APPLICATION TO AMEND STANDARD 1.3.3 OF THE AUSTRALIA AND NEW ZEALAND FOOD STANDARDS CODE TO INCLUDE *PENICILLIUM RUBENS* AS A SOURCE ORGANISM FOR GLUCOSE OXIDASE

PREPARED FOR:
Standards Management Officer
Food Standards Australia New Zealand
Ground Floor, Boeing House
55 Blackall Street
Barton ACT 2600
Australia

PREPARED BY:

[redacted]

Application to Amend Standard 1.3.3 of the Australia and New Zealand Food Standards Code to Include *Penicillium rubens* as a Source Organism for Glucose Oxidase

TABLE OF CONTENTS

List of Appendices

List of Figures and Tables

Application to Amend Standard 1.3.3 of the Australia and New Zealand Food Standards Code to Include *Penicillium rubens* as a Source Organism for Glucose Oxidase

A. GENERAL REQUIREMENTS

In accordance with Section 3.1.1 – General Requirements of the Food Standards Australia New Zealand (FSANZ) *Application Handbook* (FSANZ, 2019), the following general information must be provided:

1. Format of the application;
2. Applicant details;
3. Purpose of the application;
4. Justification for the application;
5. Information to support the application;
6. Assessment procedure;
7. Confidential commercial information;
8. Other Confidential information;
9. Exclusive capturable commercial benefit;
10. International and other national standards;
11. Statutory declaration; and
12. Checklist.

Each point is addressed in the following subsections.

A.1 Format of the Application

1. Information Related to Changes to Standard 1.3.3 – Processing Aids

This application for an amendment to Standard 1.3.3 and related Schedules is prepared pursuant to Section 3.3.2 – Processing Aids of the FSANZ *Application Handbook* (FSANZ, 2019), which requires the following structured format to assess an application for a new processing aid:

A. General information on the application;

B. Technical information on the processing aid;

C. Information related to the safety of an enzyme processing aid;

D. Additional information related to the safety of an enzyme processing aid derived from a microorganism; and

E. Information related to the dietary exposure to the processing aid.

The application is presented in this format. At the start of each section (A to E) the information that must be addressed therein is specified in more detail. Additionally, an executive summary for the application is provided as a separate electronic document to this application. The application has been prepared in English and submitted electronically, as required by the FSANZ *Application Handbook* (FSANZ, 2019).

A.2 Applicant Details

Shin Nihon Chemical Co., Ltd. is a manufacturer of enzymes used in the food industry in the production of food ingredients and finished food products. The contact information of Akio Ichihara, Sales Division, of Shin Nihon Chemical Co., Ltd., the company's representative, are as follows.

A.3 Purpose of the Application

Shin Nihon Chemical Co., Ltd. ("Shin Nihon") is submitting this application to FSANZ to request an amendment to Standard 1.3.3 of the Food Standards Code ("the Code") to include glucose oxidase (EC 1.1.3.4) derived from non-genetically modified *Penicillium rubens* as a processing aid to reduce the residual glucose and/or oxygen content during the production of a variety of foods and beverages. The trade name for the glucose oxidase enzyme preparation as described herein is "Sumizyme PGO". The enzyme preparation contains the ultra-filtered enzyme concentrate that is formulated with maltodextrin. Glucose oxidase derived from *Aspergillus niger* and genetically modified *Aspergillus oryzae* are currently permitted for use under Schedule 18 of the Code for use as a processing aid in baking applications (FSANZ, 2002, 2020a). Recently, an application to amend the Code to include genetically modified *Trichoderma reesei* as a source of glucose oxidase for use in the manufacturing of cereal-based products and egg processing was approved and gazetted as of 14 May 2020 (Amendment No. 192, Application A1182) (FSANZ, 2020b,c).

Therefore, as Schedule 18 of the Code currently only permits glucose oxidase from *Aspergillus* species and *T. reesei*, this application is submitted to amend the Code to include a different source organism, *P. rubens*, as a source of glucose oxidase.

A.4 Justification for the Application

A.4.1 Technological Function for the Processing Aid

Glucose oxidase catalyses the oxidation of β-D-glucose to D-glucono-1,5-lactone (D-glucono-δ-lactone) in the presence of molecular oxygen, which, at the same time, converts oxygen to hydrogen peroxide. The enzyme therefore performs its catalytic function directly on β-D-glucose molecules present in various food matrices during processing of the foods. The technological function of glucose oxidase in the reduction of the residual glucose and oxygen in foods and beverages is needed in the food processing industry for preservative and stabilising purposes, thus prolonging shelf-life (as reviewed by Crueger and Crueger, 1990; Wong *et al*., 2008; Bankar *et al*., 2009). The removal of glucose prevents the non-enzymatic browning of foods induced by the Maillard reaction, which may occur during food processing. Glucose oxidase also functions to strengthen the protein complexes contained in wheat flour- or starch-based products through increased formation of cross-links between proteins, thereby improving the texture of such foods as breads and other baked products (Vemulapalli and Hoseney, 1998; Vemulapalli *et al.*, 1998; Rasiah *et al.*, 2005). The cross-links formed may be achieved through increased formation of disulphide cross-links between amino acid residues as a result of decreasing the sulfhydryl content of the proteins through oxidation (Vemulapalli and Hoseney, 1998). Oxidation is carried out by the hydrogen peroxide formed as a result of glucose oxidase activity. In this instance, hydrogen peroxide oxidises the cysteine residues of proteins to form cystine, thus converting the sulfhydryl groups to a disulphide bond.

A.4.2 Costs and Benefits for Industry, Consumers, and Government Associated with Use of the Processing Aid

The inclusion of glucose oxidase derived from *P. rubens* as a processing aid will provide food manufacturers and food producers with an alternative source of glucose oxidase that is derived from a non-pathogenic and non-toxigenic source organism. The inclusion of *P. rubens* as a source organism will not result in any additional cost to the regulator as the food enzyme is already approved for use as a processing aid.

A.5 Information to Support the Application

FSANZ previously reviewed applications to include glucose oxidase from *A. niger*, *A. oryzae* (Application A458), and *T. reesei* (Application A1182) for use as a processing aid in the production of various foods (FSANZ, 2002, 2020a,c). As part of their evaluation, FSANZ reviewed safety information on glucose oxidase derived from the various sources, including pre-clinical and human safety data, and raised no safety concerns with the use of glucose oxidase as a processing aid. Technical information specific to Shin Nihon's glucose oxidase derived from *P. rubens* strain PGO 19-162, including product-specific safety data for the food enzyme, are presented in the sections that follow. The information is presented to support the safety of glucose oxidase derived from *P. rubens* in accordance with the requirements listed in Section 3.3.2 (Processing Aids) of the FSANZ *Application Handbook* (FSANZ, 2019). The information presented herein pertains to the commercial product, Sumizyme PGO, containing glucose oxidase from *P. rubens* for which approval is being sought.

A.6 Assessment Procedure

Shin Nihon considers the most appropriate assessment procedure for assessing the application to include *P. rubens* as a source organism for glucose oxidase to be the General Procedure. It is anticipated that this application will involve amending Standard 1.3.3 of the Code to include glucose oxidase derived from *P. rubens* as a processing aid. As noted, glucose oxidase derived from *A. niger* and *A. oryzae* is already permitted as a processing aid as listed in Schedule 18 of the Code.

A.7 Confidential Commercial Information (CCI)

Shin Nihon requests that certain proprietary information required for Sections B.3 (Chemical and Physical Properties), B.4 (Manufacturing Process), B.5 (Batch Analysis), and C.5.1 (Taxonomic Identity of the Production Strain) be considered confidential commercial information (CCI). General summaries of the proprietary data are provided within this application, and all details considered to be CCI have been removed and are provided in Appendix A. All information presented in Appendices A-1, A-2, and A-3 is requested to remain confidential, as it holds significant commercial value to the company, including proprietary details on the manufacture of the food enzyme, the amino acid sequence, enzyme activity, protein calculation, and extrolite analysis.

A.8 Other Confidential Information

The identity of the companies that perform analytical testing on the food enzyme are requested to remain confidential. Shin Nihon does not wish to disclose the identity of the companies and would agree to disclose the identity by location (*e.g.*, "a lab in Japan").

A.9 Exclusive Capturable Commercial Benefit (ECCB)

It is not anticipated that this application would confer Exclusive Capturable Commercial Benefit (ECCB) in accordance with Section 8 of the FSANZ Act based on the following:

> "*An exclusive, capturable commercial benefit is conferred upon a person who applies for the development of a food regulatory measure or the variation of food regulatory measure under Section 22 if:*
>
> *(a) the applicant can be identified as a person or body that may derive a financial gain from the coming into effect of the draft standard to draft variation of the standard that would be prepared in relation to the application; and*

(b) any other unrelated persons or bodies, including unrelated commercial entities, would require the agreement of the applicant in order to benefit financially from the approval of the application".

A.10 International and Other National Standards

Glucose oxidase from *P. rubens* complies with the internationally recognised specifications for enzyme preparations as established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (JECFA, 2006a,b,c) and the Food Chemicals Codex (FCC, 2018). Glucose oxidase from various bacterial and fungal species, including *Penicillium* sp., are permitted as food additives in Australia and New Zealand, Canada, China, France, South Korea, Japan, and the European Union (EU) (JORF, 2006; Ministry of Health of the PRC, 2014; EFSA, 2018, 2019; MHLW, 2018; FSANZ, 2002, 2020c; Health Canada, 2020; MFDS, 2020). Standards for glucose oxidase exist in Japan (MHLW, 2018).

A.11 Statutory Declaration

Signed Statutory Declarations for Australia and New Zealand are provided in Appendix B.

A.12 Checklist

Completed checklists relating to the information required for submission with this application based on the relevant guidelines in the FSANZ *Application Handbook* are provided in Appendix C.

B. TECHNICAL INFORMATION ON THE PROCESSING AID

In accordance with Section 3.3.1 – Food Additives of the FSANZ *Application Handbook* (FSANZ, 2019), the following technical information must be provided:

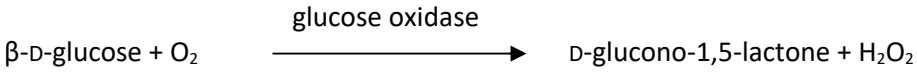
1. Information on the type of processing aid;
2. Information on the identity of the processing aid;
3. Information on the chemical and physical properties of the processing aid;
4. Manufacturing process;
5. Specification for identity and purity; and
6. Analytical method for detection.

Each point is addressed in the following subsections.

B.1 Information on the Type of Processing Aid

Shin Nihon's glucose oxidase from non-genetically modified *P. rubens* (strain PGO 19-162) is a food enzyme derived from a microbial source. Glucose oxidase catalyses the oxidation of β-D-glucose to D-glucono-1,5-lactone (D-glucono-δ-lactone) in the presence of molecular oxygen which acts as an electron acceptor, converting oxygen to hydrogen peroxide in foods and beverages (Figure B.1-1). D-Glucono-1,5-lactone is subsequently hydrolysed to gluconic acid by non-enzymatic means.

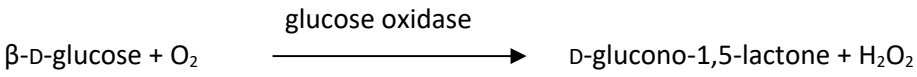
Figure B.1-1 Enzymatic Reaction of Glucose Oxidase Catalysing Oxidation of β-D-Glucose to D-Glucono-1,5-lactone



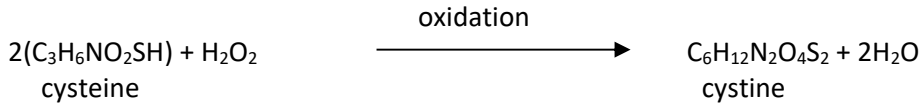
The formation of hydrogen peroxide may also lead into a secondary oxidation reaction in various food matrices, particularly in wheat flour- or starch-based products. In this secondary reaction, hydrogen peroxide oxidises the cysteine residues of proteins to form cystine, thus converting the sulfhydryl groups to a disulphide bond. The mechanism of action is described by the series of reactions in Figure B.1-2 below.

Figure B.1-2 Secondary Reaction in the Oxidation of β-D-Glucose to D-Glucono-1,5-lactone

1.



2.



The enzyme reaction does not require any co-factors. The glucose oxidase food enzyme does not exhibit any significant secondary enzymatic activities.

Shin Nihon's glucose oxidase from *P. rubens* is manufactured as an ultra-filtered enzyme concentrate. The commercial enzyme preparation, Sumizyme PGO, contains the enzyme concentrate that is formulated with maltodextrin. Based on the foregoing, glucose oxidase from *P. rubens* would be categorised as an enzyme of microbial origin under Schedule 18. The maximum use level of the enzyme preparation is 30 mg total organic solids (TOS)/kg in solid foods and 10 mg TOS/kg in non-milk beverages (see Section D.1 for further details).

B.2 Information on the Identity of Glucose Oxidase from *Penicillium rubens* (Strain PGO 19-162)

Information on the identity of glucose oxidase from non-genetically modified *P. rubens* (strain PGO 19-162), including the source organism, common and systematic name of the food enzyme, synonyms, enzyme classification and chemical abstracts registry numbers, are presented below. Shin Nihon's glucose oxidase from *P. rubens* (strain PGO 19-162) has not been protein-engineered and is not modified by a post-translational process.

B.2.1 Identity of the Food Enzyme

Source (strain):	*Penicillium rubens* strain PGO 19-162
Common/Accepted Name:	Glucose oxidase
Shin Nihon Enzyme Name/Abbreviation:	PGO
Other Names:	Glucose oxyhydrase; glucose aerodehydrogenase; β-D-glucose oxidase; D -glucose oxidase; D-glucose-1-oxidase; glucose oxhydrase; GOX; GOD
Enzyme Classification Number of Enzyme Commission (EC) of the International Union of Biochemistry and Molecular Biology (IUBMB)]:	1.1.3.4
Chemical/Systematic Name:	β- D-glucose:oxygen-1-oxidoreductase
Chemical Abstracts Service (CAS) Number:	9001-37-0
European Inventory of Existing Chemical Substances (EINECS) Number or European List of Notified Chemical Substances (ELINCS) Number:	232-601-0

B.3 Information on the Chemical and Physical Properties of Glucose Oxidase from *Penicillium rubens* (Strain PGO 19-162)

B.3.1 Molecular Mass

The glucose oxidase food enzyme produced with *P. rubens* strain PGO 19-162 is manufactured as an ultra-filtered liquid concentrate and does not contain any added diluents. The molecular weight of 3 non-consecutive batches of the glucose oxidase food enzyme were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The approximate molecular weight of the glucose oxidase food enzyme is provided in Appendix A.

