Petition to Amend Schedule 18 of the Australia New Zealand Food Standards Code to Include Triacylglycerol lipase from *Candida cylindracea* as a Processing Aid

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GENERAL REQUIREMENTS

1.0 APPLICANT DETAILS

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d) TEL:[redacted]
e) [redacted]
f) Enzyme manufacturer
g) Contact person: [redacted]

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 triacylglycerol lipase from *Candida cylindracea* as a processing aid intended for use in baking, milk and dairy processing and fats and oil processing.

3.0 JUSTIFICATION FOR THE APPLICATION

3.1.1 Regulatory Impact Information

3.1.1.1 Cost and Benefit of the Proposed Change

The inclusion of triacylglycerol lipase derived from *Candida cylindracea* in the Australia New Zealand Food Standards Code as a processing aid will no cost or benefits to consumers associated with this application. The use of triacylglycerol lipase is one of a number of commercial methods in the manufacture of certain food products. It will allow producers to add a triacylglycerol lipase step to their production process. There will be no additional cost to the regulator if the processing aid is approved as the use of triacylglycerol lipase derived from *Candida cylindracea* and will not impact the regulation of these food products since processing aid are machinery in nature and their use is voluntary. Triacylglycerol lipase acts as a biocatalyst: with the help of the enzyme, a certain substrate is converted into a certain reaction product. It is not the food enzyme itself, but the result of this conversion that determines the effect in the food or food ingredient. After the conversion has taken

place, the enzyme no longer performs a technological function.
The effect of the enzymatic conversion with the help of triacylglycerol lipase is the conversion of the substrate fats and oil in various food raw materials, which may result in improvement of organoleptic properties (flavor). Triacylglycerol lipase can preferentially separate fatty acids besides EPA and DHA from oil to be able to produce high-content EPA and DHA oil.

3.1.1.2 Impact on International Trade

The approval of triacylglycerol lipase derived from *Candida cylindracea* as a processing aid may, in the future, promote international trade and reduction of technical barriers to trade, while continuing to protect public health and safety.

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 triacylglycerol lipase derived from *Candida cylindracea* 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 triacylglycerol lipase derived from *Candida cylindracea* 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 triacylglycerol lipase derived from *Candida cylindracea*, 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 triacylglycerol lipase 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 triacylglycerol lipase 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, the use of triacylglycerol lipase derived from *Candida cylindracea* has a history of use in China and Japan. It is expected that the introduction of triacylglycerol lipase derived from *Candida cylindracea* 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 triacylglycerol lipase derived from *Candida cylindracea* 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 had approved processing aids derived from assessed and granted approval to the same enzyme (Triacylglycerol lipase: EC 3.1.1.3) derived from various kinds of microorganisms, and that Amano Enzyme's triacylglycerol lipase product has already been approved and marketed in several other major jurisdictions for food uses that are similar to those proposed in Australia/New Zealand.

6.0 CONFIDENTIAL COMMERCIAL INFORMATION

None of the information presented in this application are considered to be confidential commercial information.

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:

- Lipase is listed on the Food Additive Index of CODEX General Standard for Food Additives (GSFA) (INS: 1104). Also, this food enzyme, triacylglycerol lipase, complies with the internationally accepted JECFA specifications for chemical and microbiological purity of food enzymes (FAO/WHO, 2006). (See also A.5.1)
- Triacylglycerol lipase from *Candida cylindracea* is permitted as a food additive that may be used as food enzymes in China.
- Triacylglycerol lipase from *Candida cylindracea* is on the “List of Existing Food Additives” published by the Ministry of Health and Welfare of Japan (MHLW, 2014).

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.

SECTION A: TECHNICAL DESCRIPTION OF Triacylglycerol lipase

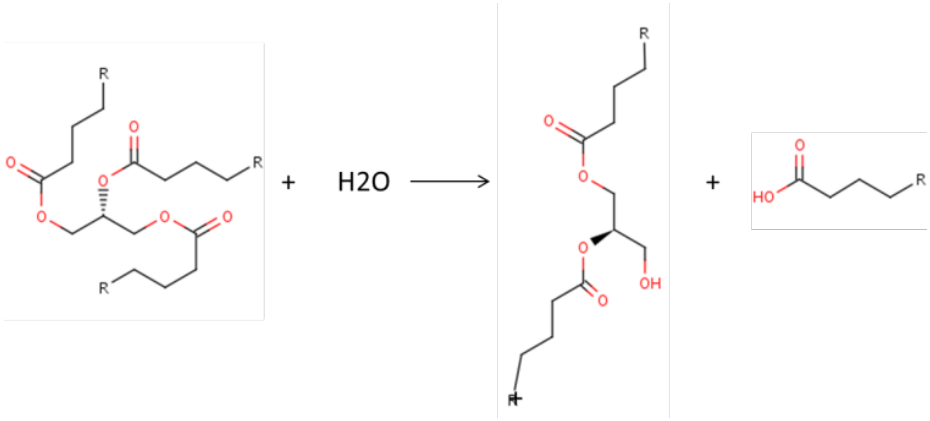
Triacylglycerol lipase is an enzyme of microbial origin that is proposed for use as a processing aid in Australia and New Zealand. A full description of the processing aid including the identity, enzymatic properties, manufacturing process, and purity is presented in this section.

