Petition to Amend Schedule 18 of the Australia New Zealand Food Standards Code to Include β-Galactosidase from *Papiliotrema terrestris* as a Processing Aid

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The materials are provided separately to this Application document.

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Petition to Amend Schedule 18 of the Australia New Zealand Food Standards Code to Include β-Galactosidase from *Papiliotrema terrestris* as a Processing Aid

GENERAL REQUIREMENTS

1.0 APPLICANT DETAILS

a)
b) Amano Enzyme Inc. (Enzyme manufacturer)
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d) TEL: +81-52-211-3032
e)
f) Enzyme manufacturer
g)

2.0 PURPOSE OF THE APPLICATION

The purpose of the application is to amend Schedule 18 of the Food Standards Code to permit the use of β-Galactosidase from *Papiliotrema terrestris* (EC 3.2.1.23) as a processing aid.
β-Galactosidase is an enzyme and is used as a processing aid for lactose processing. It is an enzyme that catalyzes the hydrolysis of β-Galactosides and is known as an enzyme to produce galactooligosaccharide (GOS) from lactose. β-Galactosidase, derived from various microorganisms, is already permitted in the Code. But, the β-Galactosidase in this submission has excellent acid resistance and temperature resistance as compared with other β-Galactosidase. Therefore, permission is required for β-Galactosidase from this source, *Papiliotrema terrestris.*

3.0 JUSTIFICATION FOR THE APPLICATION

3.1.1 Regulatory Impact Information

3.1.1.1 Cost and Benefit of the Proposed Change

Cost and Benefit to consumers:
The inclusion of β-Galactosidase derived from *Papiliotrema terrestris* in the Australia New Zealand Food Standards Code as a processing aid will no cost or benefits to consumers associated with the inclusion of β-Galactosidase in the Schedule 18. The use of β-Galactosidase is a commercial method available to produce galactooligosaccharide (GOS) from lactose. The availability of a range of food products is the same, irrespective of the method employed to achieve the results.

Cost and benefit to Industry:
It will allow producers to produce galactooligosaccharide (GOS) by using β-Galactosidase from lactose. The β-Galactosidase in this submission has excellent acid resistance and temperature

resistance as compared with other β-Galactosidase. Transgalactosylation reaction occurs more effective under the higher concentration of substrate. In order to increase the concentration of the substrate (lactose), it is necessary to dissolve the substrate at a higher temperature. Furthermore, in the production of GOS, isomerization tends to occur easily in an environment of high pH, and browning of the product is liable to occur. For the production of high quality products, it is necessary to use the enzyme at lower pH. Thus, the enzyme in this submission is more useful than other β-Galactosidases.
GOS belongs to the group of prebiotics and can be utilized to various foods. Prebiotics are defined as non-digestible food ingredients that beneficially affect the host by stimulating the growth and/or activity of beneficial bacteria in the colon. GOS occurs in commercial available products such as food for both infants and adults.

Cost and benefit to Government;
There will be no additional cost to the regulator if the processing aid is approved as the use of β-Galactosidase derived from *Papiliotrema terrestris* will not impact the regulation of these food products since processing aids are machinery in nature and their use is voluntary.

3.1.1.2 Impact on International Trade

The application of β-Galactosidase derived from *Papiliotrema terrestris* has been lodged in Denmark and will be lodged in France and applied for GRAS Notification (US). Therefore, the approval of β-Galactosidase derived from *Papiliotrema terrestris* as a processing aid may, in the future, promote international trade and reduction of technical barriers to trade. The amendment would bring Australia and New Zealand into line with other countries.

4.0 INFORMATION TO SUPPORT THE APPLICATION

Sections A through F of this application contain detailed data that supports the quality, efficacy, and safety of β-Galactosidase derived from *Papiliotrema terrestris* under the proposed conditions of use as a processing aid in Australia and New Zealand, as presented in accordance with the information requirements listed in Section 3.3.2 (Processing Aids) of the Food Standards Australia New Zealand (FSANZ) Application Handbook (FSANZ, 2016). The data pertaining to the β-Galactosidase derived from *Papiliotrema terrestris* presented in this application is representative of the commercial product for which approval is being sought.
The information is provided in this application to enable the objectives specified in Section 18 of the FSANZ Act to be addressed as follows:

a) The protection of public health and safety: Information to support objective (a) is provided in Section C of the application, in which the safety of β-Galactosidase derived from *Papiliotrema terrestris*, based on the available pre-clinical and human safety data, is discussed in detail.
b) The provision of adequate information relating to food to enable consumers to make informed choices: Data to support objective (b) are provided in Section F, in which the impact and purpose of β-Galactosidase are described in detail.
c) The prevention of misleading or deceptive conduct: Information supporting objective (c) is provided in Section F, in which the consumer awareness and potential behaviour in response to

products manufactured using β-Galactosidase are described in detail. This objective can also be further supported by human safety data contained in Section C.

Additionally, as *per* the FSANZ Application Handbook (FSANZ, 2016), any evidence that the food industry generally or other specific companies have an interest, in, or support, the proposed changes to the Code is mandatory for applications to change the Food Standards Code. As discussed in Section C, β-Galactosidase from *Papiliotrema terrestris* in this submission is Amano's new-developed product, there is no record of history of use. However, *Cryptococcus laurentii* is known as a closely related species with *Papiliotrema terrestris*. It is reported that β-Galactosidase from *Cryptococcus laurentii* has been used for manufacturing GOS for over 25years in Japan (Daniel Obed Otieno, 2010). Therefore, it is expected that the introduction of β-Galactosidase derived from *Papiliotrema terrestris* to the Australia/New Zealand market will be well received.

5.0 ASSESSMENT PROCEDURE

Amano Enzyme considers the most appropriate assessment procedure for the application herein, which relates to an amendment Schedule 18 of the Food Standards Code to include β-Galactosidase derived from *Papiliotrema terrestris* as a processing aid, to be the General Procedure (Subdivision D), Cost Category Level 1 (up to 350 hours). This is based on the fact that FSANZ has approved the same food enzymes; β-Galactosidase from various microorganisms.

6.0 CONFIDENTIAL COMMERCIAL INFORMATION

This application does contain information that is confidential commercial information (CCI). This information is provided separately and clearly labeled as CCI. There is no confidential information in the Executive Summary. Therefore, a non-confidential summary is not prepared.

7.0 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 Food Standards Australia New Zealand (FSANZ) Act, which states:

> 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

8.0 INTERNATIONAL AND NATIONAL STANDARDS

The following national and international standards are relevant to the current application:

- This food enzyme, β-Galactosidase, complies with the internationally accepted JECFA specifications for chemical and microbiological purity of food enzymes (FAO/WHO, 2006). (See also A.5.1)
- Beta galactosidase is listed in IPA Database by CCFA[1].

9.0 STATUTORY DECLARATION

A signed statutory declaration is appended to this application.

10.0 CHECKLIST

A completed checklist relating to the information required for submission is appended to this application.

[1] http://ipa.ccfa.cc/substance?task=detail&substance_id=589

SECTION A: TECHNICAL DESCRIPTION OF β-Galactosidase

β-Galactosidase is an enzyme of microbial origin that is proposed for use as a processing aid in Australia and New Zealand. A full description of the processing aid including the identity, enzymatic properties, manufacturing process, and purity is presented in this section.

A.1 Information on the Type of Processing Aid

β-Galactosidase is powdered enzyme and is an enzyme that catalyzes the hydrolysis of beta-1, 4 glycosidic bonds in beta-galactosides and releases galactose and residual organic molecule. When the substrate is lactose, it releases galactose and glucose (Figure A-1).

The enzyme also catalyzes the transgalactosylation of galactose to acceptor under the high substrate concentration condition (Figure A-2). In the case of lactose as the primary substrate, galactose out of lactose is bonded to lactose or lactose related galactooligosaccharide (GOS) as an acceptor by transgalactosylation in β-1,4, β-1,6, β-1,3 or β-1, 2 binding mode. The reaction mode of the enzyme is indicated in the Figure below. The enzyme activity is inhibited by Fe^{2+} but is not affected by K^{+}, Na^{+}, Mg^{2+}, Ca^{2+}, Mn^{2+}, and $Co2^{+}$. The Isoelectric point is pH3.95 and the Km value is 3.03 mM.

Since this application is for the β-Galactosidase to be used for the production of galactooligosaccharide (GOS), the reaction of transgalactosylation is commercial importance.