The glucose oxidase food enzyme is characterised by its glucose oxidase activity. Batch analyses demonstrating the composition of the food enzyme are provided in Section B.5.2, indicating that the food enzyme is composed of approximately 98% water, 0.6% protein, and 0.4% ash. The TOS content is

calculated to be approximately 1.9% based on batch analyses but can range from 1.5 to 2.5% based on the TOS specification for the food enzyme.

B.3.2 Amino Acid Sequence

The full amino acid sequence of the glucose oxidase enzyme from *P. rubens* strain PGO 19-162 is provided in Appendix A.

B.3.3 Properties of the Food Enzyme

B.3.3.1 Information on the Principal Enzymatic Activity

As shown in Figure B.1-1 above, the glucose oxidase food enzyme catalyses the oxidation of β-D-glucose to D-glucono-1,5-lactone (D-glucono-δ-lactone) in the presence of molecular oxygen, which, at the same time, converts oxygen to hydrogen peroxide. D-Glucono-1,5-lactone is subsequently hydrolysed to gluconic acid by non-enzymatic means.

The glucose oxidase food enzyme is specifically characterised by its glucose oxidase activity. Shin Nihon has established a specification limit for glucose oxidase activity of the food enzyme, and analytical data supporting the enzyme activity level of the food enzyme is provided in Appendix A. Glucose oxidase activity is measured using an internal method developed by Shin Nihon (see Appendix A for details of the method of analysis). Glucose oxidase activity is reported on an enzyme unit per mL or g basis. One unit of activity, expressed as U, is defined as the amount of enzyme that oxidises 1 µmole of β-D-glucose per minute under the conditions of the assay.

B.3.3.2 Activity of the Food Enzyme Under the Conditions of Intended Use

The optimal pH and temperature conditions for the glucose oxidase food enzyme produced by *P. rubens* was determined to be over a pH range of 5 to 7 and a temperature range of 30 to 75°C, respectively. The information supporting the optimal reaction conditions are presented below.

Optimal Temperature and Thermostability Data

The optimum temperature for the glucose oxidase activity of the glucose oxidase food enzyme produced with *P. rubens* PGO 19-162 has been established as 37°C. The optimum temperature was determined experimentally in a test in which 0.2 U/mL enzyme solutions were added to glucose substrate solutions at pH 7. The enzyme/substrate solutions were incubated for 5 minutes at temperatures of 30, 37, 40, 45, 50, 55, 60, 65, or 70°C. The assay method described above was used to measure glucose oxidase activity. As shown in Table B.3.3.2-1 and Figure B.3.3.2-1a below, the glucose oxidase food enzyme was determined to have the greatest glucose oxidase activity at a temperature of 30 and 45°C, with maximum activity observed at 37°C. In comparison, enzyme activity is reduced substantially at temperatures in excess of 50°C, and at temperatures of ≥65°C, the enzyme exhibited no activity. Thus, the enzyme may be inactivated by heating to a temperature of 65°C and holding for 15 minutes.

The thermostability of the glucose oxidase activity of the glucose oxidase food enzyme has been determined experimentally. In this test, 20 U/mL enzyme solutions were pre-incubated for 15 minutes at pH 5.5 and at the following temperatures: 0, 30, 40, 45, 50, 55, 60, 65, or 70°C. The enzyme solutions were then diluted to 0.2 U/mL and added to glucose substrate solutions of pH 7. These enzyme/substrate solutions were then incubated at 30°C for 5 minutes. Enzyme stability was measured by measuring glucose oxidase activity by the glucose oxidase assay method indicated above. As shown in Table B.3.3.2-2 and Figure B.3.3.2-1b, glucose oxidase activity was stable at temperatures of 0 to 45°C. Enzyme stability decreased with increasing temperatures; the enzyme activity was completely unstable at temperatures of ≥65°C.

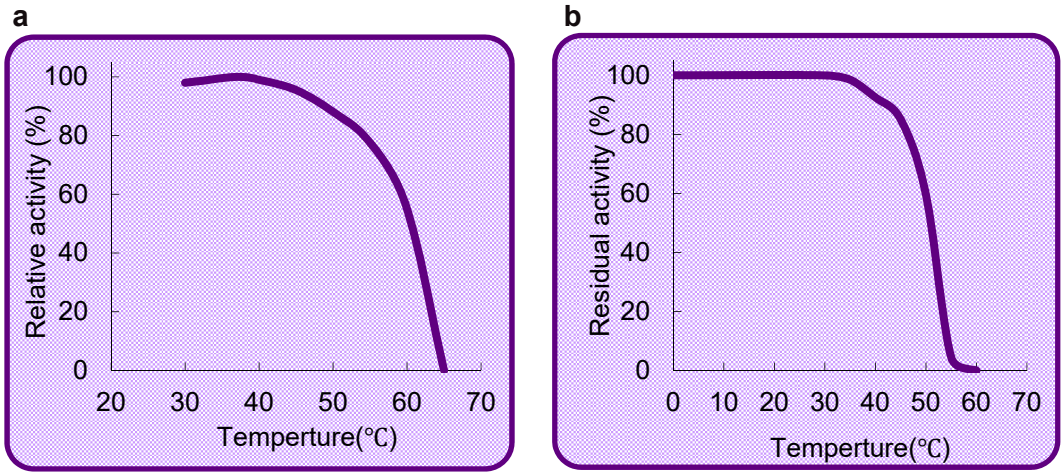
Table B.3.3.2-1 Relative Glucose Oxidase Activity of the Glucose Oxidase Food Enzyme from *Penicillium rubens* PGO 19-162 as a Function of Temperature at pH 7.0 after Incubation for 5 Minutes

Temperature (°C)	Relative Glucose Oxidase Activity (% of maximum activity)
30	98
37	100
40	99
45	96
50	88
55	78
60	55
65	0
70	0

Table B.3.3.2-2 Thermostability of the Glucose Oxidase from *Penicillium rubens* PGO 19-162 at pH 5.5 after Incubation for 15 Minutes

Temperature (°C)	Residual Glucose Oxidase Activity (%)
0	100
30	100
40	99
45	93
50	85
55	60
60	5
65	0
70	0

Figure B.3.3.2-1 Effect of Temperature on Glucose Oxidase Activity of the Glucose Oxidase Food Enzyme from *Penicillium rubens* PGO 19-162: a) Relative Enzyme Activity as a Function of Temperature and b) Thermostability of the Enzyme (Residual Activity)



Optimum pH and pH Stability

The optimum pH for the glucose oxidase activity of the glucose oxidase food enzyme has been established as 6. In this test, 0.2 U/mL enzyme solutions were added to glucose substrate solutions that were pre-adjusted to pH 3, 4, 5, 6, 7, 8, or 9. The enzyme/substrate solutions were incubated for 5 minutes at a temperature of 37°C. The glucose oxidase assay method indicated above was used to measure glucose oxidase activity. As shown in Table B.3.3.2-3 and Figure B.3.3.2-2a, maximum glucose oxidase activity was observed at pH 6. In comparison, enzyme activity is reduced substantially at pH below 5 and at pH above 7, with little to no enzyme activity observed at pH 3 and pH 9.

The effect of pH on the stability of the glucose oxidase activity of the of the glucose oxidase food enzyme also has been determined. In this test, 20 U/mL enzyme solutions were prepared with the pH adjusted to 3 to or 9. The enzyme solutions were pre-incubated at 30°C for 1 hour. The enzyme solutions were then diluted to 0.2 U/mL and added to glucose substrate solutions of pH 7. These enzyme/substrate solutions were then incubated at 30°C for 5 minutes. Enzyme stability was measured by measuring glucose oxidase activity by the glucose oxidase assay method indicated above. As shown in Table B.3.3.2-4 and Figure B.3.3.2-2b, glucose oxidase activity was stable at pH 4 to 8. Under acidic conditions of pH 3, glucose oxidase activity remained relatively stable with 83% residual activity remaining; however, the enzyme was completely unstable at pH 9.

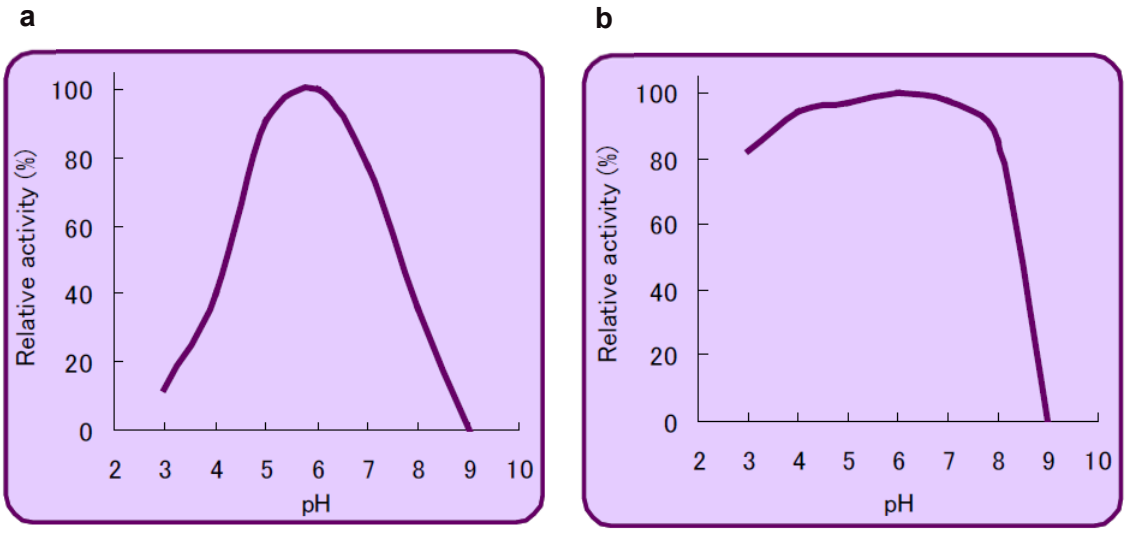
Table B.3.3.2-3 Relative Glucose Oxidase Activity of the Glucose Oxidase Food Enzyme from *Penicillium rubens* PGO 19-162 as a Function of pH at 37°C

pH	Relative Glucose Oxidase Activity (% of maximum activity)
3	12
4	40
5	91
6	100
7	78
8	36
9	0

Table B.3.3.2-4 pH Stability of the Glucose Oxidase from *Penicillium rubens* PGO 19-162 at 30°C after Pre-Incubation for 1 Hour

pH	Residual Glucose Oxidase Activity (%)
3	83
4	94
5	97
6	100
7	97
8	85
9	0

Figure B.3.3.2-2 Effect of pH on Glucose Oxidase the Glucose Oxidase Food Enzyme from *Penicillium rubens* PGO 19-162: a) Relative Enzyme Activity as a Function of pH and b) pH Stability of the Enzyme (Residual Activity)



B.3.3.3 Subsidiary and/or Side Activities

The glucose oxidase food enzyme does not exhibit any significant secondary enzymatic activities.

B.3.3.4 Stability of the Food Enzyme During Storage and Before Use

Shin Nihon has established that the shelf-life of the activity of glucose oxidase in final formulated glucose oxidase enzyme preparations is a minimum of 12 months when stored at temperatures of 5 to 20°C under dry conditions in the original packaging.

Following manufacture of the glucose oxidase food enzyme produced with *P. rubens* strain PGO 19-162, the enzyme is precipitated and the product dried and made into a powdered concentrate (containing 60,000 U/g of glucose oxidase activity) which is then formulated with the addition of maltodextrin to produce glucose oxidase enzyme preparations, which are the intended products marketed for use in food processing. Maltodextrin is added to adjust for glucose oxidase activity, thus generating less concentrated, but standardised, glucose oxidase enzyme preparations. These glucose oxidase enzyme preparations are stored under cool, dry conditions in fibre drums lined with polyethylene bags and sealed with a ring seal. Under these conditions, glucose oxidase activity is stable for at least 6 months.

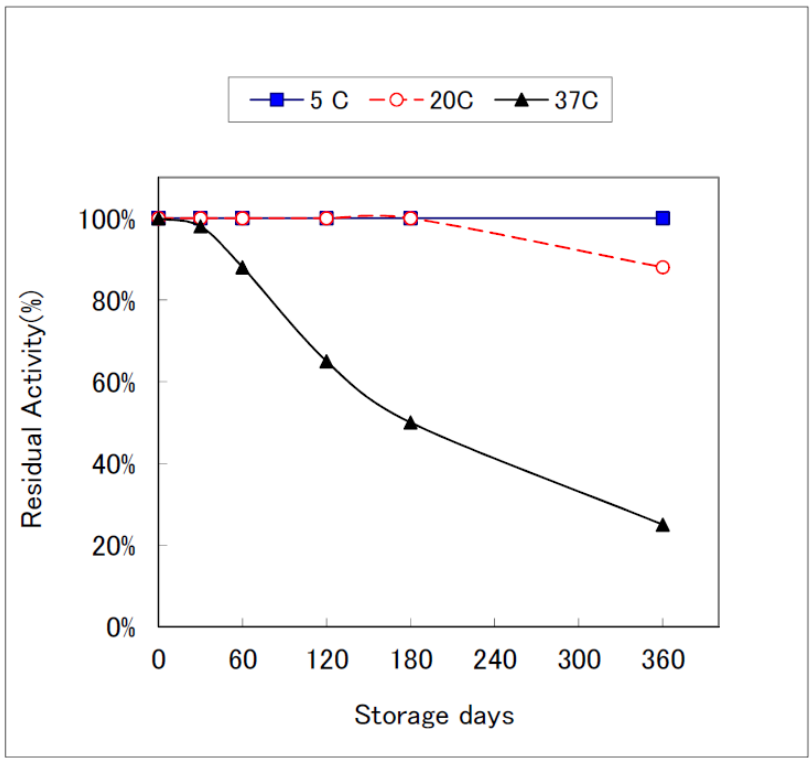
The product also may be stored under refrigerated conditions (*i.e.*, 5°C), which may extend the storage life.

Stability data supporting the 6-month shelf-life of the bulk material under the intended storage conditions are provided for a representative glucose oxidase enzyme preparation containing 15,000 U/g of glucose oxidase activity. These studies were performed on samples from 1 batch of the enzyme preparation stored at the recommended storage temperature of 20°C, under refrigerated conditions (*i.e.*, 5°C), or under accelerated conditions at 37°C over periods of up to 360 days (12 months) (1 sample per time point). Enzyme activity was measured at regular intervals using the assay method indicated above. The results of these studies are presented in Table B.3.3.4-1 and Figure B.3.3.4-1. No significant losses in glucose oxidase activity were observed during storage at the recommended storage temperature for 12 months or under refrigerated conditions. In the accelerated stability study conducted at 37°C, significant losses in glucose oxidase activity were observed after 2 months of storage, with progressive losses observed over the remaining 12-month period. The stability data therefore support a shelf-life of 12 months for glucose oxidase when the enzyme preparation is stored under the recommended storage conditions or at lower temperatures.