A.1 Information on the Type of Processing Aid

Triacylglycerol lipase is powdered enzyme. triacylglycerol lipase hydrolyses lipids (triglycerides) into fatty acids and mono-, di-glycerides or finally glycerol.
The triacylglycerol lipase catalyses the hydrolysis of short and medium chain fatty acids in preference to long chain ones from 1 and 3 positions of triacylglycerol.
Reaction formula is as follows:

triacylglycerol + H_2O = diacylglycerol + fatty acid
triacylglycerol + $2H_2O$ = monoacylglycerol + 2 fatty acid
triacylglycerol + $3H_2O$ = glycerol + 3 fatty acid

<u>The general reaction scheme</u>



Amano Enzyme has prepared triacylglycerol lipase enzyme preparation that is derived from *Candida cylindracea* 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, triacylglycerol lipase derived from *Candida cylindracea* would fall under the following classification within Schedule 18 (Processing Aids):

18-4 (5) Permitted enzymes of microbial origin

The maximum proposed level of triacylglycerol lipase to food products use is 0.082%. (Normally the enzyme is diluted with dextrin (lipase: 7%, dextrin: 93%). Therefore, the maximum proposed level of

the enzyme preparation including dextrin to food products use is 1.17% (0.082/0.7).

A.2 Information on the Identity of the Processing Aid

Common name:	Triacylglycerol lipase
Systematic name:	Triacylglycerol acylhydrolase
EC number:	3.1.1.3
CAS registration number:	9001-62-1
EINECS number:	232-619-9

The triacylglycerol lipase preparation is produced by *Candida cylindracea* LAYH. Strain LAYH is not genetically modified organism but a chemically mutated production strain derived from the original strain (See also section D.1). *Candida cylindracea* has been used for many years for food or feedstuffs purposes or in the production of enzymes processing aids in China and Japan.

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

A.3.1 Technological Function and Enzymatic Properties

A.3.1.1 Assay for Measuring Triacylglycerol lipase Activity

An analytical method for the detection and quantification of triacylglycerol lipase activity is presented in Appendix A - 1. In brief, lipase activity can be obtained by back titration of the fatty acid released during the hydrolysis of the ester linkage, when lipase acts on the olive oil. One lipase activity unit is defined as the quantity of enzyme that will liberate 1μmole of fatty acid per minute under the conditions of the assay.

A.3.1.2 Characterization of Triacylglycerol lipase Activity

The technical function of triacylglycerol lipase is to catalyze lipids (triglycerides) into fatty acids and mono-, di-glycerides or glycerols. The effects of temperature and pH on the activity of the triacylglycerol lipase 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.3.1 were employed with the only modifications affecting the temperature of the water bath or the pH of the triacylglycerol lipase 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 37°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 7.0. Based on the assays conducted, exhibits activity from pH 5 till pH 10, and from 20°C till 60°C.

Figure A - 1: Effect of the pH

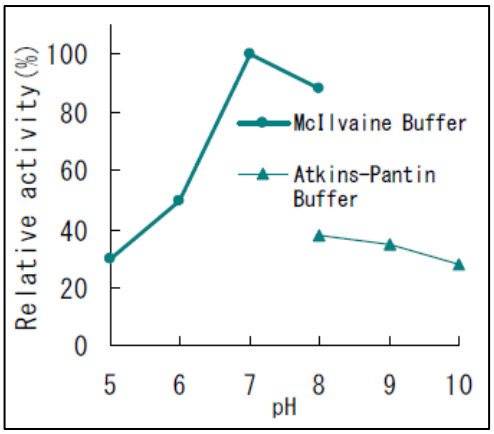
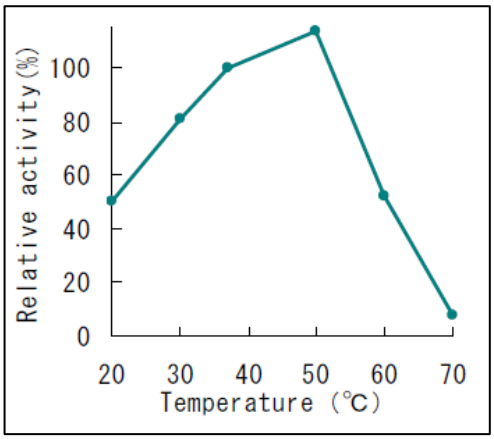