Figure A- 1: Hydrolysis reaction of the enzyme

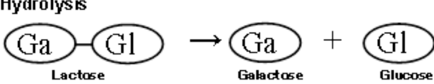
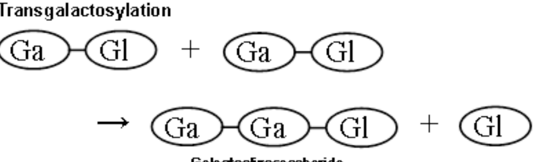


Figure A- 2: Transgalactosylation of the enzyme



Amano Enzyme has prepared β-Galactosidase enzyme preparation that is derived from *Papiliotrema terrestris* by means of a fermentation process. The enzyme intended for use as a processing aid in food. A full description of the manufacturing procedures is provided in Section A.4.
Based on the foregoing description, β-Galactosidase derived from *Papiliotrema terrestris* would fall under the classification within Schedule 18 (Processing Aids):

Typically, the β-galactosidase is added to substrate (lactose) up to **0.03%**.

A.2 Information on the Identity of the Processing Aid

IUBMB name:	β-Galactosidase
Synonyms:	lactase; β-lactosidase; maxilact; hydrolact; β-D-lactosidase; S 2107; lactozym; trilactase; β-D-galactanase; oryzatym; sumiklat
EC number:	3.2.1.23
CAS registration number:	9031-11-2
EINECS number:	232-864-1

The β-Galactosidase preparation is produced by *Papiliotrema terrestris* is not genetically modified organism but a chemically mutated production strain derived from the original strain (See also section D.1).

A.3 Information on the Chemical and Physical Properties of the Processing Aid

A.3.1 Purpose of using the processing aid

As mentioned earlier, β-Galactosidase catalyzes the hydrolysis of β-Galactosides and can produce galactooligosaccharide (GOS) from lactose. In other words, β-Galactosidase will be used in lactose processing.

The β-Galactosidase in this submission has excellent acid resistance and temperature resistance as compared with other β-Galactosidase. Transgalactosylation reaction occurs more effective under the higher concentration of substrate. In order to increase the concentration of the substrate (Lactose), it is necessary to dissolve the substrate at a higher temperature. Furthermore, in the production of GOS, isomerization tends to occur easily in an environment of high pH, and browning of the product is liable to occur. For the production of high quality products, it is necessary to use the enzyme at lower pH. Thus, the enzyme in this submission is more useful than other β-Galactosidases.

The GOS process flows given below comprise typical applications of the food enzyme and show the conditions under which the food enzyme is used. In the production flows, the enzyme after the enzyme reacting process is exposed to high temperature which is inactivatable and removed. Such process in the application is inactivation process and ion exchange process, respectively.

Figure A- 3: Process flow of GOS manufacturing by using β-Galactosidase

(This Figure is considered as CCI and provided in the separate document.)

BENEFIT OF GOS

The benefit of the use of this enzyme in certain food processes is to obtain GOS which is a beneficial food ingredient for the human, by the transgalactosylation and providing various advantages/benefits over chemical synthesis. GOS belongs to the group of prebiotics. Prebiotics are defined as non-digestible food ingredients that beneficially affect the host by stimulating the growth and/or activity of beneficial bacteria in the colon. GOS occurs in commercial available products such as food for both infants and adults.

The resulted product, GOS largely resists hydrolysis by salivary and intestinal digestive enzymes because of the configuration of their glycosidic bonds. Therefore, they reach the end of the intestine virtually intact. The human intestine contains about 300-500 different species of bacteria that can be divided into health-promoting ones, like Bifidobacteria and Lactobacilli and into harmful ones like Clostridia. The increased activity of these health-promoting bacteria results in a number of health-related benefits both directly by the bacteria themselves or indirectly by the organic acids they produce via fermentation. Examples of potential health-promoting benefits are inhibition of the growth of harmful bacteria, stimulation of immune functions, absorption of essential nutrients and syntheses of certain vitamins (Gibson, 1998, Roberfroid, 2000, Macfarlane et al, 2008).

A.3.2 Technological Function and Enzymatic Properties

A.3.2.1 Assay for Measuring β-Galactosidase Activity

An analytical method for the detection and quantification of β-Galactosidase activity is presented in Appendix A - 1.

When lactose is used as a substrate, glucose is released by using β-Galactosidase. β-Galactosidase activity is obtained by measuring the gulucose. One β-Galactosidase unit is defined as the quantity of enzyme required to liberate 1 µmol of glucose per 1 minute under the conditions described in the Appendix.

A.3.2.2 Characterization of β-Galactosidase Activity

The technical function of β-Galactosidase is to catalyze the hydrolysis of lactose specificity. The effects of temperature and pH on the activity of the β-Galactosidase were examined and the results are presented in Figures A-1 and A-2.

In all assays the same experimental procedures described in Section A.3.2.1 were employed with the only modifications affecting the temperature of the water bath or the pH of the β-Galactosidase solution. The effect of temperature and pH on the activity were compared to the activity measured under standard conditions. For the assessment of the impact of temperature on activity, the standard conditions were considered to be a water bath temperature of 40°C. The activity of the sample at a given pH was compared to the activity measured when the reaction was run at a pH of 6.0.

Based on the assays conducted, the enzyme exhibits activity from pH 3.0 till pH 8.0, and from 40°C till 75°C. The optimum pH is pH 5.0 and the optimum temperature is 70°C.

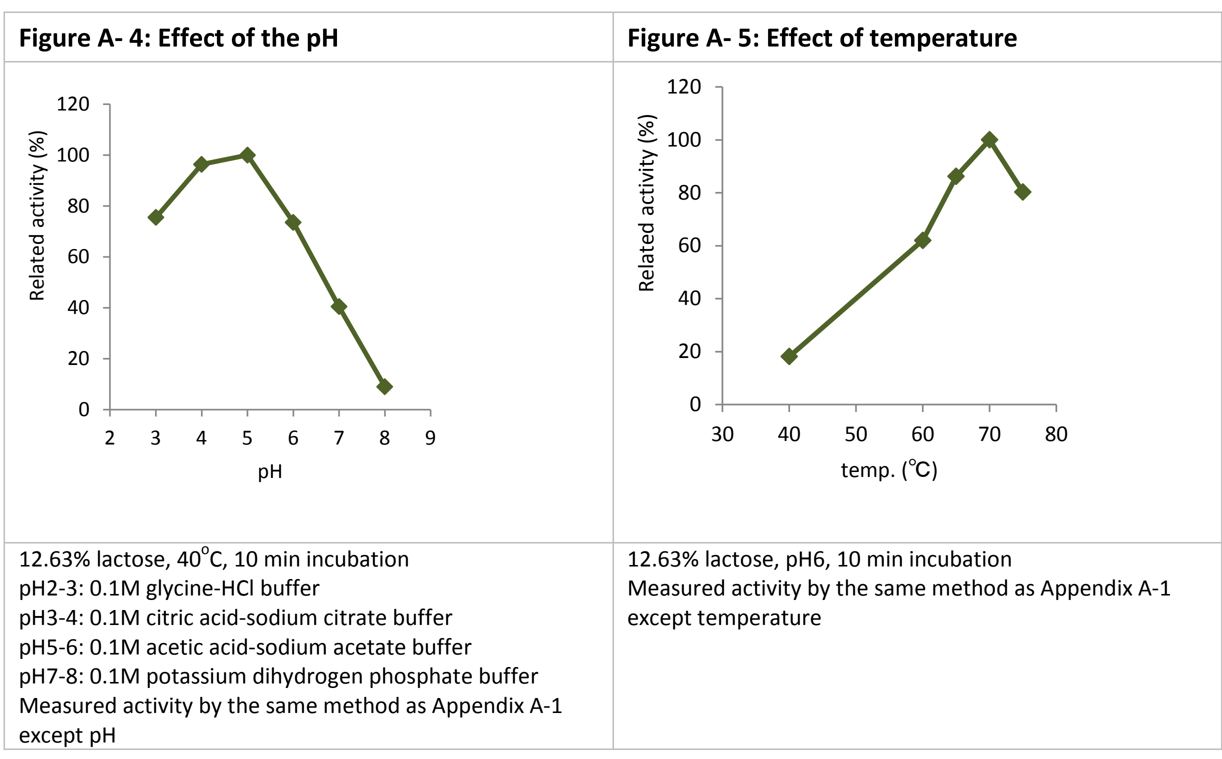


Figure A- 4: Effect of the pH	**Figure A- 5: Effect of temperature**
12.63% lactose, 40°C, 10 min incubation pH2-3: 0.1M glycine-HCl buffer pH3-4: 0.1M citric acid-sodium citrate buffer pH5-6: 0.1M acetic acid-sodium acetate buffer pH7-8: 0.1M potassium dihydrogen phosphate buffer Measured activity by the same method as Appendix A-1 except pH	12.63% lactose, pH6, 10 min incubation Measured activity by the same method as Appendix A-1 except temperature

A.3.3 Stability

pH and THERMAL STABILITY

The stability of β-Galactosidase has been assayed. As the enzyme activity was considered the primary marker of the stability of β-Galactosidase, the experimental procedures described in Section A.3.2.1 were employed to assess the stability.

