised for 7 days prior to the study start. Rats were assigned to treatment groups, five rats/group. The vehicle and negative control article was water. Doses administered were 0, 48.5, 96.7 and 193 mg TOS/kg bw/day. Rats were dosed once daily for three consecutive days by oral gavage. A positive control group was administered 200 mg/kg bw/day ethyl methanesulfonate by oral gavage. During the in-life phase, rats were observed for clinical signs and mortality. Three hours after the last dose administration, rats were weighed and then killed by carbon dioxide inhalation. The stomach, liver and femur were removed from each rat. Bone marrow cells were obtained from the femur, and cell suspensions were prepared and fixed in 10% neutral buffered formalin. Slides, coated with acridine orange, were prepared for examination of micronucleated immature erythrocytes by fluorescence microscopy. Two thousand immature erythrocytes per animal were analysed, and the number of micronucleated immature erythrocytes recorded. To assess effects on bone marrow cell proliferation, the number of immature erythrocytes out of a total 500 erythrocytes was counted. The frequency of micronucleated immature erythrocytes relative to the total number of immature erythrocytes analysed was calculated. For the comet assay, cell suspensions were prepared from the epithelium of the stomach and from the liver. Three slides per organ per animal were prepared with low-melting agarose, and cells were lysed overnight. Two slides/rat were then subjected to electrophoresis, stained and examined microscopically. Images were captured and analysed by a Comet assay analyser. One hundred cells per organ from each animal (50 cells per slide; i.e., 500 cells per group) were examined and scored for DNA damage (tail DNA) by determining the amount of damaged DNA relative to the total DNA content based on fluorescence intensity. The induction of DNA damage was determined based on the presence or absence of a significant difference in the percentage of tail DNA between the negative control and each test group.

All rats survived to scheduled termination, and no treatment-related effects on clinical signs, group mean bodyweights or gross appearance of liver or stomach were observed. In the micronucleus test, no significant differences were observed between the negative control and the enzyme-treated groups in the frequency of micronucleated cells. A significant increase in the ratio of immature erythrocytes to the total number of erythrocytes was observed in the 96.7 mg TOS/kg bw/day group, but not in the 193 mg TOS/kg bw/day group. Exposure to ethyl methanesulfonate was associated with a marked and significant increase in the frequency of micronucleated cells and a significant decrease in the ratio of immature erythrocytes to the total number of erythrocytes compared to the negative control, confirming the validity of the assay. In the comet assay, the mean percentages of tail DNA relative to total DNA content in hepatocytes and stomach cells following administration of the enzyme were comparable to the group mean values for the negative control group. Significant increases in tail DNA were observed in both cell types in the positive control group.

3.2.4 Potential for allergenicity

Recent (2020) bioinformatics searches were conducted to assess the potential of the enzyme to elicit an allergic response. using the AllergenOnline database version 20[10].
A full-length alignment search using default settings (E value cut-off = 1 and maximum alignments of 20) identified one hit to a putative allergen—the Mala s 12 allergen precursor produced by the fungal species *Malassezia sympodialis*, with a sequence alignment of 31% identity and a corresponding E-value of 4.5 e-21.

[10] http://www.allergenonline.org

The sequence identity was less than 50% over the length of the glucose oxidase sequence. A sliding window of 80-amino acid sequences conducted using default settings (E value cut-off = 1 and maximum alignments of 20) resulted in one hit, the same Mala s 12 allergen precursor. A third homology search conducted using the exact 8-mer approach did not produce any matches.

Malassezia sympodialis is a yeast that can colonize human skin, and Mala s 12 is identified as a contact allergen[11] rather than a food allergen. The sequence alignment is low (31% identity). In interpreting homology with known allergens, the threshold for identification as a positive sequence alignment is generally set at >35% identity (Codex 2003). Therefore, it is considered unlikely that the homology of the enzyme with Mala s 12 is of toxicological concern.

Searches of the scientific literature by the applicant and by FSANZ did not identify any evidence that glucose oxidase is known to act as a food allergen.

3.2.5 Assessments by other regulatory agencies

No assessments of this glucose oxidase by other regulatory agencies are available.

The US FDA responded with a No Questions letter to GRAS Notice 509 concerning this enzyme, but this is not a regulatory assessment.

3.3 Dietary exposure assessment

The objective of the dietary exposure assessment was to review the budget method calculation presented by the applicant as a 'worse-case scenario' approach to estimating the likely levels of dietary exposure, assuming all added glucose oxidase remained in the food.