Figure A - 2: Effect of temperature



A.3.2 Stability

pH and THERMAL STABILITY

The stability of triacylglycerol lipase has been assayed. As the enzyme activity was considered the primary marker of the stability of triacylglycerol lipase, the experimental procedures described in Section A.3.1 were employed to assess the stability. The only change to the experimental procedures was the duration of the incubation. The results of the assessment of the thermal and pH stability are presented in Figures A-3 and A-4.

The results of the assessment of stability under varying temperature and pH conditions indicate that triacylglycerol lipase is stable at 40-50°C and in a pH range of 7.

Figure A - 3: pH stability

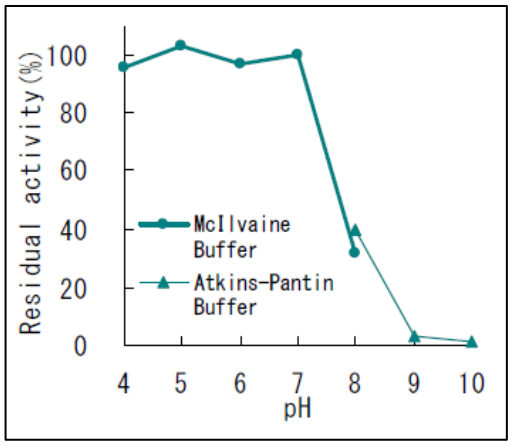
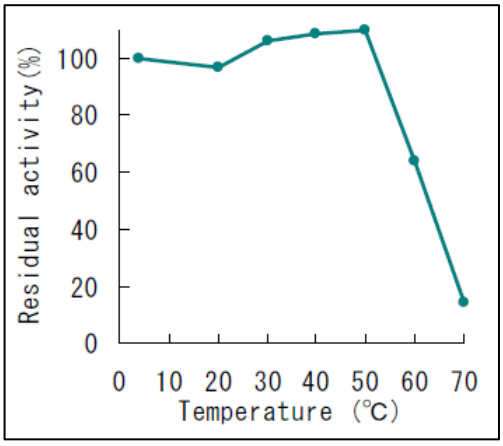


Figure A - 4: Thermal stability



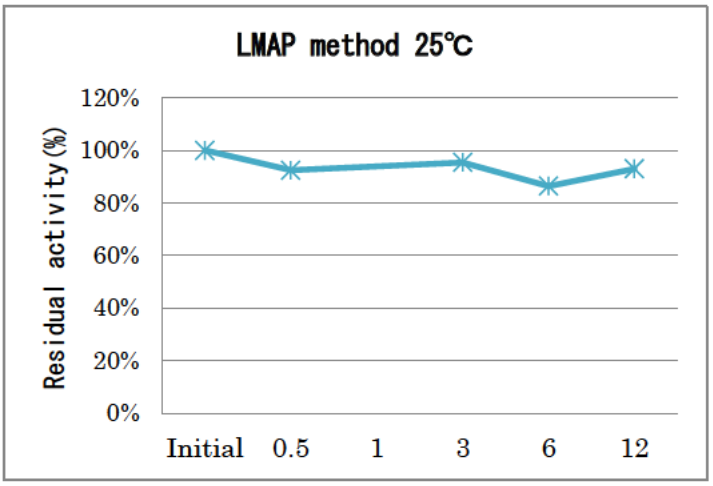
LONG TERM STABILITY

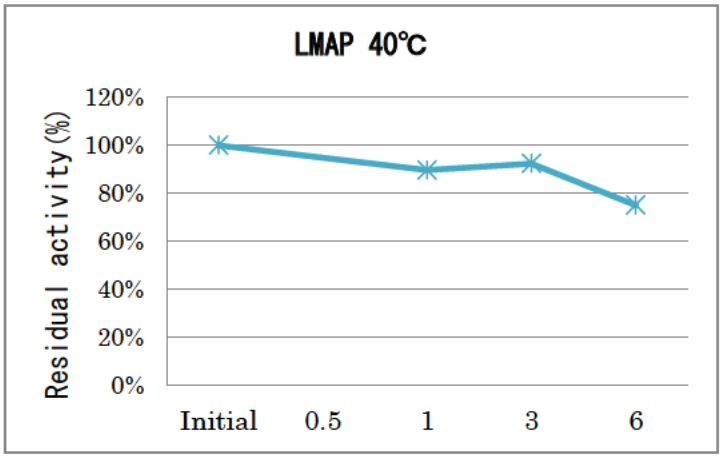
The stability of the triacylglycerol lipase was assayed by the Amano Enzyme Inc. Samples were putted into an airtight bag and kept at 25°C and 40°C.