The only change to the experimental procedures was that there was an incubation process before analyzing enzyme activity. Then the enzyme activity is assayed in accordance with the normal assay method described in Section A.3.2.1 The results of the assessment of the thermal and pH stability are presented in Figures A-6 and A-7.

The enzyme activity is markedly decreased at over pH 9.0 and no enzyme activity is left at 70°C under the buffered conditions.

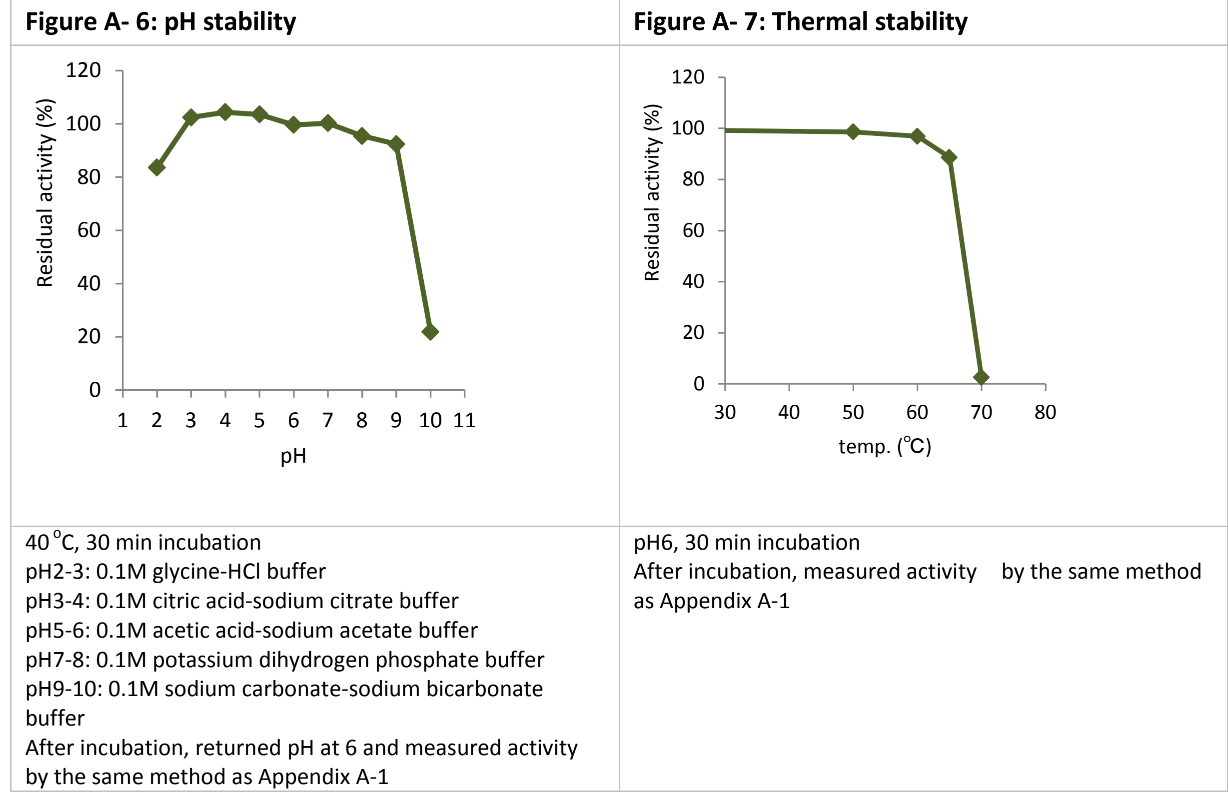


Figure A- 6: pH stability	**Figure A- 7: Thermal stability**
40 °C, 30 min incubation pH2-3: 0.1M glycine-HCl buffer pH3-4: 0.1M citric acid-sodium citrate buffer pH5-6: 0.1M acetic acid-sodium acetate buffer pH7-8: 0.1M potassium dihydrogen phosphate buffer pH9-10: 0.1M sodium carbonate-sodium bicarbonate buffer After incubation, returned pH at 6 and measured activity by the same method as Appendix A-1	pH6, 30 min incubation After incubation, measured activity by the same method as Appendix A-1

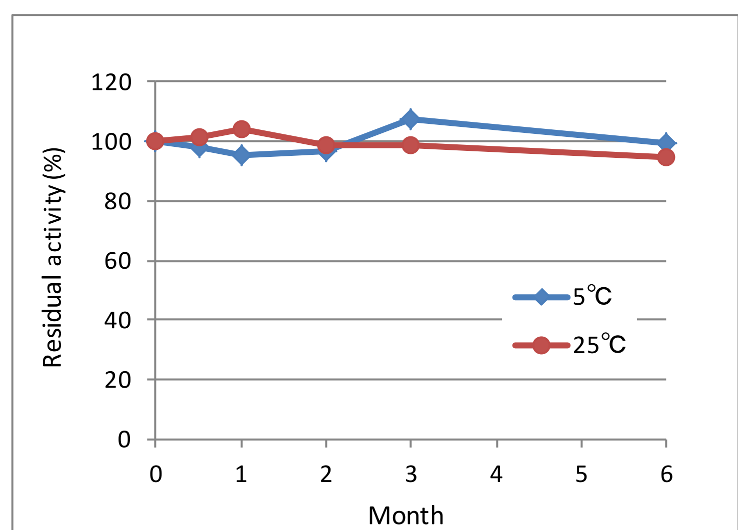
LONG TERM STABILITY

The stability of the β-Galactosidase was assayed by the Amano Enzyme Inc. Sample was β-Galactosidase enzyme powder and was putted into an airtight bag and kept at 5 or 25°C.

The enzyme activity was periodically measured by the method described in Section A.3.2.1 Results are summarized in the Figure below.

It could be concluded that the β-Galactosidase activity remained over 90% of the initial activity after at least 6 months under 25°C or below.

Figure A- 8: The stability of the enzyme preparation



A.3.4 Possible Interactions with Food Constituents

β-Galactosidase is an enzyme which acts on single substrate and would therefore, not be expected to act on other constituents in the food. The enzyme preparation must be inactivated either by temperature or pH changes.

Amano Enzyme recommends that the inactivation be accomplished by increasing the temperature above 70°C. Food manufacturers conforming to the recommended conditions of use will ensure that the enzyme is inactivated in the final food product and therefore, unable to react with any substrate present in non-target foods.

A.3.5 Characterisation of Secondary Activities

As far as Amano Enzyme is aware, the β-Galactosidase described in this dossier has no side activities. To demonstrate this, the analyses were performed for α-amylase activity, protease activity and lipase activity. The results showed that no side activities were detected at significant level.

Table A - 1: Main and subsidiary activities in β-Galactosidase

Enzyme activity	Unit	GFE(SDS)-S69-001	GFE69-001	GFE69-003	Method
β-Galactosidase	U/g	5870	5720	5740	Lactose substrate method
α-amylase	U/g	ND	ND	ND	VSEFA
Protease	U/g	250	230	310	Folin
Lipase	U/g	6.9	4.8	8.0	LMAP

As indicated in the Table A - 1, the way to allocate the lot number is different. The difference is only the production facility. All the production processes and the raw material used are the same.

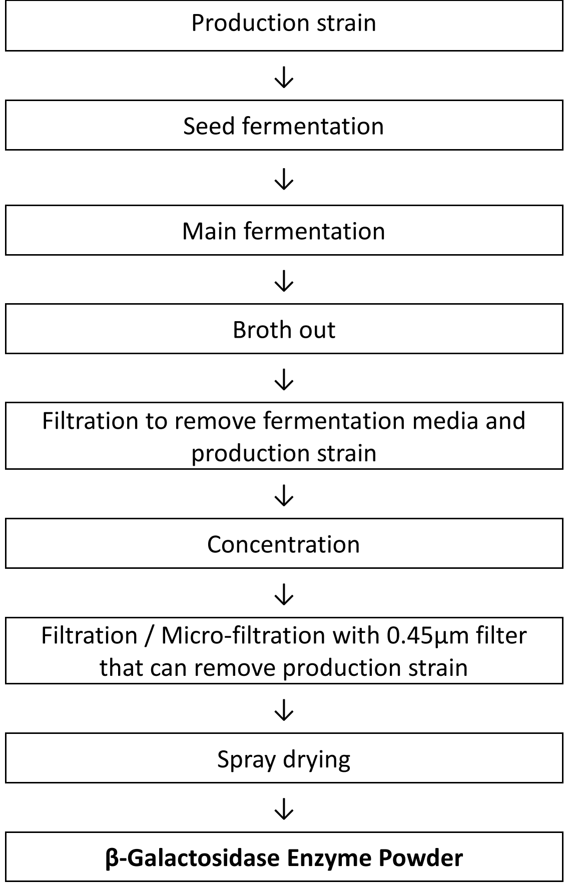
GFE(SDS)-S69-001: Shiga Plant of Amano Enzyme Inc.
GFE69-001 & GFE69-003: Yoro Plant of Amano Enzyme Inc.