Table B.3.3.4-1 Stability of a Representative Sample of a Glucose Oxidase Enzyme Preparation (Prepared with the Glucose Oxidase Food Enzyme from *Penicillium rubens* PGO 19-162) with a Glucose Oxidase Activity of 15,000 U/g

Temperature (°C)	Residual Activity (% of initial activity)					
	Day 0	Day 30	Day 60	Day 120	Day 180	Day 360
5	100	100	100	100	100	100
20	100	100	100	100	100	88
37	100	98	88	65	50	25

Figure B.3.3.4-1 Stability of a Representative Sample of a Glucose Oxidase Enzyme Preparation (Prepared with the Glucose Oxidase Food Enzyme from *Penicillium rubens* PGO 19-162) with a Glucose Oxidase Activity of 15,000 U/g



B.3.3.5 Fate of the Food Enzyme During Food Processing and its Behaviour in the Food Matrix

The glucose oxidase food enzyme is intended for use during food and beverage processing to reduce the residual glucose and/or oxygen content during the production of a variety of foods and beverages. The enzymatic reaction catalysed by glucose oxidase leading to this effect is the oxidation of β-D-glucose to D-glucono-1,5-lactone (D-glucono-δ-lactone) in the presence of molecular oxygen, which, at the same time, converts oxygen to hydrogen peroxide. The enzyme therefore performs its catalytic function directly on β-D-glucose molecules present in various food matrices during processing of the foods.

As mentioned, the reaction products of glucose oxidase activity are D-glucono-1,5-lactone and hydrogen peroxide. D-Glucono-1,5-lactone is subsequently hydrolysed to gluconic acid by non-enzymatic means. No safety concerns are raised with respect to the D-glucono-1,5-lactone and gluconic acid products. With regards to hydrogen peroxide, the level of this substance that would be produced under the intended conditions of use of the glucose oxidase food enzyme would be equivalent to the levels produced by the current uses of glucose oxidase derived from other sources, which are considered safe. In addition, the enzyme will be used only at the level required to achieve the intended effect, limiting any excessive production of hydrogen peroxide. Furthermore, glucose oxidase is often used in conjunction with catalase (Crueger and Crueger, 1990; Wong *et al*., 2008), which catalyses the dismutation of hydrogen peroxide to oxygen and water. On this basis, no safety concerns are raised with respect to the production of hydrogen peroxide from the use of the glucose oxidase food enzyme in food production. No safety concerns are therefore raised with respect to the nature of the reaction products of the glucose oxidase food enzyme under the proposed use.

Any unused residual glucose oxidase following its addition during food processing is typically heat-denatured. While the enzyme may not necessarily be removed from the foods to which it is applied, the enzyme is inactivated (*i.e.*, the enzyme is denatured) at a temperature of 65°C (see Section B.3.3.2), and therefore, residues of the enzyme in finished food applications will be inactivated under food processing conditions consisting of high temperatures, such as those which occur during baking/cooking and pasteurisation/sterilisation. To confirm that the enzyme is denatured/inactivated under the intended conditions of use in the final foods for consumption, analytical testing for residual glucose oxidase activity was conducted on a representative food product, consisting of wheat flour for breadmaking. The glucose oxidase food enzyme produced with *P. rubens* PGO 19-162 was added to wheat flour [redacted] The flour was then used to make bread dough. The dough was baked at a temperature of 200 to 250°C. Enzyme extraction was performed on 1 g of the baked bread product (in 10 mL water) and glucose oxidase activity was measured. The level of glucose activity was below the limit of detection [redacted] for the glucose oxidase assay, thus confirming the absence of enzyme activity in the final product for consumption. Therefore, on the basis that the enzyme is denatured during food processing, the enzyme would have no technological effect on the final foods as consumed in these instances.

B.4 Manufacturing Process

B.4.1 Manufacturing Process

B.4.1.1 Identity of Raw Materials and Processing Aids

The agents, reagents, and materials used in the manufacture of the glucose oxidase food enzyme are provided in Appendix A. All processing aids used in the manufacture of the food enzyme are of high quality and considered acceptable for use in the manufacture of food enzymes. All filtration aids are those commonly used by the food industry in the purification of food ingredients.

B.4.1.2 Description of the Key Steps Involved in the Production Process

The glucose oxidase food enzyme produced with *P. rubens* PGO 19-162 is manufactured in compliance with current Good Manufacturing Practice (cGMP) and the principles of Hazard Analysis and Critical Control Points (HACCP). A detailed overview of the manufacturing process for the glucose oxidase food enzyme is provided in Appendix A, while a brief summary of the pertinent steps of the manufacturing process is provided below. The food enzyme is produced using food-grade materials and using quality-controlled fermentation and purification/recovery processes. All culture media are sterilised prior to use.

Both the seed inoculum and main culture, consisting of the production strain, are grown in culture media containing anti-foaming agents under submerged fermentation. The *P. rubens* PGO 19-162 seed inoculum is first cultivated then the main liquid culture medium is inoculated with the seed inoculum and cultivated. The glucose oxidase enzyme is secreted from the production strain into the culture medium during fermentation.

Fermentation is followed by a recovery/purification process consisting of solid/liquid separation, concentration, and filtration. The production organism is removed by solid/liquid separation and filtration; the filtrate containing the food enzyme is concentrated by ultrafiltration, removing proteins, saccharides, lipids, salts, and other compounds less than 5,000 MW. A final series of filtration steps is then applied to remove insoluble materials and any potential contaminating microorganisms and residual amounts of the production strain. The liquid supernatant is then passed through a ceramic filter. The product obtained at this stage is the food enzyme in an ultra-filtered liquid concentrate form. Prior to release for further processing and for the formulation of the final enzyme preparations, the food enzyme is tested to ensure compliance with the specifications established for the food enzyme. This step is set as a critical control point in the manufacture of the food enzyme. Batches that do not meet the specifications for the food enzyme are not released for further processing or for the formulation of final enzyme preparations.

It should be noted that Shin Nihon's glucose oxidase is currently manufactured outside of Australia/New Zealand. Since the food enzyme will not be manufactured in Australia or New Zealand, the fermentation substrates, production organisms, and all processing aids used in the manufacturing process will not enter the territory.

B.4.1.3 Process Controls and Quality Assurance Procedures

The glucose oxidase food enzyme is manufactured in compliance with the principles of HACCP and Shin Nihon has established appropriate quality control procedures to ensure production of a high-purity ultra-filtered liquid concentrate that is free of contaminants, including the use of an established safe production strain (*P. rubens*).

A HACCP plan is in place for the manufacture of the glucose oxidase food enzyme produced with *P. rubens* PGO 19-162 in which the critical control points have been identified and measures set in place for the prevention of the identified hazards. Quality control steps for these critical control points have been included as part of this plan in order to ensure adherence with the established manufacturing process and to produce a high quality and consistent product. These include measures to ensure that residual amounts of the *P. rubens* production strain are not transferred to the food enzyme. Furthermore, each manufactured batch of the food enzyme is analysed for conformity to the specifications set out in Section B.5.1. Batches that do not meet the specifications for the food enzyme are not released for further processing or for the formulation of final enzyme preparations.

B.5 Specification for Identity and Purity of Glucose Oxidase from *Penicillium rubens* (Strain PGO 19-162)

B.5.1 Product Specifications for Glucose Oxidase from *Penicillium rubens* (Strain PGO 19-162)

Shin Nihon has established food-grade specifications for glucose oxidase derived from *P. rubens* strain PGO 19-162 (Table B.5.1-1). The product specifications for the food enzyme comply with the current purity and microbial limits established for enzyme preparations by JECFA (2006a,b) and FCC (2018). In addition to the specifications listed in Table B.5.1-1, Shin Nihon has also established an internal specification for the glucose oxidase activity of the food enzyme. This specification has been established as not less than 1,400 U/g or U/mL. Glucose oxidase activity is measured using an internal method developed by Shin Nihon (see Appendix A for details of the method of analysis). All methods of analysis are internationally recognised or validated methods.

Table B.5.1-1 Specifications for the Glucose Oxidase Food Enzyme from *Penicillium rubens* Strain PGO 19-162

Specification Parameter	Specification	Method of Analysis
Compositional Parameters		
Total organic solids	1.5 to 2.5%	Calculation[a] (JECFA, 2006b)
Heavy Metals		
Arsenic	NMT 3 mg/kg	Japan's Specifications and Standards for Food Additives (7th Edition), B General Tests, Arsenic Limit Test (MHLW, 2000)
Lead	NMT 5 mg/kg	Japan's Specifications and Standards for Food Additives (7th Edition), B General Tests, Lead Limit Test (AAS) (MHLW, 2000)
Microbiological Parameters		
Total aerobic plate count	NMT 50,000 CFU/g	BAM – Chapter 3: Conventional plate count method (U.S. FDA, 2001)
Coliforms	NMT 30 CFU/g	BAM – Chapter 4: Conventional method for coliforms (U.S. FDA, 2002a)
Escherichia coli	Negative in 25 g	BAM – Chapter 4: Conventional method for *E. coli* (U.S. FDA, 2002a)
Salmonella species	Negative in 25 g	AOAC Method 989.13 (AOAC, 2000)
Antibacterial activity	Negative	Antibacterial activity (JECFA, 2006b)

AAS = atomic absorption spectrophotometry; AOAC = Association of Analytical Communities; BAM = Bacteriological Analytical Manual; CFU = colony forming units; NMT = not more than.

[a] Total organic solids = 100% - (A+W+D), where A = % ash, W = % water, and D = % diluents and/or other formulation ingredients.

B.5.2 Product Analysis

B.5.2.1 Batch Analyses

Analytical data on 3 non-consecutive batches (Lot No. 110818R, 111214R, and 120229R) of the glucose oxidase food enzyme are presented below in Table B.5.2.1-1 and demonstrate that the manufacturing process produces a consistent product that meets the product specifications defined in Section B.5.1. Heavy metal contaminants (*i.e.*, arsenic and lead) were below the limit of detection of 3 mg/kg and 5 mg/kg, respectively. In addition, microbiological contaminants were also below the limit of detection and/or in compliance with the specifications for the food enzyme. All batches also were negative for antibacterial activity, which is consistent with the fact that *P. rubens* PGO 19-162 does not produce any detectable levels of antibacterial activity despite potential production of meleagrin and xanthocillin. The certificates of analysis are provided in Appendix D.

Table B.5.2.1-1 Batch Analyses for the Glucose Oxidase Food Enzyme from *Penicillium rubens* PGO 19-162 Demonstrating Compliance with the Specifications for the Food Enzyme

Specification Parameter	Specification	Manufacturing Lot No.		
		110818R	**111214R**	**120229R**
Compositional Parameters				
Total organic solids (%)	1.5 to 2.5	1.9	2.0	1.8
Heavy Metals				
Arsenic (mg/kg)	NMT 3 mg/kg	NMT 3	NMT 3	NMT 3
Lead (mg/kg)	NMT 5 mg/kg	NMT 5	NMT 5	NMT 5
Microbiological Parameters				
Total aerobic plate count (CFU/g)	NMT 50,000	NMT 10	NMT 10	NMT 10
Coliforms (CFU/g)	NMT 30	NMT 30	NMT 30	NMT 30
Escherichia coli (CFU/25 g)	Negative in 25 g	Negative	Negative	Negative
Salmonella species (CFU/25 g)	Negative in 25 g	Negative	Negative	Negative
		060722-02	**111102-02**	**120229-01**
Antibacterial activity	Negative	Negative	Negative	Negative

CFU = colony forming units; NMT = not more than.

Further detailed compositional data on the same 3 non-consecutive batches of the glucose oxidase food enzyme are provided in Table B.5.2.1-2. All methods of analysis are either internationally recognised methods or internally developed by Shin Nihon and validated. As mentioned in Section B.3.1, the majority of the food enzyme is composed of water (approximately 98%) and the protein and ash contents are approximately 0.6 and 0.4%, respectively, based on batch analyses. The TOS[1] content of the food enzyme is calculated to be approximately 1.9% based on batch analyses but can range from 1.5 to 2.5%. The TOS content is based on the ash and water contents (the food enzyme does not contain any added diluents). The certificates of analysis are provided in Appendix D.

[1] TOS = 100% - (A+W+D), where A= % ash, W= % water and D= % diluents and/or other additives and formulation ingredients.

Table B.5.2.1-2 Additional Compositional Analyses for the Glucose Oxidase Food Enzyme from *Penicillium rubens* PGO 19-162

Parameter	Methods of Analysis	Manufacturing Lot No.		
		110818R	111214R	120229R
Water (%)	Loss on drying (JECFA, 2006c)	97.7	97.6	97.9
Protein (%)	Nitrogen determination (Kjeldahl method) (JECFA, 2006c)	0.6	0.6	0.5
Ash (%)	Ash (total) (JECFA, 2006c)	0.4	0.4	0.4

B.5.2.2 Mycotoxins and Secondary Metabolites

Mycotoxin analyses for 3 non-consecutive batches of the glucose oxidase food enzyme demonstrate the absence of aflatoxins (B1, B2, G1, and G2), sterigmatocystin, zearalenone, ochratoxin A, T-2 toxin, chrysogine, and roquefortine C in the final product. These batch analyses were conducted on the dried powdered concentrate (containing 60,000 U/g of glucose oxidase activity) produced from the ultra-filtered liquid concentrate. All mycotoxins and secondary metabolites were determined to be below the limit of detection in all 3 batches tested (Table B.5.2.2-1). These mycotoxin parameters do not form part of the specifications for the food enzyme but were included as part of the batch analyses to confirm absence of potential mycotoxin production. The full study report is provided in Appendix D.

Table B.5.2.2-1 Mycotoxin Analyses for the Dried Glucose Oxidase Food Enzyme from *Penicillium rubens* PGO 19-162

Parameter	Method of Analysis	Limit of Detection	Manufacturing Lot No.		
			110504R1-01	100119R1-01	070606-03
Ochratoxin A	HPLC (external method)	0.5 µg/kg	ND	ND	ND
Aflatoxin (B_1, B_2, G_1, G_2)	HPLC (external method)	0.5 µg/kg each	ND	ND	ND
Zearalenone	HPLC (external method)	50 µg/kg	ND	ND	ND
Sterigmatocystin	HPLC (external method)	20 µg/kg	ND	ND	ND
T-2 toxin	LC/MS (external method)	50 µg/kg	ND[a]	ND	ND
Roquefortine C	HPLC [Modified method of O'Brien *et al*. (2006)]	0.25 mg/kg	**130418Z1-01**	**130214R1-01**	**121107R1-01**
			ND	ND	ND
Chrysogine	UHPLC-DAD-TOFMS (Klitgaard *et al*., 2014)	9 µg/kg	**121107R1-01**	**120418R1-01**	**130123R1-01**
			ND	ND	ND

HPLC = high-performance liquid chromatography; ND = not detected; TOFMS = time-of-flight mass spectroscopy; ULPLC-DAD = high-performance liquid chromatography-diode array detection.
[a] New limit of detection = 0.1 ppm

B.6 Analytical Method for Detection

According to Section 3.3.2 of the FSANZ *Application Handbook* (FSANZ, 2019), an analytical method for detection is not required in the case of an enzymatic processing aid, and this section is therefore not relevant to glucose oxidase derived from *P. rubens*.