The lipase activity was periodically measured for 12 months at 25°C and 6 month at 40°C. Results are shown at the below figure.

It could be concluded that the lipase activity remained over 90% of the initial activity after 12 months at 25°C.

Figure A - 5: The stability of the enzyme preparation





A.3.3 Possible Interactions with Food Constituents

Triacylglycerol lipase 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 fats and oil present in non-target foods.

A.3.4 Characterisation of Secondary Activities

As far as Amano is aware, the triacylglycerol lipase described in this dossier does not possess any enzymatic side activities which might cause adverse effects.

Microbial food enzymes are concentrates typically containing minor amounts of other enzyme activities (side activities) naturally produced by the microorganism. However, these activities are not relevant from an application or safety point of view, even if it concerns proteases and phospholipases. Proteases and phospholipases, like many other enzymes, are widely sold as digestive aids, both as over-the-counter registered pharmaceutical products and as dietary supplements. Some of these are available even as chewable dietary supplements. No effects on mucous membranes have been reported, although the enzymes in digestive aids are ingested in their active form and the oral exposure is orders of magnitude higher than the insignificant exposure from food enzymes used as processing aids in food manufacturing.

Furthermore, a wide range of food enzymes, including proteases and phospholipases, have been on the market for decades and have been approved on the market for use in food on basis of safety documentation.

Finally proteases and phospholipases are natural constituents of foods. For instance, bromelain is a protease that is ingested in its active form by consumers eating raw pineapples. Phospholipase is a normal constituent of wheat flour (Nolte et al., 1974) and is one of the digestive enzymes present in the pancreatic juice of mammals, including humans (de Haas et al., 1968; Rossiter, 1968; Johnson and McDermott, 1974).

In order to demonstrate above, the analyses were performed for Amylase activity (Starch dextrinizing activity) and Protease activity. The results showed that no side activities were detected.

Table A - 1: Subsidiary enzyme activities

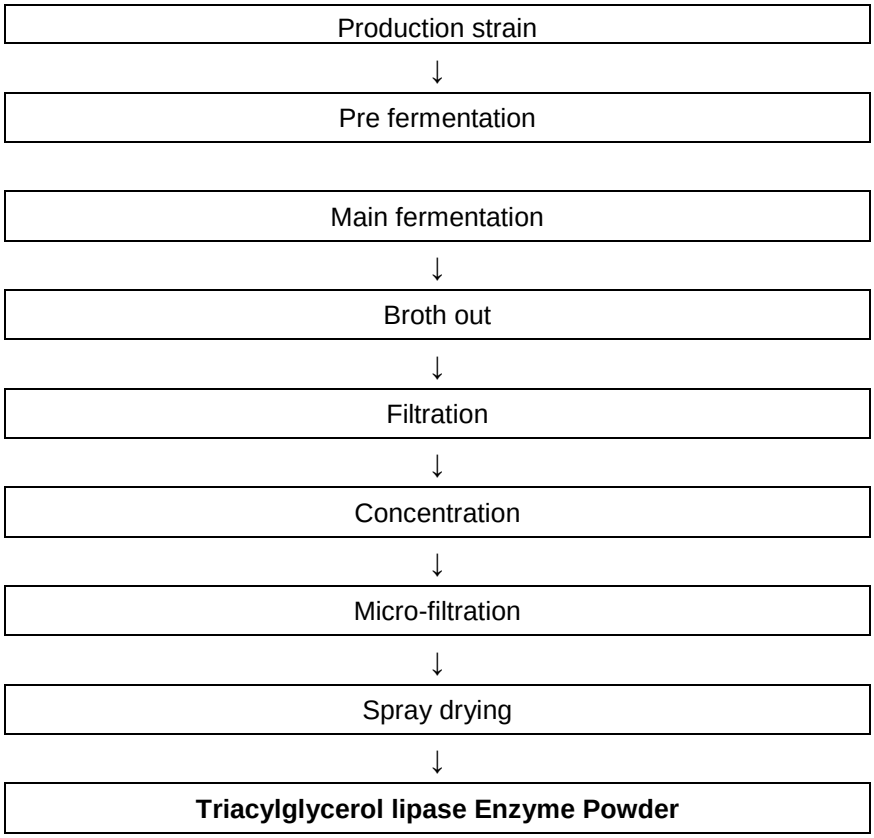
Item	Unit	LAY(SDY)-Y64-006@K	LAY(SDY)-Y64-007@K	LAY(SDY)-Y64-901@K
Amylase	u/g	Not detected	Not detected	Not detected
Protease	u/g	Not detected	Not detected	Not detected
Lipase	u/g	331,000	383,000	467,000