A.4 Manufacturing Process

A.4.1 Manufacturing Steps

A schematic overview of the overall manufacturing process for β-Galactosidase is provided in Figure below.

Figure A- 9: Manufacturing Process



In brief, the production begins with the fermentation of *Papiliotrema terrestris* under standard culturing conditions. Recombinant DNA technology is not used to obtain this strain. Once the fermentation is complete, the broth is then submitted to a series of separation and concentration steps at the end of which the food enzyme concentrate can be formulated into a commercial preparation that will be used in food processing. More specifically, after the β-Galactosidase Enzyme Powder has been spray dried, it has a chance to be blended with lactose to obtain a final enzyme preparation. There is a range of different grade products available. However, we would like FSANZ to evaluate the β-Galactosidase Enzyme Powder itself.

The enzyme preparation is produced according to the FSSC22000 quality control system and complies with international guidelines for the safe handling of microbial enzyme preparations published by the Association of Manufacturers of Fermentation Enzyme Products (AMFEP). The Good Manufacturing Practices (GMP) for food additives certification and certificate of conformity to FSSC22000 are provided in Appendix A - 2.

A.4.2 Raw Materials

The raw materials employed in the production of β-Galactosidase is listed in following table along with the grade of material employed, the function in the production process, and the status of the raw material in Australia and New Zealand. All of the raw materials employed in the production of β-Galactosidase enzyme are of appropriate quality for use in foods.

The raw materials are all approved for use in the food supply in Australia and New Zealand either as food ingredients, raw materials in used in the production of processing aids or foods additives, or as food additives themselves.

Table A - 2: Raw Materials and Processing aids used for the production

(This table is considered as CCI and provided in the separate document.)

A.4.3 Residual Allergens from the Culture Medium

Although lactose is used for the fermentation media, the fermentation media are removed during the purification process. The residual milk allergen in β-Galactosidase Enzyme Powder was analyzed and resulted as less than 1.0μg/g.

Furthermore, as described in Section F.1, β-Galactosidase is added only at low levels (0.03% at the maximal*) to raw material for enzyme reaction.

The exposure to any potential residual milk allergen in final food products consumed will be negligible and extremely unlikely to be of any allergenic concern.

*: Maximal use level for food processing: 256.3 mgTOS/kg (See section F.1)

= 277.3 mg/kg (TOS: 92.4%, see section A.5.2)

= 0.03 %

A.5 Specification for Identity and Purity

A.5.1 Product Specification

The Chemical and Microbiological Specification

It is proposed that the food enzyme β-Galactosidase should comply with the internationally accepted JECFA specifications for chemical and microbiological purity of food enzymes (FAO/WHO, 2006) and FCC specifications:

Table A - 3: Specification for β-Galactosidase

The Chemical and Microbiological Specification as defined by JECFA	
Lead	Not more than 5 mg/kg
Salmonella spp.	Absent in 25 g of sample
Total coliforms	Not more than 30 per gram
Escherichia coli	Absent in 25 g of sample
Antimicrobial activity	Not detected
Mycotoxins	Not detected
General requirements as defined by Food Chemical Codex (FCC)	
Lead	Not more than 5 mg/kg
Coliforms	Not more than 30 cfu per gram
Salmonella	Negative in 25g
Enzyme Activity	
β-Galactosidase activity	More than 2,000 u/g
General Properties	
Appearance	Off white to brown powder

A.5.2 Batch Analysis

The proof that the food enzyme β-Galactosidase complies with these specifications is shown by the analyses on various different batches, see Appendix A - 3.

Protein content and relative purity of the food enzyme β-Galactosidase from *Papiliotrema terrestris* was measured, and the TOS values were calculated, in 3 batches. The result is shown in the following Table.

Table A - 4: Batch Analysis

Batch No	Spec.	GFE(SDS)-S69-0	GFE69-001	GFE69-003	Mean
Heavy metals					
Lead	NMT 5mg/kg	0.031 mg/kg	0.015 mg/kg	0.036 mg/kg	-
Microbiology					
Salmonella sp.	Negative in 25g	Negative	Negative	Negative	-
Total coliforms	NMT 30/g	< 10cfu/g	< 10cfu/g	< 10cfu/g	-
Escherichia coli	Negative in 25g	Negative	Negative	Negative	-
Antimicrobial activity					
Antimicrobial	Negative	Negative	Negative	Negative	-
Other items					
Ash (%)	—	2.04	2.24	2.17	2.15
Water (%)	—	5.6	6.0	4.8	5.5
TOS (%)	—	92.4	91.8	93.0	**92.4**
Enzyme activity (u/g)	NLT 2,000	5,870	5,720	5,740	5,777
Units/mg TOS	—	6.35	6.23	6.17	6.25
Protein (%)	—	53.6	53.4	54.5	53.8

ABSENCE OF TOXINS

Papiliotrema terrestris is not known to produce any toxins. However, several major mycotoxins were analysed for reference. Results are indicated in the table below. Original report is given in Appendix A-3. These analyses were performed at the third party.

These results have indicated that the β-Galactosidase contains no significant amount of major mycotoxins.

Table A - 5: Search for major mycotoxins in 3 lots of β-Galactosidase

Mycotoxins	Lot No.			Unit
	GFE(SDS)-S69-001	GFE69-001	GFE69-003	
Aflatoxin B_1	< 0.2	< 0.2	< 0.2	µg/kg
Aflatoxin B_2	< 0.2	< 0.2	< 0.2	µg/kg
Aflatoxin G_1	< 0.2	< 0.2	< 0.2	µg/kg
Aflatoxin G_2	< 0.2	< 0.2	< 0.2	µg/kg
Total Aflatoxin	< 0.8	< 0.8	< 0.8	µg/kg
Ochratoxin A (OTA)	< 0.5	< 0.5	< 0.5	µg/kg
HT-2 Toxin	< 10	< 10	< 10	µg/kg
T-2 Toxin	< 10	< 10	< 10	µg/kg
Zearalenone	< 2	< 2	< 2	µg/kg
Sterigmatocystin	< 10	< 10	< 10	µg/kg
Fumonisin B_1	< 5	< 5	< 5	µg/kg
Fumonisin B_2	< 5	< 5	< 5	µg/kg

A.6 Analytical Method for Detection

In accordance with Section 3.3.2 of the FSANZ Application Handbook, an analytical method for detection is not required for an enzymatic processing aid (FSANZ, 2016). Therefore, this section is not relevant to the use of β-Galactosidase derived from *Papiliotrema terrestris.*

SECTION B: INFORMATION RELATING TO THE SAFETY OF A CHEMICAL PROCESSING AID

This section is not relevant to the current processing aid and therefore is not included in this application.

SECTION C: INFORMATION RELATING TO THE SAFETY OF AN ENZYME PROCESSING AID

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

- This food enzyme, β-Galactosidase, complies with the internationally accepted JECFA specifications for chemical and microbiological purity of food enzymes (FAO/WHO, 2006). (See also A.5.1)
- Beta galactosidase is listed in IPA Database by CCFA[2].
- *Cryptococcus laurentii* is known as a closely related species with *Papiliotrema terrestris.* It is reported that β-Galactosidase from *Cryptococcus laurentii* has been used for manufacturing of GOS for over 25years in Japan (Daniel Obed Otieno, 2010).

Since the enzyme preparation in this submission; β-Galactosidase from *Papiliotrema terrestris* is Amano Enzyme's new-developed product, there is no approval status in other countries.

[2] http://ipa.ccfa.cc/substance?task=detail&substance_id=589

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

Several toxicity studies have been conducted to assess the enzyme safety. The potential mutagenic and genotoxic activity of the β-Galactosidase were conducted through in vitro assessment, as well as a repeat-dose 13-week oral toxicity study conducted in rats. These studies are described below in Section C.2.1.