C. INFORMATION RELATED TO THE SAFETY OF AN ENZYME PROCESSING AID

In accordance with Parts C and D of Section 3.3.2 – Processing Aids of the FSANZ *Application Handbook* (FSANZ, 2019), the following safety information must be provided for enzyme processing aids:

1. General information on the use of the enzyme as a food processing aid in other countries;
2. Information on the potential toxicity of the enzyme processing aid;
3. Information on the potential allergenicity of the enzyme processing aid;
4. Safety assessment reports prepared by international agencies or other national government agencies, if applicable;
5. Information on the source microorganism;
6. Information on the potential pathogenicity and toxicity of the source microorganism; and
7. Information on the genetic stability of the source organism.

Each point is addressed in the following subsections.

C.1 General Information on the Use of the Enzyme as a Food Processing Aid in Other Countries

Glucose oxidase derived from microbial and fungal sources, including *A. niger*, *A. oryzae*, *P. chrysogenum*[2], and *T. reesei*, are permitted for use as a processing aid in food processing (*e.g.*, baking processes) or as a food additive (uses not specified) in Australia and New Zealand, China, France, South Korea, and Japan (JORF, 2006; Ministry of Health of the PRC, 2014; MHLW, 2018; FSANZ, 2002 2020c; MFDS, 2020). In addition, glucose oxidase from *P. rubens* PGO 19-162 has been determined to be Generally Recognized as Safe (GRAS) for use in a variety of food categories in the United States (U.S.). The enzyme is specifically GRAS for use in alcoholic beverages; baked goods and baking mixes; beverages and beverage bases; coffee and tea; condiments and relishes; egg products; gelatines, puddings and fillings; grain products and pastas; processed fruits and fruit juices; processed vegetables and vegetable juices; and snack foods at a maximum level of use of 30 mg TOS/kg in foods and 3 mg TOS/kg in beverages. Shin Nihon has received a "no questions letter" from the U.S. Food and Drug Administration (FDA) regarding the company's conclusion that glucose oxidase from *P. rubens* PGO 19-162 is GRAS under the intended conditions of use (U.S. FDA, 2014). The European Food Safety Authority (EFSA) recently evaluated the safety of glucose oxidase produced from genetically modified *A. niger* and *A. oryzae* and concluded that there are no safety concerns under their intended conditions of use in baking processes (EFSA, 2018, 2019).

In addition, according to the Association of Manufacturers and Formulators of Enzyme Products (AMFEP), glucose oxidase derived from non-genetically modified *P. chrysogenum* is listed for use in food processing (Amfep, 2015). A general non-exhaustive list of evaluations and authorisations for glucose oxidase derived from various sources is presented below in Table C.1-1.

Table C.1-1 General Non-Exhaustive List of Evaluations and Authorisations for Glucose Oxidase Derived from Various Sources

Jurisdiction	Evaluating/ Authoritative Body	Enzyme Source	Permitted Uses	Reference
Australia and New Zealand	FSANZ	*Aspergillus niger*, *Aspergillus oryzae* genetically modified to carry the gene for glucose oxidase from *Aspergillus niger*, *Trichoderma reesei* genetically modified to carry the gene for glucose oxidase from *Penicillium amagasakiense* (Gazette Amendment No. 192, 14 May 2020)	Processing aid	FSANZ (2002; 2020a,b)
Canada	Health Canada Food Directorate	*Aspergillus niger J39, Aspergillus niger* ZGL528-72, *Aspergillus niger* var., *Aspergillus oryzae* Mtl-72 (pHUda107), *Trichoderma reesei* RF11400	Bread; Flour; Whole wheat flour; Pasta; Surface of shredded cheese; Unstandardized bakery products; Soft drinks; Liquid egg white, whole egg, or yolk destined for drying	Food and Drug Regulations – List of Permitted Food Enzymes Health Canada (2020)
China	Ministry of Health	*Aspergillus niger*, *Aspergillus oryzae* genetically modified to carry the gene for glucose oxidase from *Aspergillus niger*, *Penicillium chrysogenum*	In food processing (uses not specified)	National Standard on Food Safety – Standard for Use of Food Additives GB 2760-2014, Table C.3

[2] Also known as *Penicillium rubens.*

Table C.1-1 General Non-Exhaustive List of Evaluations and Authorisations for Glucose Oxidase Derived from Various Sources

Jurisdiction	Evaluating/ Authoritative Body	Enzyme Source	Permitted Uses	Reference
				(Ministry of Health of the PRC, 2014)
France	L'Agence francaise de sécurité sanitatire des aliments	*Aspergillus niger*	Biscuits; Pastries; Bread making (with the exception of traditional French bread); Eggs	Arrêté du 19 octobre 2006 relatif à l'emploi d'auxiliaires technologiques dans la fabrication de certaines denrées alimentaires (JORF, 2006)
		Aspergillus oryzae genetically modified to carry the gene encoding glucose oxidase from *Aspergillus niger*	Bread making (with the exception of traditional French bread); Fine bakery	
South Korea	Korean Food and Drug Administration	*Aspergillus niger* var., *Penicillium chrysogenum* var.	Food additive	MFDS (2020)
Japan	Ministry of Health, Labour, and Welfare	*Penicillium* sp.	Food additive	MHLW (2018)
United States	U.S. FDA	*Aspergillus niger*	Food additive	GRN 89 (U.S. FDA, 2002b)
		Aspergillus oryzae	Baking applications; Manufacture of cheese; Beer, carbonated beverages, and fruit juice	GRN 106 (U.S. FDA, 2002c)
		Penicillium chrysogenum	Various foods and powdered, dried eggs	GRN 509 (U.S. FDA, 2014)
		Trichoderma reesei	Baking processes	GRN 707 (U.S. FDA, 2017)
European Union	EFSA	Genetically modified *Aspergillus oryzae*	Baking processes	EFSA (2018)
		Genetically modified *Aspergillus niger*	Baking processes	EFSA (2019)
International	JECFA	*Aspergillus niger*	In food processing (uses not specified) ADI: not specified[a]	JECFA (1974)

ADI = acceptable daily intake; EFSA = European Food Safety Authority; FSANZ = Food Standards Australia New Zealand; GRN = Generally Recognized as Safe (GRAS) notice; JECFA = Joint FAO/WHO Expert Committee on Food Additives; U.S. FDA = United States Food and Drug Administration.

[a] An ADI of not specified is a term applicable to a food substance of very low toxicity which, on the basis of the available data (chemical, biochemical, toxicological, and other), the total dietary intake of the substance arising from its use at the levels necessary to achieve the desired effect and from its acceptable background in food does not, in the opinion of JECFA, represent a hazard to health.

C.2 Information on the Potential Toxicity of the Enzyme Processing Aid

C.2.1 Toxicological Studies

Shin Nihon's glucose oxidase food enzyme produced with *P. rubens* PGO 19-162 was assessed in a series of *in vitro* genotoxicity tests (bacterial reverse mutation test and an *in vitro* mammalian chromosomal aberration test), as well as in a repeated-dose 90-day oral toxicity study conducted in rats (Konishi *et al*., 2013). While not a required test, a combined *in vivo* mammalian erythrocyte micronucleus test and comet assay also has been performed on the food enzyme. All tests were performed in compliance with the Organisation of Economic Co-operation and Development (OECD) Principles of Good Laboratory Practice (GLP) (OECD, 1998a) and in accordance with the OECD Guidelines for the testing of chemicals, where applicable. All toxicology tests were performed on a batch representative of the food enzyme

(Lot No. 090506R) before the addition of other components of the food enzyme preparation. The certificate of analysis for this batch is provided in Appendix D. The studies are summarised in the sections that follow.

As discussed in Section C.1 and C.4, glucose oxidase from various fungal sources have been concluded to be GRAS and no questions have been raised by the U.S. FDA. Most recently, the GRAS status of glucose oxidase from *T. reesei* was notified to the U.S. FDA and filed without objection under GRAS notice (GRN) 707 (U.S. FDA, 2017). Thus, an updated search of the scientific literature was performed using the electronic search tool, ProQuest Dialog™, with several databases, including Adis Clinical Trials Insight, AGRICOLA, AGRIS, Allied & Complementary Medicine™, BIOSIS® Toxicology, BIOSIS Previews®, CAB ABSTRACTS, Embase®, Foodline®: SCIENCE, FSTA®, MEDLINE®, NTIS: National Technical Information Service, and ToxFile®. No relevant information was identified that have become available since 2017. Thus, the safety of Shin Nihon's glucose oxidase from *P. rubens* is supported by the product-specific toxicology studies as discussed by Konishi *et al.* (2013) and the conclusions of multiple authoritative and regulatory bodies that have reviewed the safety of glucose oxidase (see Section C.4).

C.2.1.1 Genotoxicity

Bacterial Reverse Mutation Test

The potential mutagenicity of the glucose oxidase food enzyme was evaluated in the bacterial reverse mutation test (Ames test) performed according to OECD Test No. 471 (OECD, 1997a) (Konishi *et al*., 2013). The test was conducted in *Salmonella* Typhimurium TA100, TA98, TA1535, and TA1537 and *Escherichia coli* WP2*uvr*A in the presence and absence of S9 metabolic activation. For the purposes of this test, the glucose oxidase enzyme was rendered inactive by heating to a temperature of 60°C and adjusting the pH to 2. The pH was then re-adjusted to the original pH of the test substance (approximately 4). The enzyme was inactivated on the basis that the reaction catalysed by glucose oxidase produces hydrogen peroxide as a by-product. Hydrogen peroxide is a well-known reactive oxygen species, which may cause cellular and sub-cellular oxidative damage, including damage to DNA. Thus, to ensure that secondary genetic mutations arising from potential hydrogen peroxide production would not occur, the enzyme was inactivated. A reference control [vehicle control (water) treated in the same manner as the test article during pH adjustment] was included to control for the pH adjustment treatment, and all test results were compared to the value obtained with the reference control. Appropriate positive controls also were included. A preliminary concentration-range finding test and a main 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 concentration-range finding test and the main test, no positive mutagenic responses (*i.e.,* reproducible 2-fold or dose-dependent induction in the number of revertant colonies compared to the reference control) were observed in any strain at any concentration tested in either the presence or absence of metabolic activation. In contrast, positive control substances displayed marked mutagenic activity. Based on the results of this study, it was concluded that the glucose oxidase food enzyme was non-mutagenic in the bacterial reverse mutation test.

In Vitro Mammalian Chromosomal Aberration Test

The clastogenic potential of the glucose oxidase food enzyme was investigated in an *in vitro* chromosomal aberration test conducted in cultured human lymphocytes in accordance with OECD Test No. 473 (OECD, 1997b), using both the short-term (3-hour) and continuous (24-hour) treatment methods (Konishi *et al*., 2013). The short-term assay was conducted in the presence and absence of S9 metabolic activation and the continuous assay was conducted in the absence of metabolic activation. The glucose oxidase enzyme was inactivated as in the bacterial reverse mutation test. Distilled water served as the negative control. Mitomycin C was used as the positive control in assays conducted in the absence of metabolic activation and cyclophosphamide was used as the positive

control in the presence of metabolic activation. All control and treatments were conducted in duplicate. In the short-term assays, human lymphocytes were incubated with the test article at concentrations of 0.0151, 0.0303, 0.0605, 0.121, 0.242, and 0.484 mg TOS/mL. In the 24-hour continuous treatment assay, lymphocytes were incubated with the test article at concentrations of 0.000118, 0.000236, 0.000472, 0.000945, 0.00188, and 0.00378 mg TOS/mL. The lowest test article concentration resulting in a relative mitotic index (MI) of less than 50% was 0.242 mg TOS/mL under short-term treatment with and without S9 metabolic activation and 0.000945 mg TOS/mL under continuous treatment. Thus, the test article concentrations that were assessed for structural chromosome aberrations were 0.0605, 0.121, and 0.242 mg TOS/mL in the short-term assays and 0.000236, 0.000472, and 0.000945 mg TOS/mL in the continuous assay. A concentration-dependent decrease in the relative MI was observed at concentrations of 0.0605, 0.121, and 0.242 mg TOS/mL in both short-term assays and at concentrations of 0.000236, 0.000472, and 0.000945 mg TOS/mL in the continuous assay. In all assays, no statistically significant differences in the incidence of cells with structural chromosome aberrations were observed in cells treated with glucose oxidase compared to the negative control. In addition, the incidence of polyploidy cells in cells treated with the food enzyme was not significantly different from that of the negative control group in all assays. In contrast, treatment with positive control agents produced a significant increase in the percentage of cells with structural chromosome aberrations compared to the negative control. Based on the results of this study, it was concluded that the glucose oxidase food enzyme is non-clastogenic in human lymphocytes.

Combined *In Vivo* Mammalian Erythrocyte Micronucleus test and Comet Assay

The genotoxic potential of the glucose oxidase food enzyme was further investigated in a combined *in vivo* mammalian erythrocyte micronucleus test and comet assay conducted in rats (Konishi *et al*., 2013). The mammalian erythrocyte micronucleus test portion of this study was conducted in accordance with OECD Test No. 474 (OECD, 1997c). The glucose oxidase enzyme was not inactivated for the purposes of this test. Groups of 5 male Crl:CD(SD)[SPF] rats were orally administered glucose oxidase at doses providing 48.5 (low-dose), 96.7 (mid-dose), or 193 (high-dose) mg TOS/kg body weight/day for 3 consecutive days. A negative control group received 10 mL/kg body weight/day of the vehicle control (distilled water), and a positive control group received 200 mg/kg body weight/day of ethyl methanesulphonate (EMS). Body weights were measured prior to the dosing period and before necropsy. All animals were observed for clinical signs of toxicity once daily after dosing and prior to necropsy. All rats were euthanised 3 hours following the final dose, and their glandular stomach, liver, and femur were removed. Bone marrow samples were obtained from the femur and assessed in the micronucleus assay. The stomach and liver were examined macroscopically, and cell suspensions prepared from these organs were examined for DNA damage in the comet assay.