A.4 Manufacturing Process

A.4.1 Manufacturing Steps

A schematic overview of the overall manufacturing process for triacylglycerol lipase is provided in Figure below.

Figure A - 6: Manufacturing Process



In brief, the production begins with the fermentation of *Candida cylindracea* 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. Normally, triacylglycerol lipase enzyme powder is diluted with dextrin to apply for food processing (lipase: 7%, dextrin: 93%). The proposed amount of triacylglycerol lipase to show the function in various foods is indicated in the section F.1.

The enzyme preparation is produced according to the FSSC22000 food safety 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 triacylglycerol lipase 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 triacylglycerol lipase 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

Raw Materials and Processing aids Used in the Production			
Substance	**Grade**	**Function**	**Status in Australia and New Zealand (FSANZ, 2014)**
Soybean oil	Food	Culture media	Food ingredients
Glycerol	Food additive	Culture media	Approved for use a food additive when used in accordance with GMP (Schedule 8)
Glucose	Food	Culture media	Food ingredients
Corn steep liquor	Food	Culture media	Food ingredients
Sorbitol	Food additive	Culture media	Approved for use a food additive when used in accordance with GMP (Schedule 8)
Yeast extract	Food	Culture media	Food ingredients
Ammonium phosphate dibasic	Food additive	Culture media	Approved for use a food additive when used in accordance with GMP (Schedule 8)
Dipotassium phosphate	Food additive	Culture media	Approved for use a food additive when used in accordance with GMP (Schedule 8)
Ammonium sulfate	Food additive	Culture media	Permitted for use in the production of processing aids (Schedule 18)
Magnesium sulfate	Food additive	Culture media	Approved for use a food additive when used in accordance with GMP (Schedule 8)
Potassium phosphate monobasic	Food additive	Culture media	Approved for use a food additive when used in accordance with GMP (Schedule 8)
Sodium hydroxide	Food additive	Culture media (pH adjustment)	Permitted for use in the production of processing aids (Schedule 18)
Hydrochloric acid	Food additive	Culture media (pH adjustment)	Approved for use a food additive when used in accordance with GMP (Schedule 8)

Raw Materials and Processing aids Used in the Production			
Substance	**Grade**	**Function**	**Status in Australia and New Zealand (FSANZ, 2014)**
Water	Tap water	Processing	—
Silicone compound (Polydimethylsiloxane)	Food additive	Antifoaming	Approved for use a food additive when used in accordance with GMP (Schedule 8)
Sodium chloride	Food	Purification, Filtration	Food ingredients
Dextrin	Food	Purification, Diluent	Food ingredients
Diatomaceous earth	Food additive	Filtration	Permitted for use in the production of processing aids (Schedule 18)
Hydrochloric acid	Food additive	pH adjustment	Approved for use a food additive when used in accordance with GMP (Schedule 8)
Sodium hydroxide	Food additive	pH adjustment	Permitted for use in the production of processing aids (Schedule 18)

A.4.3 Residual Allergens from the Culture Medium

Soybean product (Soybean oil) is used in the fermentation media. Residual soy protein was analyzed and resulted as “not more than the detection limit (1µg/g)” in triacylglycerol lipase enzyme powder.

Furthermore, as described in Section F.1, triacylglycerol lipase is added only at low levels (0.082% at the maximal*) to food products for enzyme reaction. The exposure to any potential residual soy allergens in final food products consumed will be negligible and extremely unlikely to be of any allergenic concern.