The food enzyme has been subjected to a standard package of toxicological tests, with the following results:

- Bacterial reverse mutation: No mutagenic activity under the given test conditions
- Chromosomal aberrations: No clastogenic activity under the given test conditions
- Systemic toxicity: The No Observed Adverse Effect Level (NOAEL) is greater than 2,000 mg/kg-bw/day (1,800mg TOS/kg-bw/day), which is the high dose in the study.

ABSENCE OF TOXINS

Papiliotrema terrestris is not known to produce any toxins. However, several major mycotoxins were analysed for reference. Results are indicated in the section A.5.2; Batch Analysis.

C.2.1 β-Galactosidase

C.2.1.1 Mutagenicity and Genotoxicity

Bacterial reverse mutation test (Appendix C- 1)

The tests were conducted according to OECD Guidelines for testing of chemicals 471, (1997) and "OECD Principles of Good Laboratory Practice (1997), at Bozo Research Center Inc. (Study Number: T-2095). The test was conducted in 5 tester strains: Salmonella typhimurium TA100, TA98, TA1535 and TA1537, and Escherichia coli (hereinafter referred to as E. coli) WP2 uvrA.

The test article was a β-Galactosidase concentrate (Lot no. GFE68-001@K) having activity of 2,550 u/g (β-Galactosidase activity, Lactose substrate method).

In this study, a dose-range finding test and main test were conducted with and without metabolic activation by the pre-incubation method. 5 dose levels (5000, 1250, 313, 78.1, 19.5 µg/plate) were set for the dose-range finding test and 6 dose levels (5000, 2500, 1250, 625, 313 and 156 µg/plate) for the main test. The main test was conducted twice at the same dose levels. In the preliminary and both two main tests, no biologically or statistically significant increases in the number of revertant colonies were observed in any tester strain, either in the absence or presence of the metabolic activation.

Based on the described above, it is conducted that test article has no reverse mutation inducing activity (negative).

Chromosomal aberration test (Appendix C- 2)

The tests were conducted according to OECD Guidelines for testing of chemicals 473, (1997) and "OECD Principles of Good Laboratory Practice (1997), at Bozo Research Center Inc. (Study Number: T-G212). The test was conducted using cultured Chinese hamster cells (CHL/IU cells). The test article was the same as the bacterial reverse mutation test. In order to select dose levels for the chromosome aberration test, a cell-growth inhibition test was conducted setting the highest dose level at 5000 µg/mL and a total of 8 concentrations. As a result, the chromosome aberration test was conducted setting the highest concentration at 5000 µg/mL for the short-term treatment with or without metabolic activation, at 2000 µg/mL for the continuous treatment. A total of 4 and 3 concentrations were set in the short-term treatment with and without metabolic activation, respectively. In the continuous treatment, a total of 5 concentrations were set.

In the chromosome aberration test, the incidence of chromosome aberrations excluding gaps (TA value), an index of structural chromosome aberrations, and the incidence of polyploid cells did not show statistically significant increases in any test article treatment group. As another result, cells forming a ski-pair were observed at a high frequency in the continuous treatment and dose dependency was observed. Since delay of cell cycle or cell division inhibition was suspected, the confirmation test that longer exposure time (48 hours) was conducted. The result indicated that no statistically significant increase in TA value and the incidence of polyploid cells. A slight increase in the incidence of a ski-pair was observed, but TA value and the incidence of polyploid cells did not increase under the condition that the cell cycle is probably turning not less than one rotation

Based on the results described above, it is concluded that the test article has no potential to induce chromosome aberrations.

C.2.1.2 Repeat Dose Toxicity Assay

<u>Sub-chronic toxicity (13-week oral toxicity study) (Appendix C- 3)</u>
The tests were conducted according to Japanese Test Guidelines (Notification No. 24 of Ordinance no. 1, MHW, 1989, Notification no. 29, MHW, 1996, Notification no. 655, MHW, 1999, Article no. 4 of Notification no. 0219, MHW, 2010) and Japanese Principles of Good Laboratory Practice (Ordinance no. 21, MHW, 1997) at Bozo Research Center Inc. (Study Number: TT-160003).

The test article is the same as the above two in vitro tests.

The test article was orally administered to Sprague-Dawley rats [Crl:CD(SD), 12/sex/group, started at 6 weeks of age] for 13 weeks. The dose solutions at dose levels of 500, 1000 and 2000 mg/kg/day were administered by oral gavage once daily at a dose volume of 20 mL/kg for 13 weeks. A control group was provided and the animals received the vehicle, water for injection. Observation of clinical signs, body weight and food consumption, ophthalmology and urinalysis (including water consumption), haematology, blood chemistry, necropsy, organ weights, and histopathology were performed.

As a result, no deaths occurred in any group and no test article-related toxic changes were noted in any examinations.

Based on the results described above, repeated oral dosing of the test article showed no toxic effects. Therefore, the no-observed-adverse-effect level (NOAEL) of the test article was considered to be 2,000 mg/kg/day (correspond to 1,800mgTOS/kg/day) in both sexes under the test conditions.

TEST Article

Lot No.	GFE68-001@K (β-Galactosidase Enzyme Powder)
Water (%)	4.9
Ash (%)	5.1
TOS (%)	90

In the section A.3.5, the analysis of three batches indicates that β-Galactosidase activity was around 5,800 u/g which is twice that given on toxicity studies. Test article used for the toxicity studies was the substance that was derived from the "M12-22-196" which is indicated in the Appendix D-1. It can be assured that the test article is derived from M12-22-196 which is obtained by mutation of the parent strain and the current strain is obtained from M12-22-196. (Appendix C- 4)

It was confirmed that there is a no difference between the β-Galactosidase structural genes of the current production strain and M12-22-196. This means that the enzyme, β-Galactosidase obtained from the current production strain is exactly the same as the one from M12-22-196.

SAFETY MARGIN

The Margin of Safety (MoS) for human consumption can be calculated by dividing the NOAEL by the Total Theoretical Maximal Daily Intake (TMDI) or Estimated Daily Intake (EDI). As was shown in Section F, the intake of adult and infant and baby are **4.75** and **2.1** mgTOS/kg-bw/day, respectively. Consequently, the MoS are:

Adult: MoS = 1,800 / 4.75 = **379**
Infant and baby: MoS = 1,800 / 2.1 = **857**

As is explained in Section F.3, the intake amount of both cases is highly exaggerated. Moreover, the NOAEL was based on the highest dose administered, and is therefore to be considered as a minimum value. Therefore, the actual MoS in practice will be some magnitudes higher. Consequently, there are no safety reasons for laying down maximum levels of use.

C.2.2 Assessment of the similarity of the amino acid sequence to known protein toxins

Homology search was carried out using MvirDB (http://mvirdb.llnl.gov/) which is provided by the Lawrence Livermore National Laboratory since it was more specific. The database includes not only toxic protein but all the enzyme expressed by drug resistance gene or protein expressed by pathogenic bacteria.

Search conditions
Score 20, E Value: less than 0.5, Match length: more than 15 amino acid sequences, Identity: More than 80%

Result
The result indicated that there was not match with known protein toxins. (Appendix C- 5)

C.3 Information on Potential Allergenicity

C.3.1 Source of the Processing Aid

Cryptococcus laurentii is known as a closely related species with *Papiliotrema terrestris*. It is reported that β-Galactosidase from *Cryptococcus laurentii* has been used for manufacturing of GOS for over 25years in Japan (Daniel Obed Otieno, 2010).

No allergenicity warnings are associated with the use of this organism in foods. Also, no adverse effects have been reported in workers exposed to *Papiliotrema terrestris*.