No clinical signs of toxicity or adverse effects on body weight gain were observed in the glucose oxidase groups. In addition, there were no macroscopic findings observed in the liver or stomach of all animals. In the micronucleus test, there were no significant differences in the frequency of micronucleated cells in the glucose oxidase groups compared to the negative control group. A significant increase in the ratio of immature erythrocytes to the total number of analysed erythrocytes was observed in the mid-dose group; however, this finding is not considered to be toxicologically significant due to the absence of a dose-response relationship. In the comet assay, no evidence of increased DNA damage was observed in liver or stomach cells of rats administered glucose oxidase compared to the negative control. Administration of EMS resulted in significant increases in the frequency of micronucleated cells in the micronucleus test and DNA damage in the comet assay compared to the negative control. Based on these findings, it was concluded that the glucose oxidase food enzyme was non-genotoxic *in vivo* in the mammalian erythrocyte micronucleus test and the comet assay.

C.2.1.2 Repeat-Dose Toxicity

The sub-chronic oral toxicity of the glucose oxidase food enzyme was assessed in a 90-day toxicity study conducted in rats, which was performed in accordance with OECD Test No. 408 (OECD, 1998b) (Konishi *et al*., 2013). The doses administered in the study were selected based on the results of a 14-day dose-range finding study in which no compound-related adverse effects were observed in Crl:CD(SD) rats (5 animals/sex/group) at doses providing 1.93 (low-dose), 19.3 (mid-dose), and 193 (high-dose) mg TOS/kg body weight/day administered by gavage. A group of 5 male and 5 female control animals were also administered the vehicle control (distilled water). Based on the results of the dose-range finding study, 193 mg TOS/kg body weight/day was selected as the high-dose to be administered in the 90-day study.

In the sub-chronic study, groups of 10 male and 10 female Crl:CD(SD) rats were orally administered the food enzyme by gavage at doses providing 1.93 (low-dose), 19.3 (mid-dose), and 193 (high-dose) mg TOS/kg body weight/day for 90 days. The control group consisted of 10 males and 10 females administered the vehicle control (water). All animals were observed twice daily for clinical signs, and individual body weights and food consumption were measured weekly. Ophthalmological examinations were conducted in control and high-dose animals prior to study initiation and on Day 83. Fasting blood samples were obtained from all animals on Days 91 or 92 for routine haematological, blood coagulation, and serum chemistry analyses. Urine samples were collected on Day 85 following overnight fasting for urinalysis. All animals were euthanised on the day after the last dose administration. Complete necropsies were conducted on all animals, and their organs were removed and weighed. Histopathological examination of organs and tissues was conducted for all animals in the control and high-dose groups.

No mortalities were observed during the 90-day study period, and no compound-related clinical signs or ophthalmological abnormalities were observed. In addition, no significant differences in body weight, body weight gain, or food consumption were observed between the control group and any of the glucose oxidase-administered dose groups throughout the course of the study.

No significant differences in any of the blood coagulation parameters were observed between the control and glucose oxidase-administered dose groups. Statistically significant differences in haematology parameters observed in the glucose oxidase groups compared to the control group included the following: increased white blood cell count, neutrophil count, and large unstained cell count in low-dose females and decreased platelet count in mid-dose males. In addition, clinical chemistry analysis revealed significantly increased γ-globulin ratio and concentration in low-dose females, as well as significantly increased blood glucose levels in high-dose males. The haematology and clinical chemistry variations observed in the low- and mid-dose groups were not observed at higher dose levels (*i.e.*, changes were non-dose-dependent) and were not consistent among sexes. Moreover, although blood glucose levels were increased in males of the high-dose group, this value (167 ± 13 mg/dL) remained within the range of historical background data (154 ± 26 mg/dL, n=70). Furthermore, none of the changes observed were associated with compound-related histopathological abnormalities, and therefore, the variations observed were considered to be incidental and not compound-related or toxicologically significant.

Several statistically significant differences in urinary electrolyte parameters were observed in the glucose oxidase groups compared to the control group and included the following changes: increased urinary sodium concentration, total sodium excretion, and total potassium excretion in high-dose males and increased urinary potassium concentration in high-dose females. In addition, a significant non-dose-dependent decrease in urinary chloride concentration was observed in mid- and high-dose females compared to control females. This latter effect was associated with a significant decrease in total chloride excretion in these groups, which appeared to be dose-dependent. All urinary electrolyte changes, however, were small in magnitude and did not result in any effects on urinary volume or blood

electrolyte concentrations and were not accompanied by any clinical findings which might lead to changes in electrolyte levels. Furthermore, changes in urinary electrolyte parameters were not accompanied by changes in clinical chemistry indices that would suggest renal toxicity (*i.e.*, blood urea nitrogen and creatinine concentrations) or any compound-related gross (including changes in kidney weights) or histopathological manifestation of nephropathy. Therefore, the urinary electrolyte changes observed were not considered to be toxicologically significant.

Absolute seminal vesicle weights in low-dose males were significantly increased compared to controls; however, this effect was not considered to be compound-related due to the lack of a dose-response relationship and no effects on relative seminal vesicle weights. Relative thymus weights of high-dose males were significantly decreased compared to controls; however, the investigators noted that this reduction was an artifact of increased thymus weights in the control group (0.073 ± 0.018%) as determined from a review of the historical control data (0.06 ± 0.04%, n=115). Moreover, the relative thymus weights in all glucose oxidase groups were within the range of historical background data (low-dose = 0.059 ± 0.013%, mid-dose = 0.060 ± 0.008%, high-dose = 0.058 ± 0.010%). Therefore, the variation in thymus weight was not considered to be compound-related or toxicologically relevant. No other significant differences in absolute or relative organ weights were observed among groups. Furthermore, no compound-related macroscopic or histopathological findings were observed. Based on the results of this study, the no-observed-adverse-effect level (NOAEL) for the oral toxicity of glucose oxidase food enzyme was determined to be 193 mg TOS/kg body weight/day, the highest dose tested.

C.2.1.3 Data Reporting

The data reported in the original study reports for all toxicological tests performed followed the recommendations for data reporting provided in the relevant OECD guidelines.

The test material used in all toxicological studies is representative of the glucose oxidase food enzyme produced with *P. rubens* PGO 19-162 as described in the present application. Analytical data demonstrating compliance with the specifications for the food enzyme presented in Section B.5.1 and consistency with compositional analyses provided in B.5.2 are summarised in Table C.2.1.3-1. The certificate of analysis for this batch is provided in Appendix D. Internal analysis for glucose oxidase activity of the test material used in the toxicological studies is provided in Appendix A.

Table C.2.1.3-1 Analysis for the Glucose Oxidase from *Penicillium rubens* PGO 19-162 Used in Toxicological Tests

Specification Parameter	Specification	Manufacturing Lot No. 090506R
Compositional Parameters		
Total organic solids	1.5 to 2.5%	1.93 %
Water	n/a (analytical data = 97.6 to 97.9%)	97.7%
Protein	n/a (analytical data = 0.5 to 0.6%)	0.6%
Ash	n/a (analytical data = 0.4%)	0.4%
Heavy Metals		
Arsenic	NMT 3 mg/kg	<3 mg/kg
Lead	NMT 5 mg/kg	<5 mg/kg
Microbiological Parameters		
Total aerobic plate count	NMT 50,000 CFU/g	<10 CFU/g
Coliforms	NMT 30 CFU/g	<30 CFU/g
Escherichia coli	Negative in 25 g	Negative in 25 g
Salmonella species	Negative in 25 g	Negative in 25 g
Mould	NMT 100 CFU/g	<10 CFU/g
Antibacterial activity	Negative	Negative

CFU = colony forming units; n/a = not applicable; NMT = not more than.

C.2.1.4 Review of the Toxicological and Exposure Data and Conclusions

The full set of toxicological tests required for food enzymes were performed on Shin Nihon's glucose oxidase food enzyme produced with *P. rubens* PGO 19-162. The food enzyme was assessed in a series of genotoxicity tests (including a bacterial reverse mutation test, an *in vitro* mammalian chromosomal aberration test, and a combined *in vivo* mammalian erythrocyte micronucleus test and comet assay), as well as in a repeated-dose 90-day oral toxicity study conducted in rats. The food enzyme was non-mutagenic in the bacterial reverse mutation test, non-clastogenic in human lymphocytes in the *in vitro* chromosomal aberration test, and non-genotoxic *in vivo* in the mammalian erythrocyte micronucleus test and the comet assay. In the 90-day oral toxicity study conducted in Crl:CD(SD) rats, no compound-related adverse effects were observed at up to the highest dose tested of 193 mg TOS/kg body weight/day. Thus, based on the results of this study, the NOAEL for the oral toxicity of the glucose oxidase food enzyme was determined to be 193 mg TOS/kg body weight/day, the highest dose tested. In comparison, the theoretical maximum daily intake (TMDI) for the glucose oxidase food enzyme using the Budget Method assumptions for potential exposure was calculated to be 0.63 mg TOS/kg body weight/day (see Section D.1). Thus, a large margin of safety exists between the NOAEL for the glucose oxidase food enzyme and the estimated maximum potential daily intakes on a TOS basis. No toxicity concerns are therefore raised with respect to the conditions of use of the glucose oxidase food enzyme produced with *P. rubens* PGO 19-162.

C.3 Information on the Potential Allergenicity of the Enzyme Processing Aid

C.3.1 Source of the Processing Aid

The glucose oxidase as described herein is derived from a non-genetically modified strain of *P. rubens*, a filamentous fungus with a history of safe use in Europe as a starter culture for the production of dry sausages (Sunesen and Stahnke, 2003) and in the fermentation and biopreservation of meat (Mogensen *et al.*, 2002). *P. chrysogenum*, also known as *P. rubens* based on current reclassification of the species, also has a history of safe use as a production organism used in the production of food enzymes. As indicated in Section C.1, *P. chrysogenum* is listed by AMFEP as a current source of commercialised glucose oxidase enzyme preparations used in food processing in the EU (Amfep, 2015). *Penicillium* (species not specified) also is an approved source of glucose oxidase in Japan as indicated in the list of existing food additives (MHLW, 2011). As such, the production strain *P. rubens* PGO 19-162 has been used in Japan in the commercial production of Shin Nihon's glucose oxidase food enzyme. Shin Nihon's glucose oxidase enzyme preparations derived from *P. rubens* PGO 19-162 have been commercially marketed in Japan since 1999, and have been used in the production of foods, such as baked goods, cooked rice, and mayonnaise. *P. rubens* PGO 19-162 therefore has a history of safe use in food enzyme production in Japan.

C.3.2 Allergenicity of the Enzyme

As reported by Pariza and Foster (1983), "*Allergies and primary irritations from enzymes used in food processing should be considered a low priority item of concern except in very unusual circumstances*". To confirm that glucose oxidase produced by *P. rubens* does not contain amino acid sequences similar to known allergens that might produce an allergenic response, a sequence homology search was conducted using the AllergenOnline database version 20 (available at http://www.allergenonline.org; updated 10 February 2020) maintained by the Food Allergy Research and Resource Program of the University of Nebraska (FARRP, 2020). The database contains a comprehensive list of allergenic proteins (and presumed allergens) developed *via* a peer reviewed process for the purpose of evaluating food safety. A full-length alignment search of AllergenOnline was conducted using default settings (*E* value cut-off = 1 and maximum alignments of 20). A search of the full-length amino acid sequence of glucose oxidase revealed 1 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. The sequence identity was <50% over the length of the glucose oxidase sequence and was therefore unlikely to share immunologic or allergic cross-reactivity (Hileman *et al*., 2002).

A second homology search was conducted according to the approach outlined by the Food and Agriculture Organization of the United Nations (FAO), the World Health Organization (WHO) (FAO/WHO, 2001), and the Codex Alimentarius Commission (Codex Alimentarius, 2009). In accordance with this guideline, the AllergenOnline database was searched using a sliding window of 80-amino acid sequences (segments 1–80, 2–81, 3–82, *etc*.) derived from the full-length glucose oxidase amino acid sequence from *P. rubens* PGO 19-162. The 80-amino acid alignment search was conducted using default settings (*E* value cut-off = 1 and maximum alignments of 20). According to the approach adopted by the Codex Alimentarius Commission, significant homology is defined as an identity match of greater than 35%, and in such instances, cross-reactivity with the known allergen should be considered a possibility. Using this search strategy, 1 identity match of 45% to the Mala s 12 allergen precursor produced by *M. sympodialis* was identified, consistent with the results of the full-length alignment search. However, Goodman *et al.* (2005) and Goodman and Hefle (2005) discuss this approach of evaluating segments of 80 amino acids to be quite conservative and precautionary. On a similar note, Aalberse (2000) notes that proteins with less than 50% identity over the length of the proteins are rarely cross-reactive; a 70% identity is typically required for cross-reactivity. Therefore, considering that the percent identity of the full-length amino acid sequences was low at 31%, the potential for cross-reactivity to the Mala s 12 allergen precursor is low. A third homology search conducted using the exact 8-mer approach did not produce any matches.

The sequence homology searches were performed on 05 October 2020, and the reports are provided in Appendix E.

The allergenicity of glucose oxidase was also considered through a search of the available scientific literature; no relevant information was identified.

Based on the information provided above, no evidence exists that might indicate that the glucose oxidase produced by *P. rubens* PGO 19-162 would produce an allergenic response following consumption of foods to which the enzyme is added. Additionally, there is no evidence from the available scientific literature or from the history of use of glucose oxidase enzyme preparations formulated with Shin Nihon's glucose oxidase food enzyme in Japan indicating allergenicity to the enzyme in consumers. Based on this information, no evidence exists that might indicate that glucose oxidase produced by *P. rubens* strain PGO 19-162 would produce an allergenic response following use in food processing.

C.4 Safety Assessment Reports Prepared by International Agencies or Other National Government Agencies

Glucose oxidase derived from various source organisms have been reviewed and approved for use as a processing aid in food processing or as a food additive in Australia and New Zealand, Canada, China, France, South Korea, and Japan (see Section C.1 for further details). In particular, glucose oxidase from *A. niger*, *A. oryzae*, *P. chrysogenum,* and *T. reesei* has been determined to be GRAS for use in a variety of food categories in the U.S., and the notices are briefly discussed below.

The Enzyme Technical Association's glucose oxidase from *A. niger* is intended for general use in foods at use levels in accordance with cGMP. The GRAS status was notified to the U.S. FDA (GRN 89), and the U.S. FDA responded with a "no questions" letter in April 2002 (U.S. FDA, 2002b).

Novozymes determined the use of glucose oxidase from *A. oryzae* genetically modified to express the enzyme from *A. niger* to be GRAS (GRN 106). The enzyme preparation is intended for use in baking applications and in the manufacture of cheese, beer, carbonated beverages, and fruit juice at use levels

in accordance with cGMP, with maximum suggested dosage of up to 500 U/L. The U.S. FDA responded with a "no questions" letter in October 2002 (U.S. FDA, 2002c).