*: Maximal use level to food product: 719 mgTOS/kg (See section F.1)
= 719/0.88=0.817g/kg = 0.082% (TOS: 88%, see section A.5.2)

A.5 Specification for Identity and Purity

A.5.1 Product Specification

The Chemical and Microbiological Specification

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

Table A - 3: Specification for Triacylglycerol lipase

The Chemical and Microbiological Specification	
Lead	Not more than 5 mg/kg
Salmonella sp.	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 applicable for yeast derived enzymes
Enzyme Activity	
Lipase activity	Not less than 33,000 u/g
General Properties	
Appearance	White to light yellowish brown powder

A.5.2 Batch Analysis

The proof that the food enzyme triacylglycerol lipase 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 triacylglycerol lipase from *Candida cylindracea* 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	LAY(SDY)-Y64 -006@K	LAY(SDY)-Y64-007@K	LAY(SDY)-Y64-901@K	Mean
Heavy metals				
Lead	0.155 mg/kg	0.262 mg/kg	0.242 mg/kg	-
Microbiology				
Salmonella sp.	ND/25g	ND/25g	ND/25g	-
Total coliforms	1000 cfu/g	< 1000 cfu/g	2000 cfu/g	-
Escherichia coli	ND/10g	ND/10g	ND/10g	-
Antimicrobial activity				
Antimicrobial	Negative	Negative	Negative	-
Protein content and relative purity				
Ash (%)	6.2	6.7	7.7	6.9
Water (%)	5.1	5.0	5.2	5.1
TOS (%)	88.7	88.3	87.1	88.0
Enzyme activity (u/g)	331,000	383,000	467,000	393,667
Units/mg TOS	373	433	536	448
Protein (%)	14.9	16.7	18.2	16.6

ND: Not detected

ABSENCE OF TOXINS

Candida cylindracea is not known to produce any bacterial toxins, which is why it is a common production organism for food processing enzymes. As the species *Candida* is yeast, it does not produce any mycotoxins. In addition, according to the EFSA's SCIENTIFIC OPINION[1], it was concluded that *Candida cylindracea* can be recommended for QPS status when it is used for the

[1] http://www.efsa.europa.eu/sites/default/files/scientific_output/files/main_documents/3938.pdf

production of enzymes. Therefore, it can be concluded that *Candida cylindracea* has no safety concern.

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 triacylglycerol lipase derived from *Candida cylindracea.*

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

Triacylglycerol lipase from *Candida cylindracea* has been used in Japan for many years in food processing, and it is currently on the “List of Existing Food Additives” published by the Ministry of Health and Welfare of Japan (MHLW, 2014).

Also, triacylglycerol lipase from *Candida cylindracea* is permitted as a food additive that may be used as food enzymes in China (Appendix C - 1).

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

As mentioned in Section C.1, triacylglycerol lipase has a wide history of use in food processing. To further support the safety of triacylglycerol lipase enzyme preparation, several toxicity studies have been conducted to assess the safety. The potential mutagenic and genotoxic activity of the triacylglycerol lipase 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
- 90-day oral toxicity on rats: The No Observed Adverse Effect Level (NOAEL 10.2 g/kg bw/day) is 581 mg TOS/kg bw/day, which is the high dose in the study.

ABSENCE OF TOXINS

Candida cylindracea is not known to produce any bacterial toxins, which is why it is a common production organism for food processing enzymes. As the species *Candida cylindracea* is a yeast, it does not produce any mycotoxins. In addition, according to the EFSA's SCIENTIFIC OPINION[2], it was concluded that *Candida cylindracea* can be recommended for QPS status when it is used for the production of enzymes. Therefore, it can be concluded that *Candida cylindracea* has no safety concern.

[2] http://www.efsa.europa.eu/sites/default/files/scientific_output/files/main_documents/3938.pdf

C.2.1 Triacylglycerol lipase Concentrate

C.2.1.1 Mutagenicity and Genotoxicity

The following two genotoxicity studies and a chronic toxicity study were carried out in accordance with Japanese recognized guidelines.

A bacterial reverse mutation test (Appendix C - 2)

A bacterial reverse mutation test of lipase liquid concentrate was conducted at Bozo research center Inc. Study Number: T-1056, (2012)

The test was conducted in accordance with the test guidelines of Japanese Ministry of Health and Welfare (JMHW, 1999)

The test article, lipase liquid concentrate (Lot No. 65-002# having a specific lipase activity of 71,000 u/mL) is a material which is obtained after concentration from normal commercial production. The test article contains neither the diluent nor the stabilizer.

The test used 5 tester strains, *Salmonella typhimurium* strains TA 98, TA 100, TA 1535, TA 1537 and the *Escherichia coli* strain WP2 *uvrA*.