C.3.2 Allergenicity of β-Galactosidase

Amino-acid sequence

The amino-acid sequence for the β-Galactosidase from *Papiliotrema terrestris* enzyme protein has been determined as indicated below.

```
1     ATTNQDAITP TATGPVGGQG TPAVNFTDYS SSSLEQFWND WVGEVEEPPF AYVPEPPNPY    60
61    PLPNAPPPIY PEYYTKRPKD ILPDYKFPKD FLFGWATAAQ QWEGAVKADG KGPSIWDWAS   120
121   RFPGFIADNT TSDVGDLGYY LYKEDLARIA ALGANVYSFS MFWTRIFPFG KADSPVNQAG   180
181   IDFYHDLIDY SWSLGIEPVV TLFHWDTPLA LQLEYGGFAS ERIIDDYVNY AETVFKAYNG   240
241   SVHKWVTFNE PVVFCSQMAA PVNTTLPPNL NSTIYPYTCS YHLVLAHAKT VKRFRELNIQ   300
301   GQIAFKSDNF VGIPWREGNQ EDIDAVERHQ AYQIGIFAEP IYNTGDWPDI VKNDLSPDIL   360
361   PRFTDDEIAM IKCTADFFPI DGYRDGYVQA VPGGVEACVA NISNPLWPAC NQVNFYDSTP   420
421   AGWAIGTFGN WPTTPWLQNT WQFVRPFLAD LAKRYPTEGG IYLSEFGFSE PFENDKTFIY   480
481   QITQDSGRTA YFNSYLGEVL KGIVEDGIPI KGVFGWSMVD NFEWNSGLST RFGVQYVDYN   540
541   SPTRQRTFKR SALEMSEFWN AHRCSA                                      566
```

The amino-acid sequence of β-Galactosidase enzyme protein has been determined. There are three lengths of amino-acid sequences of the enzyme protein. They depend on the amino acid sequence on the N-terminal side. The longest chain amino-acid sequence is indicated above, and it is constituted of 566 amino acids. The shortest and middle length sequences lack 11 and 6 amino acids on the N-terminal side. Therefore, these are constituted of 555 and 560 amino acids, respectively. Based on the amino acid sequences, the theoretical molecular weights of the enzyme proteins have been calculated and are estimated to be 63.9, 63.3 and 62.8 kDa in each length of enzyme proteins

The homology search was performed (Appendix C- 6). The result of the search indicated that the enzyme protein did not match any known allergens.

Literature Search

In order to address allergenicity by ingestion, it may be taken into account that:

- The allergenic potential of enzymes was studied by Bindslev-Jensen et al. (2006) and reported in the publication: "Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry". The investigation comprised enzymes produced by wild-type and genetically modified strains as well as wild-type enzymes and Protein Engineered variants and comprised 400 patients with a diagnosed allergy to inhalation allergens, food allergens, bee or wasp. It was concluded from this study that ingestion of food enzymes in general is not likely to be a concern with regard to food allergy.
- Previously, the AMFEP Working Group on Consumer Allergy Risk from Enzyme Residues in Food performed an in-depth analysis of the allergenicity of enzyme products (Dauvrin et al., 1998). The overall conclusion was that – as opposed to exposure by inhalation – there are no scientific indications that the small amounts of enzymes in food can sensitize or induce allergy reactions in consumers.
- Enzymes when used as digestive aids are ingested daily, over many years, at much higher amounts when compared to enzymes present in food (up to 1 million times more). Wüthrich (1996) published a list of enzymes used as digestive aids and concluded that they are not potent allergens by ingestion.

Thus, there are no scientific indications that small amounts of enzymes in food can sensitize or induce allergic reactions in consumers.
Additional considerations supporting the assumption that the ingestion of an enzyme protein is not a concern for food allergy should also be taken into account:

- The food enzyme is used in small amounts during food processing, resulting in very small amounts of the enzyme protein in the final food. A high concentration generally equals a higher risk of sensitization, whereas a low level in the final food equals a lower risk (Goodman et al., 2008).
- In the case where proteins are denatured, the tertiary conformation of the enzyme molecule is destroyed. In general, these alterations in conformation are associated with decrease in the antigenic reactivity in humans: in the vast majority of investigated cases, denatured proteins are much less immunogenic than the corresponding native proteins (Valenta and Kraft, 2002; Valenta, 2002; Takai et al., 1997; Takai et al., 2000; Nakazawa et al., 2005; Kikuchi et al., 2006).
- In addition, residual enzyme proteins still present in the final food will be subjected to digestion in the gastro-intestinal system, which reduces further the risk of enzyme allergenicity. While stability to digestion is considered as a potential risk factor of allergenicity, it is believed that small protein fragments resulting from digestion are less likely to be allergenic (FAO/WHO, 2001; Goodman et al., 2008).
- Finally, enzymes have a long history of safe use in food processing, with no indication of adverse effects or reactions. Moreover, a wide variety of enzyme classes (and structures) are naturally present in food. This is in contrast with most known food allergens, which are naturally present in a narrow range of foods.

Long History of Use

Since β-Galactosidase from *Papiliotrema terrestris* in this submission is Amano's new-developed product, there is no record of history of use.

However, *Cryptococcus laurentii* is known as a closely related species with *Papiliotrema terrestris.* It is reported that β-Galactosidase from *Cryptococcus laurentii* has been used for manufacturing GOS for over 25years in Japan.

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

There is no safety assessment report that can be provided.

SECTION D: ADDITIONAL INFORMATION RELATED TO THE SAFETY OF THE ENZYME PROCESSING AID

D.1 Information on the Source Microorganism

- The production microorganism is *Papiliotrema terrestris* which is obtained from the parent strain by conventional mutation using N-methyl-N'-nitro-N-nitroso-guanidine (NTG) (Appendix D - 1).

- The parent strain is originally derived from soil and it is identified as *Cryptococcus terrestris* by the third party. However, *Cryptococcus terrestris* is recommended to transfer *Papiliotrema terrestris* by the current re-evaluated taxonomy using up-to-date methods, (Liu et al, 2015). Therefore the strain name of the species in this submission is *Papiliotrema terrestris*.

- The parent strain has the following phenotypic properties and these are correspond with those of *Cryptococcus terrestris* (Crestani et al, 2009); On YM agar after 3 days at 25°C, colonies are smooth, mucous to butyrous, glistening and cream-colored with an entire margin. Vegetative cells are subspherical to elliptical or oval and budding is monopolar. Formation of sexualreproductive organ was not observed in the plate after 3 weeks of culture.

- The parent strain has been executed homology search using 26S-rDNA-D1/D2 base sequence. The result indicated that the parent strain indicated 100% of homology to the type culture, *Cryptococcus terrestris* CBS10810. The identification was conducted by the third party; TechnoSuruga Laboratory Co., Ltd. (Appendix D - 2). In addition, the identification test was also conducted on the current production strain (i.e. *Papiliotrema terrestris* AE-BLC). The current production strain was identified as *Cryptococcus terrestris* (Current name: *Papiliotrema terrestris*). (Appendix D - 3).

According to the current state of the art, the taxonomic classification of this microorganism is as follows:

Super Kingdom	Eukaryote
Kingdom	Fungus
Phylum	Basidiomycota
Order	Tremellomycetes
Class	Tremellales
Family	Tremellaceae
Genus	*Papiliotrema*
Species	*Papiliotrema terrestris*

ABSENCE OF TOXINS

Papiliotrema terrestris is not known to produce any toxins. However, several major mycotoxins were analysed for reference. Results are indicated in the section A.5.2; Batch Analysis.

D.2 Information on the Pathogenicity and Toxicity of the Source Microorganism

Conventionally, members of the genus *Cryptococcus* are found in a wide variety of habitats, and are difficult to identify by traditional methods. The genus is polyphyletic and is distributed throughout the class *Tremellomycetes*, with representatives in all orders (*Tremellales*, *Filobasidiales* and *Cystofilobasidiales*) (Crestani et al, 2009). In such conventional classification, the genus *Cryptococcus* contained two major pathogenic species, *C neoformans* and *C gatti*. These two species are known to cause cryptococcosis which is consisted with chronic lung disease, a unifocal extrapulmonary condition with cutaneous or neurologic manifestations, or a disseminated process (Negroni, 2012). However, in the current re-evaluated taxonomy using up-to-date methods, *Cryptococcus terrestris* is recommended to transfer the different genus (*Papiliotrema*) from the genus that the two pathogenic species are included (Liu et al, 2015).

Other than that, *Cryptococcus laurentii* and *Cryptococcus albidus* have been known to occasionally cause infection (Cheng et al, 2001), however it is caused to the patients with compromised immunity (owing to HIV infection, cancer chemotherapy, metabolic immunosuppression, etc.).

The source organism of the enzyme in this submission, *C. terrestris* (*P. terrestris*) does not appear on the list of pathogens in Annex III of Directive 2000/54/EC on the protection of workers from risks related to exposure to biological agent at work and it is also evaluated as safety level 1 in the public type culture collections (ATCC[3]). In addition to above, the literature search was performed (without any limit in time) in order to eventually identify academic works on the pathogenicity or toxigenicity of the *C. terrestris* (*P. terrestris*).

No reports or other information indicating any concern with regard to the safety of the organism as a source of enzyme used in food.