As mentioned previously in Section C.1, glucose oxidase from *P. rubens* PGO 19-162 has been concluded by Shin Nihon to be GRAS for its intended use in alcoholic beverages; baked goods and baking mixes; beverages and beverage bases; coffee; tea; condiments and relishes; egg products; gelatines; puddings and fillings; grain products and pastas; processed fruits and fruit juices; processed vegetables and vegetable juices; and snack foods at a maximum level of use of 30 mg TOS/kg in foods and 3 mg TOS/kg in beverages (GRN 509). Shin Nihon's GRAS conclusions were filed without objection from the U.S. FDA in 2014 under GRN 509 (U.S. FDA, 2014).

More recently, glucose oxidase from genetically modified *T. reesei* expressing the enzyme from *Penicillium* has been concluded to be GRAS by AB Enzymes GmbH (GRN 707). The enzyme is intended for use in baking processes under conditions of cGMP, with a recommended use level of 10 mg TOS/kg of raw material (*i.e.*, flour). The U.S. FDA responded with a "no questions" letter in November 2017 (U.S. FDA, 2017).

C.5 Information on the Source Microorganism

The production strain from which the glucose oxidase food enzyme is produced is a non-genetically modified strain of the filamentous fungus *Penicillium rubens*, designated as strain PGO 19-162. Strain PGO 19-162 was selected as the production strain based on its capacity to produce high levels of glucose oxidase activity, its viability, and its suitability for industrial production, including minimal production of secondary metabolites as secondary metabolite production reduces enzyme production. The production strain is therefore one that produces the maximum level of enzyme and that is free from significant secondary metabolite production.

C.5.1 Taxonomic Identity of the Production Strain

The strain was taxonomically identified as belonging to the species *P. rubens* by the CABI Microbial Identification Service using morphological methods consisting of macroscopic and microscopic analysis on diagnostic media and use of taxonomic keys. The taxonomic identification of the strain was confirmed by a lab in Japan that further identified the strain as belonging to the species *P. rubens* by phylogenetic means based on ITS-5.8S- ribosomal deoxyribonucleic acid (rDNA) sequence similarities. Briefly, genomic DNA from strain PGO 19-162 was extracted and ITS-5.8S rDNA was amplified by polymerase chain reaction (PCR) and sequenced. The sequence was then subjected to an alignment search against nucleotide sequences available through the International Nucleotide Sequence Database Collaboration (comprising the DNA DataBank of Japan, the European Molecular Biology Laboratory, and GenBank at the National Center for Biotechnology Information) using the BLAST program. The alignment search revealed a 100% sequence similarity to the ITS-5.8S rDNA of a strain *Penicillium rubens*. Based on this high sequence similarity, and on subsequent phylogenetic analysis, strain PGO 19-162 was identified as belonging to the species *Penicillium rubens*, synonym *Penicillium chrysogenum*. It should be noted that several strains of *P. chrysogenum* were recently re-identified as belonging to the species *Penicillium rubens* by Houbraken and colleagues (Houbraken *et al*., 2011, 2012). As a result of this re-identification, the lab identified Shin Nihon's strain PGO 19-162 as belonging to the group of *P. chrysogenum* strains now classified under *P. rubens*. The strain has been deposited in the National Institute of Technology and Evaluation (NITE) Biological Resource Center (NBRC) culture collection (NITE SD No. 00254).

C.5.2 Details of Documented History of Use with Absence of Human Health Adverse Effects

P. chrysogenum/*P. rubens* is a filamentous fungus with a history of safe use in Europe as a starter culture for the production of dry sausages (Sunesen and Stahnke, 2003) and in the fermentation and

biopreservation of meat (Mogensen *et al.*, 2002). *P. chrysogenum* also has a history of safe use as a production organism used in the production of food enzymes. As indicated in Section C.1, *P. chrysogenum* is listed by AMFEP as a current source of commercialised glucose oxidase enzyme preparations used in food processing in the EU (Amfep, 2015). *Penicillium* (species not specified) also is an approved source of glucose oxidase in Japan as indicated in the list of existing food additives (MHLW, 2011). As such, the production strain *P. rubens* PGO 19-162 has been used in Japan in the commercial production of Shin Nihon's glucose oxidase food enzyme described herein. Shin Nihon's glucose oxidase enzyme preparations derived from *P. rubens* PGO 19-162 have been commercially marketed in Japan since 1999, and have been used in the production of foods, such as baked goods, cooked rice, and mayonnaise. *P. rubens* PGO 19-162 therefore has a history of safe use in food enzyme production in Japan.

C.6 Information on the Pathogenicity and Toxicity of the Source Microorganism

According to the guidelines developed by Pariza and Foster (1983), Pariza and Johnson (2001), and the International Food Biotechnology Council (IFBC, 1990), the primary consideration in evaluating the safety of an enzyme preparation derived from a microbial source is the safety of the production strain. The safety of a production strain is addressed primarily by evaluating its toxigenic potential; for filamentous fungi, such as *P. rubens*, the oral toxins of concern are mycotoxins (small molecular weight organic molecules, usually less than 1,000 Da in size) (Pariza and Johnson, 2001). Additional considerations for evaluating the safety of a production strain include pathogenicity and antibiotic production. According to the established guidelines, the safety of the enzyme preparation itself also should be assessed in part *via* analytical testing to ensure absence of toxic constituents (*e.g.*, mycotoxins) and antibiotic activity. The potential toxigenicity, pathogenicity, and antibiotic production of the production strain *P. rubens* PGO 19-162 and the impact on the manufactured glucose oxidase food enzyme are discussed below.

Toxigenicity

Filamentous fungi are known as potential producers of toxic secondary metabolites known as mycotoxins. The glucose oxidase food enzyme produced with *P. rubens* PGO 19-162 has been analysed for the standard list of mycotoxins previously established by JECFA as required to be tested for all enzyme preparations [*i.e.*, ochratoxin A, aflatoxin (B1, B2, G1, and G2), zearalenone, sterigmatocystin, T-2 toxin] (JECFA, 2001). The glucose oxidase food enzyme also was analysed for roquefortine C production. Based on the available scientific literature, *P. chrysogenum* is known to produce the mycotoxin roquefortine C (Frisvad *et al.*, 2004). However, the glucose oxidase food enzyme produced with *P. rubens* PGO 19-162 has been analysed for roquefortine C, the results of which confirm that roquefortine C, is not present at detectable levels in the glucose oxidase food enzyme. Batch analyses are provided in Section B.5.2.2 and confirm that such substances are not present at detectable levels in the food enzyme.

Pathogenicity

Some *Penicillium* species have been reported to occasionally cause opportunistic infections in humans, and in particular, as fungal etiological agents in fungemia, pneumonia, peritonitis, urinary tract infections, endocarditis, and disseminated infections (Swoboda-Kopec *et al.*, 2003). Invasive *Penicillium* infections, however, are rare and the majority of these are not acquired under normal conditions but are associated with conditions which would render an individual susceptible to infection, including injury, surgery and other invasive procedures, and use of prosthetic materials (Lyratzopoulos *et al.*, 2002). Likewise, infections caused specifically by the species *P. chrysogenum* are extremely rare. Although several case reports of infection caused by *P. chrysogenum* have been identified in the scientific literature, such cases consisted of opportunistic infections occurring in patient population

groups, including in individuals with compromised immune systems [*e.g.*, acquired immunodeficiency syndrome (AIDS), undergoing chemotherapy for acute myeloid leukaemia, valve surgery)] and in those having endured injury (*i.e.,* incidental skull/scalp trauma) or surgery (Yassin *et al.*, 1978; Eschete *et al.*, 1981; Prasad and Nema, 1982; Hoffman *et al.*, 1992; D'Antonio *et al*., 1997; Keung *et al.*, 1997; Lyratzopoulos *et al.*, 2002; Swoboda-Kopec *et al.*, 2003; Barcus *et al.*, 2005). Only 2 cases of infection in non-immunocompromised individuals were identified in the available literature (López-Martínez *et al.*, 1999; Kantarcioğlu *et al.*, 2004). Strains of *P. chrysogenum* are thus generally considered to have no or little pathogenicity (Botterel *et al.*, 2002; Kantarcioğlu *et al.*, 2004; Barcus *et al.*, 2005). Therefore, the production strain *P. rubens* PGO 19-162, previously classified as *P. chrysogenum*, is therefore considered to be non-pathogenic.

Antibiotic Production

P. chrysogenum, previously classified as *P. chrysogenum*, is a well-known producer of β-lactam antibiotics, including the antibiotics meleagrin, xanthocillin, sorrentanone, and sorbicillin (Frisvad *et al.*, 2004). Consistent with this information, the secondary metabolite analysis of *P. rubens* PGO 19-162 indicates that the production strain may produce the antibiotics meleagrin and xanthocillin X when cultured on agar plates. To determine whether *P. rubens* PGO 19-162 produces antibiotic activity, the production strain was tested using the antibacterial activity assay established by JECFA (2006b). The certificates of analysis are provided in Appendix D. The analytical results demonstrate that the production strain does not produce any detectable levels of antibacterial activity. Additionally, the specifications for the glucose oxidase food enzyme ensure that no antibacterial activity is present in the manufactured food enzyme. The batch analyses demonstrating that the food enzyme lacks antibacterial activity are presented in Section B.5.2.1.

C.7 Information on the Genetic Stability of the Source Organism

The source organism is not genetically modified or self-cloned. The production strain, *P. rubens* PGO 19-162, was selected as the production strain based on its capacity to produce high levels of glucose oxidase activity, its viability, and its suitability for industrial production, including minimal production of secondary metabolites. The organism is stored at the CABI Genetic Resource Collection until needed and maintained in a microbial collection at -80°C at Shin Nihon. Shin Nihon maintains a well-defined cell bank system using master cell bank (MCB) and working cell bank (WCB) to store the production strain. The cell bank is a collection of ampoules containing a pure culture; the cell line history and the production of a cell bank, propagation, preservation, and storage, is monitored and controlled. The WCB is prepared from an ampoule of MCB; the ampoule is inoculated and grown to form spores. This cultured slant is then inoculated to seed medium for the production strain and incubated. The seed culture is macroscopically and microscopically examined for the shape of the production strain and to confirm the absence of other microbial contaminants. Likewise, the minimum CFU/mL of the production strain is measured, and if the seed culture meets the requirements, it is then prepared into an ampoule and inoculated again to seed medium for the production strain and incubated. This seed culture is grown under appropriate fermentation conditions, and the enzyme activity and pH are monitored. The seed culture is also examined macroscopically and microscopically for microbial contaminants. If the seed culture meets the requirements, it is then stored as the WCB. If the criteria are not met, then the culture is discarded and another ampoule is prepared from the MCB.

The production process of the glucose oxidase food enzyme complies with HACCP, and includes quality control steps throughout the production process, including the fermentation steps, to ensure that certain criteria are met. Deviations from these quality control steps result in cessation of the process and the material discarded.

D. INFORMATION RELATED TO THE DIETARY EXPOSURE TO THE PROCESSING AID

In accordance with Section 3.3.2 – Processing Aids of the FSANZ *Application Handbook* (FSANZ, 2019), the following dietary exposure information must be provided:

1. A list of foods or food groups likely to contain the processing aid or its metabolites;
2. The levels of residues of the processing aid or its metabolites for each food or food group;
3. For foods or food groups not currently listed in the most recent Australian or New Zealand National Nutrition Surveys (NNSs), information on the likely level of consumption;
4. The percentage of the food group in which the processing aid is likely to be found or the percentage of the market likely to use the processing aid;
5. Information relating to the levels of residues in foods in other countries; and
6. For foods where consumption has changed in recent years, information on likely current food consumption.

Each point is addressed in the following subsections.

D.1 A list of Foods or Food Groups Likely to Contain the Processing Aid

D.1.1 Intended Uses

The glucose oxidase food enzyme produced with *P. rubens* PGO 19-162 is intended for use in the processing of foods and beverages belonging to the food categories listed in Table D.1.1-1. Food uses outlined below were defined using the categories provided in Table S15–5 in Schedule 15 of the Code (FSANZ, 2020d).

Table D.1.1-1 Food Categories Associated with the Intended Uses of the Glucose Oxidase Food Enzyme from *Penicillium rubens* PGO 19-162 in Food Processing

FSANZ Schedule 15 S15—5 Food Category	Description of Proposed Food Uses
1 Dairy products (excluding butter and fats)	Yoghurt drinks
	Cheese
4 Fruits and vegetables (including fungi, nuts, seeds, herbs and spices)	Canned Fruit
	Vegetable Purees
	Fruit-based desserts
6 Cereals and cereal products	Cereal-Based Dishes
	Rice
	Snack Foods
	Pasta, Noodles
7 Breads and bakery products	Bread and rolls
	Fine bakery wares
8 Meat and meat products (including poultry and game)	Processed Meat Products
9 Fish and fish products	Fish Products
10 Eggs and egg products	Powdered Eggs
12 Salts and condiments	Meat imitates (tofu)
13 Special purpose foods	Meal Replacement Beverages
14 Non-alcoholic and alcoholic beverages	Fruit Juices & Smoothies
	Vegetable Juices
	Tea
	Coffee & Coffee Imitates
	Wine
	Liquor
	Alcoholic Mixed Drinks
Snack foods	Snack Foods
Dips and sauces	Condiments
	Dressing

D.1.2 Estimated Daily Intake

D.1.2.1 Overview of the Budget Method

The potential human exposure to glucose oxidase has been estimated using the Budget Method, which is a widely-accepted preliminary screening tool used to assess the intake of chemicals such as food additives (FAO/WHO, 2009). The Budget Method allows for the calculation of a TMDI based on assumptions regarding the maximum human physiological levels of daily food and beverage consumption, rather than on food consumption data collected from dietary surveys. Specifically, the Budget Method relies on conservative assumptions made regarding (i) the level of consumption of solid foods and of non-milk beverages; (ii) the level of presence of the substance in solid foods and in non-milk beverages; and (iii) the proportion of solid foods and of non-milk beverages that may contain the substance (FAO/WHO, 2009). The levels of anticipated exposure to food enzymes that are derived using the Budget Method are thus considered to be conservative estimates (FAO/WHO, 2009).

Assumptions applied in the Budget Method assessment and results of the assessment are described below.

Level of Consumption of Solid Foods and Non-Milk Beverages

The FAO/WHO report on the "*Principles and Methods for the Risk Assessment of Chemicals in Food*" (FAO/WHO, 2009) specifies the standard value for food intakes to be 0.05 kg/kg body weight/day for solid foods 0.1 L/kg bodyweight/day for non-milk beverages.