In this study, a dose-range finding test and main test were conducted with and without metabolic activation by the pre-incubation method. Water for injection was used as the vehicle for the test article. Seven dose levels (100, 25, 6.25, 1.56, 0.39, 0.0977, 0.0244 and 0 % solution / plate) were set for the dose-range finding test and five dose levels (100, 50, 25, 12.5, 6.25 and 0 % solution / plate) for the main test. 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 metabolic activation. It was therefore concluded that the test article does not induce gene mutations in the bacterial reverse mutation assay under the test conditions employed for this study.

Chromosome aberration test (Appendix C - 3)

Chromosome aberration in cultured Chinese hamster cells treated with lipase liquid concentrate was conducted at Bozo Research Center Inc. Study Number: T-G050, (2012).

The test was conducted in accordance with the test guidelines of Japanese Ministry of Health and Welfare (JMHW, 1999)

The test article, lipase liquid concentrate (Lot No. 65-002# having a specific lipase activity of 71,000 u/mL) is a material which is obtained after concentration from normal commercial production. The test article contains neither the diluent nor the stabilizer.

Based on the results of the cell-growth inhibition test, the dose levels for the chromosome aberration test were set at 100 % liquid concentrate (maximum dose level), 50, 25, 12.5, 6.25, 3.13, 1.56, 0.781% and 0 solution for both short-term treatment and continuous treatment.

In the chromosome aberration test, in the short-term treatment with and without metabolic activation, the percentage of cells with abnormalities excluding gaps (TA value, an index for structural chromosome aberrations) was 0, 1.0, 0 and 1.0% (with metabolic activation) and 0.5, 1.0, 2.0 and 1.5% (without metabolic activation) at 100, 50, 25, 12.5 % solution respectively. Since the TA value was less than 5% at all concentrations, the result was judged to be negative.

In the 24-hour continuous treatment, the TA value was 0, 0, 1.0 and 0.5% (24hr) at 3.13, 1.56, 0.781 and 0.391 %, respectively, which was less than 5%, and therefore the result was judged to be negative.

In the 48-hour continuous treatment, the TA value was 0, 0, 0 and 1.0 % (48hr) at 0.781, 0.391, 0.195 and 0.0977 % solution, respectively, which was less than 5%, and therefore the result was judged to be negative.

Based on the results described above, it was concluded that the test article had no chromosome aberration inducibility under the conditions of this test.

C.2.1.2 Repeat Dose Toxicity Assay

Sub-chronic toxicity (13-week oral toxicity study) (Appendix C - 4)

Reference :

A 13-week oral toxicity study of lipase concentrate (*Candida cylindracea*) in rats was conducted at Bozo Research Center Inc. Study Number: B-7279, (2013).

GLPs and QA:

The test was conducted in accordance with the test guidelines of Japanese Ministry of Health and Welfare (JMHW, 2010)

Summary:

The test article, lipase liquid concentrate (Lot No. 65-002# having a specific lipase activity of 71,000 u/mL) is a material which is obtained after concentration from normal commercial production. The test article contains neither the diluent nor the stabilizer.

Three groups, each comprising ten males and ten females, received test article at doses of 100 % (liquid concentrate), 50% and 25% solution. A similarly constituted Control group received the vehicle (purified water obtained by reverse osmosis) at the same volume-dosage as in the dose group. There were no test article-related deaths in any group. Administration of the test article was associated with no effects in clinical signs, body weight, food consumption, urinalysis, ophthalmology, haematology, blood chemistry, organ weight, necropsy or histopathological examination.

Based on the results described above, it was estimated that the no observed adverse effect level of the test article for oral administration to rats for 13 weeks was higher than 10mL/kg/day (correspond to 10.2g/kg/day, 581 mgTOS/kg/day) for both males and females.

Data reporting

All individual safety studies and reporting of data have been performed according to the respective guidelines. For further details, see the individual studies (Appendix C – 2 to C - 4).

The composition and specifications of the test article are given in the table below:

Test article: Lipase liquid concentrate

Table C - 1: The composition and specifications of the test article

Lot No.	**65-002#**
Ash (%)	1.0
Water (%)	93.3
TOS (%)	5.7
Activity (U/mL)	71,000
U/mg TOS	1246
Salmonella sp. (per 25 g)	Negative
Total coliforms (per g)	Less than 10/g
Coliforms	Negative

The test article used for the toxicity test is liquid concentrate obtained after concentration process of normal commercial production.