In addition to above, pathogenicity of the source organism is experimentally investigated (Appendix D - 4). Oral and Intravenous inoculation study of the source organism using male and female mice were conducted. The result indicated that the source organism did not cause die of animal or abnormality of clinical signs, body weight changes, necropsy findings or histopathological findings by the oral inoculation. By the intravenous inoculation, no animal die and no clinical signs were observed in each dose and in both sex. In the highest dose (2.4×10^9 CFU/mL), decreased locomotor activity on the inoculation day and inhibited body weight gain on 1 day after inoculation were observed in both sex but these changes were transient and no abnormalities were observed thereafter. In the histopathological examination, mild granuloma in the liver was observed in the highest dose of both sex but no viable test organism was observed in any examined organs included the liver. Therefore, the mild granuloma was attributable to a foreign body (killed source organism) removal reaction to a large amount of the inoculated test organism and was not judged to be a change suggesting pathogenicity.

From the above results, test organism, *C. terrestris* was not infectious or pathogenic.

[3] https://www.atcc.org/Search_Results.aspx?dsNav=Ntk:PrimarySearch%7cCryptococcus%7c3%7c-PrimarySearch%7cterrestris%7c3%7c,Ny:True,Ro:0,N:1000552&dsDimensionSearch=D:terrestris,Dxm:All,Dxp:3,N:1000552&dsCompoundDimensionSearch=N:1000552,D:terrestris,Dxm:All,Dxp:3&searchTerms=Cryptococcus&redir=1&fromsearch=true

D.3 Information on the Genetic Stability of the Source Organism

The source micro-organism is neither genetically modified nor self-cloned. The production strain was established by a repeated mutation process from the prior strain. (Mutagen used: N-methyl-N'-nitro-N-nitrosoguanidine) (Appendix D - 5 and Appendix D - 6) The mutation was carried out to obtain a strain that has higher enzyme activity.

In order to ensure the genetic stability of the enzyme, it is produced under well controlled manufacturing processes which are in compliance with AMFEP's guidelines for the safe handling of microbial enzyme preparations (see Section A.4.1).

To ensure the genetic stability of the source organism, the production strain is fermented and is divided into an ampule. They are kept at below -70°C in a locked freezer.

When ready, an ampule is used for each individual fermentation and after use the residue is inactivated prior to discarding the vial. During fermentation the genetic stability of the source organism is monitored through the changes in pH and growth rates.

In order to confirm that the strain of the source organism does not undergo strain drift and that the culture conditions can be applied consistently between batches, enzyme activities and pH of the broth obtained after completing the fermentation are confirmed.

In any instance where a deviation from normal is detected in either of these parameters, the fermentation media is removed from production and discarded. The strain is then checked to ensure that no genetic drift has occurred.

SECTION E: INFORMATION RELATING TO THE SAFETY OF AN ENZYME PROCESSING AID DERIVED FROM A GENETICALLY MODIFIED MICROORGANISM

This section is not relevant to the current processing aid and therefore is not included in this application.

SECTION F: INFORMATION RELATED TO THE DIETARY EXPOSURE TO THE ENZYME PROCESSING AID

A summary of the proposed food uses, the anticipated residue level in foods, the anticipated exposure, and anticipated market share are presented in the Section below.

F.1 Proposed Food Uses

The purpose of use of β-Galactosidase from *Papiliotrema terrestris* is to produce galactooligosaccharide (GOS) from lactose.

GOS will be used as a food ingredient of various foods.

Food enzyme preparations are used by food manufacturers according to the Quantum Satis principle, which means that food manufacturers will typically fine-tune the enzyme dosage based on a dose range recommended by the enzyme supplier.

Typically, the β-galactosidase is added to substrate (lactose) up to **256.3mgTOS/kg raw material weight**.

F.2 Anticipated Residue Levels of β-Galactosidase

In the previous section, it is stated that the β-galactosidase is added to substrate (lactose) up to **256.3mgTOS/kg raw material weight**. TOS of the β-galactosidase is 92.4%. Therefore, 256.3mgTOS/kg is equivalent to 277.3mg/kg.

Use Levels and Residue Levels of β-Galactosidase

- 256.3 mgTOS of β-galactosidase is required to treat 1kg of lactose
- 1kg of GOS mixture* can be obtained from 1kg of lactose by the enzyme reaction
- There are 60% of GOS in GOS mixtures which means 1kg of GOS contains 427.2 mgTOS/kg of β-galactosidase (256.3/0.6)

*GOS mixture: Mixture of GOS, lactose and monosaccharide (glucose, galactose etc.)

Resulted GOS product will be used in various final foods. The recommended use levels of the GOS, the ratio of GOS/final food and estimated maximal level of β-Galactosidase in various foods are shown below. However, it should be stated that actually, β-Galactosidase added to raw material (lactose) is removed during the GOS production process.

Table F - 1: Recommended Use Levels of GOS and estimated maximal level of β-Galactosidase in GOS

(This Table is considered as CCI and provided in the separate document.)

F.3 Information on the Likely Level of Consumption of β-Galactosidase

Commercial food enzyme preparations are generally used following the GMP/Quantum Satis (QS) principle, i.e. at a level not higher than the necessary dosage to achieve the desired final product – according to Good Manufacturing Practice. The amount of enzyme activity added to the raw material by the individual food manufacturer has to be determined case by case, based on the desired effect and process conditions and the raw material's quality. Therefore, the enzyme manufacturer can only issue a recommended enzyme dosage range. Such a dosage range is the starting point for the individual food producer to fine-tune their process and determine the optimal amount of enzyme that will provide the desired effect and nothing more. Consequently, from a technological point of view, there are no 'regular or maximal use levels' and **β-Galactosidase** is used according to the QS principle. A food producer who would add much higher doses than the needed ones would experience excessive costs as well as potentially negative technological consequences mainly with respect to quality.

Microbial food enzymes contain – apart from the enzyme protein in question – some substances derived from the producing micro-organism and the fermentation medium. The presence of all organic materials is expressed as Total Organic Solids (TOS). Whereas the dosage of a food enzyme depends on the enzyme activity present in the final food enzyme preparation, the dosage on basis of TOS is more relevant from a safety point of view. Therefore, the use levels are expressed in TOS.

Actually the β-Galactosidase added to raw material (lactose) is removed during the GOS production process, but EDI was calculated assuming that the all amount remained as an extreme example. However, it should be stated that actually, β-Galactosidase added to raw material (lactose) is removed during the GOS production process.

1. Adult case
1-1; In the case of other than milk beverages and dairy products

In this case, dairy product (exclude milk substitutes), infant and baby foods are excluded for the calculation. The reason of exception is described later.

GOS produced by using **β-Galactosidase from *Papiliotrema terrestris*** may be used in the manufacture of a wide variety of foods, food ingredients and beverages. Due to this wide variety of applications, the most appropriate way to estimate the human consumption in the case of food enzymes is using the so-called Budget Method (Hansen, 1966; Douglass et al., 1997) which calculates a Theoretical Maximum Daily Intake (TMDI) based on conservative assumptions regarding physiological requirements for energy from food and the energy density of food rather than on food consumption survey data.

The Budget Method was originally developed for determining food additive use limits and is known to result in conservative estimations of the daily intake.

The Budget Method is based on the following assumed consumption of important foodstuffs and beverages (for less important foodstuffs, e.g. snacks, lower consumption levels are assumed):

Average consumption over the course of a lifetime/kg body weight/day	Total solid food (kg)	Total non-milk beverages (l)	Processed food (50% of total solid food) (kg)	Soft drinks (25% of total beverages) (l)
	0.025	0.1	0.0125	0.025

For the calculation of the TMDI, the maximum use levels are chosen. Furthermore, it is assumed that all the TOS will end up in the final product.

As indicated in the section F.1, typically, the β-galactosidase is added to substrate (lactose) up to **256.3mgTOS/kg raw material weight**.

The recommended use levels of the enzyme β-Galactosidase are given below based on the raw materials used in the various food processes. For the calculation of the TMDI, the maximum use levels are chosen. Furthermore, it is assumed that all the TOS will end up in the final product.

Table F - 2: Use levels other than milk and dairy products (Adult)

(This Table is considered as CCI and provided in the separate document.)

The Total TMDI can be calculated on basis of the maximal values found in food and beverage, multiplied by the average consumption of food and beverage/kg body weight/day. Consequently, the Total TMDI will be:

TMDI in food (mg TOS/kg body weight/day)	TMDI in beverage (mg TOS/kg body weight/day)	Total TMDI (mg TOS/kg body weight/day)
142.2 x 0.0125 = 1.78	9 x 0.025 = 0.23	**2.0**

Based on the recommended use levels and the amounts of the respective ingredients that end up in the final foods, the TMDI of the food enzyme β-Galactosidase is calculated to be:

2.0 mg TOS/kg body weight/day

1-2; In the case of milk beverages and dairy products

Originally, milk and dairy products cannot be used Budget method for the calculation of TMDI. Therefore, database was used for the calculation of EDI in these applications.