Level of Presence of Food Enzyme in Solid Foods

To estimate the exposure to glucose oxidase from its intended uses in foods, it is assumed that the entire enzyme preparation added during processing will be present in the final foods as consumed (*i.e.*, assuming no removal and/or inactivation). Thus, the amount of enzyme assumed to be present in solid foods and non-milk beverages is based on the maximum level of the enzyme used in the production of foods and beverages, 30 and 10 mg TOS/kg, respectively.

Proportion of Solid Foods that May Contain the Food Enzyme

According to the FAO/WHO report, the default proportions that are typically assumed are that 12.5% of all solid foods and 25% of all non-milk beverages consumed will contain the food enzyme (FAO/WHO, 2009). However, the proportion of solid foods containing the food enzyme may be increased to 25% in cases where the substance (or in this case, the ingredients made with the enzyme) is used in a wide range of food categories (FAO/WHO, 2009). As mentioned, the glucose oxidase enzyme is intended for use in the processing of foods and beverages listed in Table D.1.1-1. Therefore, as a conservative estimate, the proportion of solid foods that are assumed to contain the enzyme was increased to 25% for the TMDI assessment.

D.1.2.2 Theoretical Maximum Daily Intake (TMDI)

Based on conservative estimates of exposure calculated using the budget method, the TMDI of glucose oxidase from all foods and beverages was calculated to be 0.63 mg TOS/kg bw/day, as show in Table D.1.2.2-1.

Table D.1.2.2-1 Estimated TMDI of Glucose Oxidase from Foods Intended for the General Population that Contain the Ingredients Made with the Enzyme Preparation

Products	A Level of Consumption of Solid Foods and Non-Milk Beverages (kg/kg bw/day)	B Maximum Level of Food Enzyme in Solid Foods and Non-Milk Beverages (mg TOS/kg)	C Proportion of Solid Foods and Non-Milk Beverages Containing the Food Enzyme (%)	Total Exposure to Food Enzyme[a] (mg TOS/kg bw/day)
Solid Foods	0.05	30	25	0.38
Non-Milk Beverages	0.1	10	25	0.25
Total Food and Beverage				0.63

bw = body weight; TMDI = theoretical maximum daily intake; TOS = total organic solids.
[a] Calculation: (A)*(B)*(C/100)

D.1.3 Summary of the Estimated Daily Intake

The potential human exposure to glucose oxidase was calculated assuming that all of the enzyme added during manufacture is present in the final food as consumed. However, the glucose oxidase will be heat-denatured and inactivated during the final stages of processing for final food products in most cases, which involves treatment at high temperatures, such as those that occur during pasteurisation or sterilisation. As such, the enzyme will not have any technological effect in most final foods as consumed. A number of other conservative assumptions are also made during the exposure assessment to ensure there is no underestimation of the potential intakes to glucose oxidase, including:

- Conservative assumptions made as part of the Budget Method used to calculate the TMDI (*e.g.*, increasing the proportion of solids foods assumed to contain the enzyme to 25% from the default value of 12.5% recommended by the FAO/WHO (2009) and assuming the maximum recommended use level of the enzyme preparation is always used in the production of foods and beverages.

Using the budget method, the TMDI of glucose oxidase from the consumption of finished foods and beverages made with the enzyme preparation was estimated to be 0.63 mg TOS/kg body weight/day in the general population.

D.2 The Levels of Residues of the Processing Aid or its Metabolites for Each Food or Food Group

Not applicable.

D.3 For Foods or Food Groups Not Currently Listed in the Most Recent Australian or New Zealand National Nutrition Surveys (NNSs), Information on the Likely Level of Consumption

Not applicable.

D.4 The Percentage of the Food Group in Which the Processing Aid is Likely to be Found or the Percentage of the Market Likely to Use the Processing Aid

Glucose oxidase from *A. niger*, *A. oryzae*, and *T. reesei* are currently permitted for use as a processing aid in Australia and New Zealand. It is anticipated that Shin Nihon's glucose oxidase from *P. rubens* will

provide food manufacturers and food producers with an alternative source of glucose oxidase for use in food production, and therefore, approval for *P. rubens* as an alternative source of glucose oxidase is not expected to increase the existing usage of the enzyme in food production.

D.5 Information Relating to the Levels of Residues in Foods in Other Countries

Glucose oxidase from *P. rubens* has GRAS status in the U.S. (GRN 509) and is currently used in the U.S. in the production of food products at levels of 30 mg TOS/kg in foods and 3 mg TOS/kg in beverages. Furthermore, glucose oxidase from genetically modified strains of *A. niger* and *A. oryzae* are currently used in the EU in the production of baked goods (EFSA, 2018, 2019) and glucose oxidase from *P. chrysogenum* is used in food processing in the EU (Amfep, 2015). Glucose oxidase is permitted for use in other countries including China, France, South Korea, and Japan. It is anticipated that the levels of residues in foods will be similar to those used in Australia and New Zealand.

D.6 For Foods Where Consumption Has Changed in Recent Years, Information on Likely Current Food Consumption

Glucose oxidase from *P. rubens* is intended for use in the processing of foods and beverages such as bread, bakery products, cheese, alcoholic and non-alcoholic beverages, and snack foods. It is not anticipated that the intakes of these food products have drastically changed in recent years.

REFERENCES

Aalberse RC (2000). Structural biology of allergens. J Allergy Clin Immunol 106(2):228-238. DOI:10.1067/mai.2000.108434.

Amfep (2015). *List of Commercial Enzymes*. Brussels, Belgium: Association of Manufacturers & Formulators of Enzyme Products (Amfep). Available at: https://amfep.org/_library/_files/Amfep_List_of_Enzymes_update_May_2015.pdf. [updated: May 2015].

AOAC (2000). AOAC Official Method 989.13. Motile *Salmonella* in all foods. *Official Methods of Analysis of the Association of Official Analytical Chemists: Vols. 1&2, 17th edition* (2002, Revision 1). Arlington (VA): Association of Official Analytical Chemists (AOAC).

Bankar SB, Bule MV, Singhal RS, Ananthanarayan L (2009). Glucose oxidase--an overview. Biotechnol Adv 27(4):489-501. DOI:10.1016/j.biotechadv.2009.04.003.

Barcus AL, Burdette SD, Herchline TE (2005). Intestinal invasion and disseminated disease associated with *Penicillium chrysogenum*. Ann Clin Microbiol Antimicrob 4:21 [4p]. DOI:10.1186/1476-0711-4-21.

Botterel F, Cordonnier C, Barbier V, Wingerstmann L, Liance M, Coste A, et al. (2002). *Aspergillus fumigatus* causes *in vitro* electrophysiological and morphological modifications in human nasal epithelial cells. Histol Histopathol 17(4):1095-1101. DOI:10.14670/HH-17.1095.

Codex Alimentarius (2009). *Foods Derived from Modern Biotechnology, 2nd edition*. (Codex Alimentarius). Geneva, Switz.: World Health Organization (WHO) / Rome, Italy: Food and Agriculture Organization of the United Nations (FAO). Available at: http://www.fao.org/docrep/011/a1554e/a1554e00.htm.

Crueger A, Crueger W (1990). Glucose transforming enzymes. In: Fogarty WM, Kelly CT, editors. *Microbial Enzymes and Biotechnology, 2nd edition*. London, Engl. / New York (NY): Elsevier Applied Science, pp. 177-226.

D'Antonio D, Violante B, Farina C, Sacco R, Angelucci D, Masciulli M, et al. (1997). Necrotizing pneumonia caused by *Penicillium chrysogenum*. J Clin Microbiol 35(12):3335-3337 [erratum, 1998, 3336(3332):3607]. DOI:10.1128/jcm.35.12.3335-3337.1997.

EFSA (2018). Scientific Opinion on the safety evaluation of the food enzyme glucose oxidase from a genetically modified *Aspergillus oryzae* (strain NZYM-KP). (EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids/CEF) (Question no EFSA-Q-2013-00687, adopted: 6 June 2018 by European Food Safety Authority). EFSA J 16(7):5319 [20pp]. DOI:10.2903/j.efsa.2018.5319. Available at: http://www.efsa.europa.eu/en/efsajournal/pub/5319.

EFSA (2019). Scientific Opinion on the safety evaluation of the food enzyme glucose oxidase from *Aspergillus niger* (strain ZGL). (EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids/CEF) (Question no EFSA-Q-2013-01005, adopted: 7 February 2019 by European Food Safety Authority). EFSA J 17(3):5629 [17pp]. DOI:10.2903/j.efsa.2019.5629. Available at: http://www.efsa.europa.eu/en/efsajournal/pub/5629.

Eschete ML, King JW, West BC, Oberle A (1981). *Penicillium chrysogenum* endophthalmitis. Mycopathologia 74(2):125-127. DOI:10.1007/BF01259468.

FAO/WHO (2001). *Evaluation of Allergenicity of Genetically Modified Foods*. Report of a Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology 22-25 January 2001. Rome, Italy: Food and Agriculture Organization of the United Nations (FAO) / Geneva, Switz.: World Health Organization (WHO). Available at: http://www.who.int/foodsafety/publications/gmo-allergenicity/en.

FAO/WHO (2009). Chapter 6: Dietary exposure assessment of chemicals in food. In: *Principles and Methods for the Risk Assessment of Chemicals in Food*. (Environmental Health Criteria, No. 240). Rome, Italy: Food and Agriculture Organization of the United Nations (FAO) / Geneva, Switz.: World Health Organization (WHO), International Programme on Chemical Safety (IPCS). Available at: https://apps.who.int/iris/bitstream/handle/10665/44065/WHO_EHC_240_9_eng_Chapter6.pdf;jsessionid=423E9F9EF415CF750F5FF0200E5A22B6?sequence=9.

FARRP (2020). *AllergenOnline Version 20: Home of the FARRP Allergen Protein Database*. Lincoln (NE): University of Nebraska-Lincoln, Food Allergy Research and Resource Program (FARRP). Available at: http://www.allergenonline.org [Released: February 10, 2020].

FCC (2018). Enzyme preparations. In: *Food Chemicals Codex, 11th edition*. Rockville (MD): United States Pharmacopeia Convention (USP), pp. 413-417.

Frisvad JC, Smedsgaard J, Larsen TO, Samson RA (2004). Mycotoxins, drugs and other extrolites produced by species in *Penicillium* subgenus *Penicillium.* Stud Mycol 49:201-241.

FSANZ (2002). *A458 – Glucose Oxidase as a Processing Aid [Final Assessment Report]*. (18 December 2002). Canberra, Australia / Wellington, New Zealand: Food Standards Australia New Zealand (FSANZ). Available at: https://www.foodstandards.gov.au/code/applications/documents/A458%20FAR%20Glucose%20Oxidase.pdf.

FSANZ (2019). 3.1.1 – General Requirements of the FSANZ / Section 3.3.2 – Processing Aid. In: *Food Standards Australia New Zealand Application Handbook*. (1 July 2019). Canberra, Australia / Wellington, NZ: Foods Standards Australia New Zealand (FSANZ). Available at: http://www.foodstandards.gov.au/code/changes/pages/applicationshandbook.aspx.

FSANZ (2020a). Schedule 18. Processing aids (F2020C00710). In: *Australia New Zealand Food Standards Code*. Canberra, Australia / Wellington, New Zealand: Food Standards Australia New Zealand (FSANZ), Australian Government. Available at: https://www.legislation.gov.au/Details/F2020C00710.

FSANZ (2020b). Food Standards (Application A1182 – Glucose Oxidase from a GM *Trichoderma reesei* as a Processing Aid (Enzyme)) Variation. Commonwealth of Australia Gazette, No. FSC 133, 14 May 2020. Canberra, Australia / Wellington, New Zealand: Food Standards Australia New Zealand (FSANZ). Available at: https://www.foodstandards.gov.au/code/changes/gazette/Documents/Gazette%20Notice%20No%20192.pdf?csf=1&e=5jb5KF.

FSANZ (2020c). *A1182 – Glucose Oxidase from GM Trichoderma reesei as a Processing Aid (Enzyme) [Approval Report]*. (4 March 2020). Canberra, Australia / Wellington, New Zealand: Food Standards Australia New Zealand (FSANZ). Available at: https://www.foodstandards.gov.au/code/applications/Pages/A1182.aspx.

FSANZ (2020d). Schedule 15 — Substances that may be used as food additives (F2020203). In: *Australia New Zealand Code*. Canberra, Australia / Wellington, NZ: Foods Standards Australia New Zealand (FSANZ), Australian Government. Available at: https://www.legislation.gov.au/Details/F2020C00203.

Goodman RE, Hefle SL (2005). Gaining perspective on the allergenicity assessment of genetically modified food crops. Expert Rev Clin Immunol 1(4):561-578. DOI:10.1586/1744666x.1.4.561.

Goodman RE, Hefle SL, Taylor SL, Van Ree R (2005). Assessing genetically modified crops to minimize the risk of increased food allergy: a review. Int Arch Allergy Immunol 137(2):153-166. DOI:10.1159/000086314.

Health Canada (2020). *List of Permitted Food Enzymes*. (Lists of Permitted Food Additives, No. 5). Ottawa (ON): Health Canada. Available at: https://www.canada.ca/en/health-canada/services/food-nutrition/food-safety/food-additives/lists-permitted/5-enzymes.html [Date Modified: 2020-10-22].

Hileman RE, Silvanovich A, Goodman RE, Rice EA, Holleschak G, Astwood JD, et al. (2002). Bioinformatic methods for allergenicity assessment using a comprehensive allergen database. Int Arch Allergy Immunol 128(4):280-291. DOI:10.1159/000063861.

Hoffman M, Bash E, Berger SA, Burke M, Yust I (1992). Fatal necrotizing esophagitis due to *Penicillium chrysogenum* in a patient with acquired immunodeficiency syndrome. Eur J Clin Microbiol Infect Dis 11(12):1158-1160. DOI:10.1007/bf01961135.

Houbraken J, Frisvad JC, Samson RA (2011). Fleming's penicillin producing strain is not *Penicillium chrysogenum* but *P. rubens*. IMA Fungus 2(1):87-95. DOI:10.5598/imafungus.2011.02.01.12.

Houbraken J, Frisvad JC, Seifert KA, Overy DP, Tuthill DM, Valdez JG, et al. (2012). New penicillin-producing *Penicillium* species and an overview of section *Chrysogena*. Persoonia 29:78-100. DOI:10.3767/003158512X660571.

IFBC (1990). Chapter 4: Safety evaluation of foods and food ingredients derived from microorganisms (International Food Biotechnology Council - IFBC). Regul Toxicol Pharmacol 12(3, Part 2):S114-S128. DOI:10.1016/S0273-2300(05)80080-7.