The budget method is a valid screening tool for estimating the theoretical maximum daily intake (TMDI) of a food additive (Douglass et al 1997). The calculation is based on physiological food and liquid requirements, the food additive concentration in foods and beverages, and the proportion of foods and beverages that may contain the food additive (Douglass et al 1997). The TMDI can then be compared to an ADI or a NOAEL to estimate a margin or exposure for risk characterisation purposes. Whilst the budget method was originally developed for use in assessing food additives, it is also appropriate to use for estimating the TMDI for processing aids (FAO/WHO 2020). The method is used by international food regulatory bodies and the FAO/WHO Joint Expert Committee on Food Additives (JECFA) (FAO/WHO 2021) for dietary exposure assessments for processing aids.

In their budget method calculation, the applicant made the following assumptions:

- the maximum physiological requirement for solid food (including milk) is 0.05 kg/kg body weight/day
- 25% of solid food is processed
- the maximum physiological requirement for liquid is 0.1 L/kg body weight/day (the standard level used in a budget method calculation)
- 25% of non-milk beverages are processed
- all the enzyme remains in the final food
- all solid foods contain the maximum use level of 30 mg TOS/kg
- all non-milk beverages contain the maximum use level of 10 mg TOS/kg.

Based on these assumptions, the applicant calculated the TMDI of the enzyme to be 0.63 mg

[11] http://www.allergen.org/viewallergen.php?aid=435

TOS/kg body weight/day.

As assumptions made by the applicant differ from those that FSANZ would have made in applying the budget method, FSANZ independently calculated the TMDI using the following assumption that is conservative and reflective of a first tier in estimating dietary exposure:

- FSANZ would generally assume 12.5% of solid foods (including milk) contain the enzyme based on commonly used default proportions noted in the FAO/WHO Environmental Health Criteria (EHC) 240 Chapter 6 on dietary exposure assessment (FAO/WHO 2009). However, the applicant has assumed a higher proportion of 25% based on the extent of use of the enzyme and, therefore, FSANZ has also used this proportion for solid food (including milk) as a worse-case scenario.

Additionally, the applicant confirmed that the enzyme is intended to be used only in the manufacture of cooked products made from a dough (such as bread); pasta; noodles; and dried egg powder. As these are solid foods, FSANZ did not include non-milk beverages in the TMDI calculation.

All other inputs and assumptions used by FSANZ remained as per those used by the applicant. The TMDI based on FSANZ's calculations for solid food is 0.38 mg TOS/kg body weight/day. FSANZ concluded that this estimate of the TMDI was appropriate for use in the risk characterisation due this assessment aligning with the confirmation of the proposed uses of the enzyme.

Both the FSANZ and applicant's estimates of the TDMI will be overestimates of the dietary exposure given the conservatism of the budget method. This includes that it was assumed that the enzyme remains in the final foods. The applicant has stated that it is likely to be inactivated and perform no function in the final food.

4 Discussion

No public health and safety concerns were identified in the assessment of this glucose oxidase produced from a non-genetically modified strain of *P. rubens* under the proposed use conditions. The *P. rubens* production organism has a long history of safe use in food, is not pathogenic, and does not produce toxicologically significant amounts of mycotoxins. Bioinformatic analyses indicated that the enzyme shows no significant homology with any known toxins or allergens. Glucose oxidases from different source organisms are already permitted in the Code.

Glucose oxidase was not genotoxic *in vitro*. The NOAEL determined in a 90-day oral gavage study in rats was the highest dose level tested, 193 mg TOS/kg bw/day. The TMDI was calculated to be 0.38 mg TOS/kg bw/day. A comparison of the NOAEL and the TMDI gives a Margin of Exposure (MOE) of approximately 500.

5 Conclusion

FSANZ concludes that the proposed use of this glucose oxidase is technologically justified and has been demonstrated to be effective in achieving its stated purpose.

Glucose oxidase performs its technological purpose during the production of the nominated foods and is not performing a technological purpose in the final food. It is, therefore, appropriately categorised as a processing aid as defined in the Code.

There are relevant identity and purity specifications for the enzyme in the Code, and the

applicant provided evidence that the enzyme meets these specifications.

The enzyme production organism, *P. rubens*, is non-pathogenic, does not produce toxicologically significant amounts of mycotoxins or other extrolites of concern, and does not present an unacceptable food safety risk.

No public health and safety concerns were identified in the assessment of enzyme under the proposed use conditions.

Based on the reviewed data it is concluded that, in the absence of any identifiable hazard, an acceptable daily intake (ADI) ‘not specified’ is appropriate.

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