From The EFSA Comprehensive European Food Consumption Database[4] (g/kg bw per day, consumers only), the maximum value of robust 95th percentile intake amount of the application and calculated enzyme intake were summarized in the Table F - 3.

[4] http://www.efsa.europa.eu/en/food-consumption/comprehensive-database

Table F - 3: Use levels of milk and dairy products (Adult)

(This Table is considered as CCI and provided in the separate document.)

1-3; Total intake

Total estimated intake in adult by Budget method (partially including public food consumption survey data) is estimated as **4.75 mgTOS/kg-bw/day**

Table F - 4: Total estimated intake in adult

Method	**Intake (mgTOS/kg-bw/day)**
Budget (except milk beverages and dairy product)	2.00
Food Consumption Database (milk beverages and dairy product)	2.75
Total	**4.75**

It should be stressed that this Total TMDI is based on conservative assumptions and represents a highly exaggerated value because of the following reasons:

- It is assumed that ALL producers of the above mentioned foodstuffs and beverages use the specific enzyme **β-Galactosidase from *Papiliotrema terrestris***;
- It is assumed that ALL producers apply the HIGHEST use level per application;
- For the calculation of the TMDI's in food as well as in beverage, only THOSE foodstuffs and beverages were selected containing the highest theoretical amount of TOS. Thus, foodstuffs and beverages containing lower theoretical amounts were not taken into account;
- It is assumed that the amount of TOS does not decrease as a result of the food production process;
- It is assumed that the final food containing the calculated theoretical amount of TOS is consumed DAILY over the course of a lifetime;

Assumptions regarding food and beverage intake of the general population are overestimates of the actual average levels (Douglass et al., 1997).

2. Infant case

Although the guidance of the EFSA CEF panel on the submission of a dossier for food enzymes (EFSA, 2009) proposes the Budget Method for calculation of the daily intake, it also mentions that where the food enzyme is used for the production of foods specifically designed for infants (0-12 months), ad hoc conservative exposure estimates must be provided.

The dietary exposure for infant was therefore calculated by means of the Estimated Daily Intake (EDI). The EDI can be calculated based on the maximal dose levels and consumption data.

From The EFSA Comprehensive European Food Consumption Database, the maximum value of robust 95 percentile intake amount of the applications for infant and the calculated enzyme intake are shown in Table F - 5. As a result, intake was **2.1 mgTOS/kg-bw/day**.

Table F - 5: Use levels (Infant)

(This Table is considered as CCI and provided in the separate document.)

It should be stressed that this Total EDI is based on conservative assumptions and represents a highly exaggerated value because of the following reasons:

- It is assumed that ALL producers of the above mentioned foodstuffs and beverages use the specific enzyme **β-Galactosidase from *Papiliotrema terrestris***;
- It is assumed that ALL producers apply the HIGHEST use level per application;
- For the calculation of the EDIs in food as well as in beverage, only THOSE foodstuffs and beverages were selected containing the highest theoretical amount of TOS. Thus, foodstuffs and beverages containing lower theoretical amounts were not taken into account;
- It is assumed that the amount of TOS does not decrease as a result of the food production process;
- It is assumed that the final food containing the calculated theoretical amount of TOS is consumed DAILY.

F.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

There is no information on the expected use of this enzyme preparation in Australia/New Zealand or imported product currently sold in Australia/New Zealand.

F.5 Information relating to the levels of residues in foods in other countries

The enzyme does not have any function (activity) in final foods since the enzyme is only used as a processing aid and is inactivated or removed during food production process.
Maximal level described in the section F.3 is the level when we assume if all enzymes remain in final foods.

F.6 For foods where consumption has changed in recent years, information on likely current food consumption

Not applicable.

REFERENCES

Bindslev-Jensen C, Stahl Skov P, Roggen EL, Hvass P and Sidelmann Brinch D (2006). Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry. Food and Chemical Toxicology, 44, 1909–1915

Cheng MF, Chiou CC, Liu YC, Wang HZ, Hsieh KS (2001) Cryptococcus laurentii fungemia in a premature neonate. J Clin Microbiol, vol. 39(4):1608–1611.

Crestani J, Landell M, Faganello J, Vainstein MH, Vishniac HS and Valente P, (2009). Cryptococcus terrestris sp. nov., a tremellaceous, anamorphic yeast phylogenetically related to Cryptococcus flavescens, Int J Syst Evol Microbiol, vol. 59, p631-636.

Daniel Obed Otieno (2010). Synthesis of β-Galactooligosaccharides from Lactose Using Microbial β-Galactosidases

Dauvrin T, Groot G, Maurer K-H, de Rijke D, Ryssov-Nielsen H, Simonse M and Sorensen TB (1998). Working group on consumer allergy risk from enzyme residues in food. An Amfep expert group evaluation study

Directive 2000/54/EC (2000). Official Jounal of the European Communities

Douglass JS, Barraj LM, Tennant DR, Long WR and Chaisson CF (1997). Evaluation of the Budget Method for screening food additive intakes. Food Additives and Contaminants, 14, 791-802

EFSA (2009). Guidance of the Scientific Panel of Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF) on the Submission of a Dossier on Food Enzymes for Safety Evaluation by the Scientific Panel of Food Contact Material, Enzymes, Flavourings and Processing Aids

FAO/WHO (2001). Evaluation of Allergenicity of Genetically Modified Foods

FAO/WHO (2006). COMBINED COMPENDIUM OF FOOD ADDITIVE SPECIFICATIONS, GENERAL SPECIFICATIONS AND CONSIDERATIONS FOR ENZYME PREPARATIONS USED IN FOOD PROCESSING

Gibson GR (1998), Dietary modulation of the human gut microflora using prebiotics, Br J Nutr, 80 (4), S209–S212.

Goodman RE, Vieths S, Sampson HA, Hill D, Ebisawa M, Taylor SL and van Ree R (2008). Allergenicity assessment of genetically modified crops--what makes sense? Nat. Biotechnol., 26 (1), 73-8

Hansen, S. C. (1966). Acceptable daily intake of food additives and ceiling on levels of use. Food and cosmetics toxicology, 4, 427-432.

Kikuchi Y, Takai T, Kuhara T, Ota M, Kato T, Hatanaka H, Ichikawa S, Tokura T, Akiba H, Mitsuishi K, Ikeda S, Okumura K and Ogawa H (2006). Crucial commitment of proteolytic activity of a purified recombinant major house dust mite allergen Der p 1 to sensitization towards IgE and IgG responses. J Immunol., 177, 1609-1617

Liu XZ, Wang QM, Göker M, Groenewald M, Kachalkin AV, Lumbsch HT, Millanes AM, Wedin M and Yurkov AM, (2015), Towards an integrated phylogenetic classification of the Tremellomycetes, St Mycol, vol. 81, p85-147.

Macfarlane GT, Steed H, Macfarlane S, (2008), Bacterial metabolism and health-related effects of galactooligosaccharides and other prebiotics, J. Appl. Microbiol. 104 (2), p305–344.

Nakazawa T, Takai T, Hatanaka H, Mizuuchi E, Nagamune T, Okumura K and Ogawa H (2005). Multiple-mutation at a potential ligand-binding region decreased allergenicity of a mite allergen Der f 2 without disrupting global structure. FEBS Lett., 579, 1988–1994

Negroni RMD, (2012), Cryptococcosis, Clin Dermatol, vol. 30, p599–609.

Roberfroid MB (June 2000), Prebiotics and probiotics: are they functional foods?, Am J Clin Nutr, 71 (6), p1682S–1687S.

Takai T, Ichikawa S, Yokota T, Hatanaka H, Inagaki F and Okumura Y (2000). Unlocking the allergenic structure of the major house dust mite allergen Der f 2 by elimination of key intramolecular interactions. FEBS Lett., 484, 102–107

Takai T, Yokota T, Yasue M, Nishiyama C, Yuuki T, Mori A, Okudaira H and Okumura Y (1997). Engineering of the major house dust mite allergen Der f2 for allergen-specific immunotherapy. Nat. Biotechnol., 15, 754–758

Valenta R (2002). The future of antigen-specific immunotherapy of allergy. Nat. Rev. Immunol., 2, 446–453

Valenta R and Kraft D (2002). From allergen structure to new forms of allergen specific immunotherapy. Curr. Opin. Immunol., 14, 718–727

Wüthrich B (1996). Enzyme als ingestive Allergene. Allergologie für die Praxis, 4, p74-91.