JECFA (1974). 5.3. Food enzymes. In: *Evaluation of Certain Food Additives*. 18th Meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), June 4-13, 1974, Rome, 1974. (WHO Technical Report Series, No. 557; WHO Food Additives Series No. 6; FAO Nutrition Meetings Report Series No. 54). Rome, Italy: Food and Agriculture Organization of the United Nations (FAO) / Geneva, Switz.: World Health Organization (WHO), pp. 19-20, 34. Available at: https://apps.who.int/iris/bitstream/handle/10665/41117/WHO_TRS_557.pdf;jsessionid=C263E9122FAF778AC8C6F393A102C215?sequence=1.

JECFA (2001). T-2 and HT-2 toxins [T-2]. *Safety Evaluation of Certain Mycotoxins in Food*. 56th Meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), Feb. 6-15, 2001, Geneva, Switz. (WHO Food Additives Series, No. 47 / FAO Food and Nutrition Paper, No. 74). Rome, Italy: Food and Agriculture Organization of the United Nations (FAO) / Geneva, Switz.: World Health Organization (WHO), International Programme on Chemical Safety (IPCS), pp. 557-652. Available at: http://www.inchem.org/documents/jecfa/jecmono/v47je06.htm.

JECFA (2006a). General specifications and considerations for enzyme preparation used in food processing [prepared by the Committee at its sixty-seventh meeting (2006)]. In: *Combined Compendium of Food Additive Specifications [Online Edition]. General Specifications for Enzymes Analytical Methods, Volume 4: Analytical Methods, Test Procedures and Laboratory Solutions Used by and Referenced in the Food Specifications*. 1st to 67th JECFA Meetings, 1956-2006. (FAO JECFA Monographs, No. 1). Rome, Italy: Food and Agriculture Organization of the United Nations (FAO) / Geneva, Switz.: Joint FAO/WHO Expert Committee on Food Additives (JECFA), xxi-xxv. Available at: http://www.fao.org/docrep/009/a0691e/a0691e00.htm ; http://www.fao.org/food/food-safety-quality/scientific-advice/jecfa/jecfa-additives/enzymes/en/.

JECFA (2006b). General specifications and considerations for enzymes used in food processing / Antibacterial activity / Total organic solids [prepared by the Committee at its sixty-seventh meeting (2006)]. In: *Combined Compendium of Food Additive Specifications*, Joint FAO/WHO Expert Committee on Food Additives, 67th Meeting, June 20-29, 2006, Rome, Italy. (FAO JECFA Monographs, No. 3). Rome, Italy: Food and Agriculture Organization of the United Nations (FAO) / Geneva, Switz.: Joint FAO/WHO Expert Committee on Food Additives (JECFA), pp. 63-67. Available at: http://www.fao.org/3/a-a0675e.pdf.

JECFA (2006c). Loss on drying / Nitrogen determination / Ash. In: *Combined Compendium of Food Additive Specifications, Volume 4 [Online Edition]. Analytical Methods, Test Procedures and Laboratory Solutions Used by and Referenced in the Food Specifications. 1st to 65th JECFA Meetings, 1965-2005*. (FAO JECFA Monographs, No. 1). Rome, Italy: Food and Agriculture Organization of the United Nations (FAO), Joint FAO/WHO Expert Committee on Food Additives (JECFA). Available at: http://www.fao.org/docrep/009/a0691e/A0691E06.htm#6.3.9.

JORF (2006). Arrêté du 19 octobre 2006 relatif à l'emploi d'auxiliaires technologiques dans la fabrication de certaines denrées alimentaires NOR: ECOC0600115A [Article Annexe I C, enzymes]. J Off Repub Fr (Oct.). Available at: http://www.legifrance.gouv.fr/affichTexte.do;jsessionid=D5E8EE6DCDB0FE77B51BD116BC4DD1F7.tpdjo09v_2?cidTexte=LEGITEXT000020667468&dateTexte=20121002#LEGIARTI000025176716. [Version consolidée au 14 mars 2014].

Kantarcioğlu AS, Apaydın H, Yücel A, de Hoog GS, Samson RA, Vural M, et al. (2004). Central nervous system infection due to *Penicillium chrysogenum*. Mycoses 47(5/6):242-248. DOI:10.1111/j.1439-0507.2004.00974.x.

Keung Y-K, Kimbrough R III, Yuen K-Y, Wong W-C, Cobos E (1997). *Penicillium chrysogenum* infection in a cotton farmer with acute myeloid leukemia. Infect Dis Clin Pract 6(7):482-483.

Klitgaard A, Iversen A, Andersen MR, Larsen TO, Frisvad JC, Nielsen KF (2014). Aggressive dereplication using UHPLC-DAD-QTOF: screening extracts for up to 3000 fungal secondary metabolites. Anal Bioanal Chem 406(7):1933-1943. DOI:10.1007/s00216-013-7582-x.

Konishi T, Aoshima T, Mizuhashi F, Choi SSH, Roberts AS (2013). Safety evaluation of glucose oxidase from *Penicillium chrysogenum*. Regul Toxicol Pharmacol 66(1):13-23 [corrigendum, 66(3):300]. DOI:10.1016/j.yrtph.2013.02.012.

López-Martínez R, Neumann L, González-Mendoza A (1999). Case report: cutaneous penicilliosis due to *Penicillium chrysogenum*. Mycoses 42(4):347-349. DOI:10.1046/j.1439-0507.1999.00464.x.

Lyratzopoulos G, Ellis M, Nerringer R, Denning DW (2002). Invasive infection due to *Penicillium* species other than *P. marneffei*. J Infect 45(3):184-195. DOI:10.1053/jinf.2002.1056.

MFDS (2020). [II. Food additives and mixed preparations 4. Component Specification. A. Food Additives. Glucose oxidase]. In: *Korea Food Additives Code*. (All designated chemicals and some natural additives are currently regulated by Food Additives Code. It includes specifications, standards and general test methods for each additives). Chungcheongbuk-do, South Korea: Ministry of Food and Drug Safety (MFDS). Available at: https://www.foodsafetykorea.go.kr/foodcode/04_03.jsp?idx=48.

MHLW (2000). Arsenic, Lead. In: *Japan's Specifications and Standards for Food Additives, 7th edition*. Tokyo, Japan: Ministry of Health, Labor and Welfare, Japan (MHLW) and Translated by Japan Food Chemical Research Foundation. Available at: http://www.ffcr.or.jp/zaidan/FFCRHOME.nsf/pages/spec.stand.fa [2000, Last updated: 2006/11/30].

MHLW (2011). *List of Existing Food Additives*. Tokyo, Japan: Ministry of Health, Labor and Welfare, Japan (MHLW) and Translated by Japan Food Chemical Research Foundation. Available at: http://www.ffcr.or.jp/zaidan/FFCRHOME.nsf/pages/list-exst.add [Compiled: April 16, 1996, Effective: May 6, 2011].

MHLW (2018). Glucose oxidase. In: *Japan's Specifications and Standards for Food Additives*, 9th edition. Tokyo, Japan: Ministry of Health, Labour and Welfare (MHLW), Consumer Affairs Agency, pp. 422-424. Available at: http://www.nihs.go.jp/dfa/dfa-j/shokuten_kikaku_j.html.

Ministry of Health of the PRC (2014). *[Food Safety National Standard Food Additive Standard]*. (National Food Safety Standard, No. GB:2760-2014; [Announcement No. 11, Notice No. 17, 2014]). Beijing, People's Republic of China: China Bureau of Scientific Standards, Standards Press of China / Ministry of Health of the People's Republic of China (PRC). Information available at: http://bz.cfsa.net.cn/staticPages/E13798C0-F243-4ACB-B541-C4E543349BE5.html.

Mogensen G, Salminen S, O'Brien J, Ouwehand A, Holzapfel W, Shortt C, et al. (2002). Inventory of microorganisms with a documented history of use in food. In: *Health Benefits and Safety Evaluation of Certain Food Components*. (Bulletin of the International Dairy Federation, No. 377). Brussels, Belgium: International Dairy Federation (IDF), pp. 10-19.

O'Brien M, Nielsen KF, O'Kiely P, Forristal PD, Fuller HT, Frisvad JC (2006). Mycotoxins and other secondary metabolites produced in vitro by *Penicillium paneum* Frisvad and *Penicillium roqueforti* Thom isolated from baled grass silage in Ireland. J Agric Food Chem 54(24):9268-9276. DOI:10.1021/jf0621018.

OECD (1997c). Mammalian erythrocyte micronucleus test. In: *OECD Guidelines for the Testing of Chemicals*. (OECD Guideline, No.474) [Updated & Adopted: 21st July 1997]. Paris, France: Organisation for Economic Co-operation and Development (OECD). Available at: http://www.oecd-ilibrary.org/environment/test-no-474-mammalian-erythrocyte-micronucleus-test_9789264071285-en;jsessionid=60s3nuot5c3s1.x-oecd-live-01. [New Versions Updated & Adopted: 26 September 2014, 29 July 2016].

OECD (1997a). Bacterial reverse mutation test. In: *OECD Guidelines for the Testing of Chemicals*. (OECD Guideline, No. 471) [Updated & Adopted: 21 July 1997]. Paris, France: Organisation for Economic Co-operation and Development (OECD). Available at: https://www.oecd-ilibrary.org/fr/environment/test-no-471-bacterial-reverse-mutation-test_9789264071247-en [New Version Updated & Adopted: 26 June 2020].

OECD (1997b). *In Vitro* mammalian chromosome aberration test. In: *OECD Guidelines for the Testing of Chemicals*. (OECD Guideline, No. 473) [Updated & Adopted: 21 July 1997]. Paris, France: Organisation for Economic Co-operation and Development (OECD). Available at: http://www.oecd-ilibrary.org/environment/test-no-473-in-vitro-mammalian-chromosomal-aberration-test_9789264224223-en. [New Versions45 Updated & Adopted: 26 September 2014, 29 July 2016].

OECD (1998a). *OECD Principles of Good Laboratory Practice*. (Series on Principles of Good Laboratory Practice and Compliance Monitoring, No. 1 [ENV/MC/CHEM(98)17]). Paris, France: Organisation for Economic Co-Operation & Development (OECD), Environment Directorate, Chemicals Group and Management Committee, OECD Environmental Health and Safety Publications. Available at: http://www.oecd-ilibrary.org/environment/oecd-principles-on-good-laboratory-practice_9789264078536-en [As revised in 1997].

OECD (1998b). Repeated dose 90-day oral toxicity study in rodents. In: *OECD Guidelines for the Testing of Chemicals*. (OECD Guideline, No. 408) [Updated & Adopted: 21 September 1998]. Paris, France: Organisation for Economic Co-operation and Development (OECD). Available at: http://www.oecd-ilibrary.org/environment/test-no-408-repeated-dose-90-day-oral-toxicity-study-in-rodents_9789264070707-en;jsessionid=1m6bvwspup322.delta [New Version Updated & Adopted: 27 June 2018].

Pariza MW, Foster EM (1983). Determining the safety of enzymes used in food processing. J Food Prot 46(5):453-468 [plus addendum]. DOI:10.4315/0362-028X-46.5.453.

Pariza MW, Johnson EA (2001). Evaluating the safety of microbial enzyme preparations used in food processing: update for a new century. Regul Toxicol Pharmacol 33(2):173-186. DOI:10.1006/rtph.2001.1466.

Prasad S, Nema HV (1982). Mycotic infections of cornea. (Drug sensitivity study). Indian J Ophthalmol 30(2):81-85.

Rasiah IA, K.H. S, Low FL, Lin H-M, Gerrard JA (2005). Crosslinking of wheat dough proteins by glucoseoxidase and the resulting effects on bread and croissants. Food Chem 89(3):325-332. DOI:10.1016/j.foodchem.2004.02.052.

Sunesen LO, Stahnke LH (2003). Mould starter cultures for dry sausages-selection, application and effects. Meat Sci 65(3):935-948. DOI:10.1016/S0309-1740(02)00281-4.

Swoboda-Kopec E, Wroblewska MM, Rokosz A, Luczak M (2003). Mixed bloodstream infection with *Staphylococcus aureus* and *Penicillium chrysogenum* in an immunocompromised patient: case report and review of the literature. Clin Microbiol Infect 9(11):1116-1117. DOI:10.1046/j.1469-0691.2003.00718.x.

U.S. FDA (2001). Chapter 3. Aerobic plate count. In: *Bacteriological Analytical Manual (BAM)*. College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN). Available at: https://www.fda.gov/food/laboratory-methods-food/bam-aerobic-plate-count.

U.S. FDA (2002a). Chapter 4. Enumeration of Escherichia coli and the coliform bacteria. In: *Bacteriological Analytical Manual (BAM)*. College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN). Available at: https://www.fda.gov/food/laboratory-methods-food/bam-4-enumeration-escherichia-coli-and-coliform-bacteria.

U.S. FDA (2002b). *Agency Response Letter GRAS Notice No. GRN 89 [Five enzyme preparations from Aspergillus niger: Carbohydrase enzyme preparation, catalase enzyme preparation, glucose oxidase enzyme preparation, pectinase enzyme preparation, and protease enzyme preparation, Washington (DC): Enzyme Technical Association]*. (Additional Correspondence in 2004). Washington (DC): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=89 [Apr. 3, 2002 - FDA response - no questions; additional correspondence available].

U.S. FDA (2002c). *Agency Response Letter GRAS Notice No. GRN 106 [Glucose oxidase enzyme preparation from Aspergillus oryzae carrying a gene encoding a glucose oxidase from Aspergillus niger, Franklinton (NC): Novozymes North America, Inc.]*. College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=106 [Oct. 3, 2002 - FDA response - no questions].

U.S. FDA (2014). *Agency Response Letter GRAS Notice No. GRN 509 [Glucose oxidase enzyme preparation derived from Penicillium chrysogenum, Anjou-city Aichi prefecture Japan: Shin Nihon Chemical Co., Ltd.]*. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=509 [Nov. 6, 2014 - FDA response - no questions].

U.S. FDA (2017). *Agency Response Letter GRAS Notice No. GRN 707 [Glucose oxidase from Penicillium produced in Trichoderma reesei, Darmstadt, Germany: AB Enzymes GmbH]*. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=707 [Nov. 24, 2017 - FDA response - no questions].

Vemulapalli V, Hoseney RC (1998). Glucose oxidase effects on gluten and water solubles. Cereal Chem 75(6):859-862. DOI:10.1094/CCHEM.1998.75.6.859.

Vemulapalli V, Miller KA, Hoseney RC (1998). Glucose oxidase in breadmaking systems. Cereal Chem 75(4):439-442. DOI:10.1094/CCHEM.1998.75.4.439.

Wong CM, Wong KH, Chen XD (2008). Glucose oxidase: natural occurrence, function, properties and industrial applications. Appl Microbiol Biotechnol 78(6):927-938. DOI:10.1007/s00253-008-1407-4.

Yassin A, Maher A, Moawad MK (1978). Otomycosis: a survey in the eastern province of Saudi Arabia. J Laryngol Otol 92(10):869-876. DOI:10.1017/s0022215100086242.