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). As was shown in Section F.8, the Total TMDI of the food enzyme is 0.102 mg TOS/kg body weight/day. Consequently, the MoS is:

MoS = 581 / 0.102 = 5,696

As is explained in Section F.8, the Total TMDI 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.3 Information on Potential Allergenicity

C.3.1 Source of the Processing Aid

Candida cylindracea is used and approved for use as a source organism in many countries (refer to the section 8.0). No allergenicity warnings are associated with the use of this organism in food in these countries. Amano's *Candida cylindracea* in this submission has been sold and used safely for the production of food enzymes for over 30 years in Japan. Meanwhile no pathogenic or toxic accident has been arisen in the workers exposed to the strain. Furthermore, it has a long history of use in various industries (see the section C.3.2: long history of use).

C.3.2 Allergenicity of Triacylglycerol lipase

Amino-acid sequence

Amino-acid sequence of Triacylglycerol lipase was determined. The homology search of EFSA CEF Guidance document on Food enzymes (EFSA, 2009b) was performed. As a result, there was no match with any allergens (Appendix C - 5).

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 is that exposure to enzyme proteins by ingestion, as opposed to exposure by inhalation, are not potent allergens and that sensitization to ingested enzymes is rare.

- 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 assumptions that the ingestion of an enzyme protein is not a concern for food allergy should also be taken into account:

- The majority of proteins are not food allergens and based on previous experience, the enzyme industry is not aware of any enzyme proteins used in food that are homologous to known food allergens.
- 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 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[3]; 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

Triacylgricerol lipase from *Candida cylindracea* has been used in wide variety of industrial uses such as medicines, cosmetics, detergents, and also food industry areas.
In the food industry areas, the enzyme has been used in the production of bakery products, flavour, dairy products, beverage and edible fats and oils, etc. (Hasan et al 2006, kim and Hou, 2006; Arnold et al 1975; Pandey et al, 1999). Triacylgricerol lipase from Candida cylindracea has been used safely for a couple of decades, therefore, *Candida cylindracea* has a long history of safe use.

[3] http://www.who.int/foodsafety/publications/biotech/en/ec_jan2001.pdf

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

Triacylglycerol lipase from *Candida cylindracea* was evaluated in China. The enzyme received the approval for use in 2012[4]. (English translation is attached in Appendix C - 6)

[4] http://www.nhfpc.gov.cn/sps/s7891/201205/70b499205c984b0b87d50c69346fea90.shtml

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

D.1 Information on the Source Microorganism

The production organism for this enzyme preparation is a strain of *Candida cylindracea*. The wild type strain, *Candida cylindracea*, is very common and widely distributed in soil.
Amano's *Candida cylindracea* has been used safely for the production of food enzymes for many years.

The production strain LAYH-1 was obtained by several mutations of the original strain that was found in Japanese soil. The production strain is derived via selection by conventional mutagenesis using NTG (N-methyl-N'-nitro-N-nitrosoguanidine), UV (Ultraviolet), EMS (Ethyl methanesulfonate) and CI(Cell isolation) (Appendix D - 1). Recombinant DNA technology is not used to obtain this strain. It has been identified as *Candida cylindracea*.

The microorganism that is used for the production of triacylglycerol lipase, is the Yeast, *Candida cylindracea*. According to the current state of the art, the taxonomic classification of this microorganism is as follows:

Genus	*Candida*
Species	*Candida cylindracea*

The strain is identified by the third party (TechnoSuruga Laboratory Co., Ltd. Japan) from the following assay results (Appendix D - 2):
(Test material is described as "SIID6376" which is the same strain as "LAY-3" as described in Appendix D - 1.)
Based on the results of the homology search using BLAST, the 26S rDNA-D1/D2 sequence of the original strain was the closest to that of an anamorphic ascomycete yeast, *Candida cylindracea* NRRL Y-17506T with 100％ similarity.
The colonies in this strain were crater-like. Cell reproduction is predominantly pseudohypahe composed of elongate cells with few ellipsoidal cells by budding. These characteristics agreed well will those of *C. cylindracea*（Kurtzman and Fell, 1998）.

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

Candida cylindracea was evaluated as safety level 1 in the public type culture collections (ATCC[5] and DSMZ[6]).

In addition to the above, by the literature search performed in order to eventually identify academic works on the pathogenicity or toxigenicity of the Genus *Candida* and *Candida cylindracea* (performed without any limit in time), no reports of mycotoxin production or other information indicating any concern with regard to the safety of the organism as a source of enzyme preparations used in food. Also, according to the EFSA's SCIENTIFIC OPINION[7], no clinical reports for *Candida cylindracea* have been reported and it can be recommended for QPS status when it is used for the production of enzymes.

[5] http://www.atcc.org/products/all/14830.aspx
[6] http://www.dsmz.de/catalogues/details/culture/DSM-2031.html
[7] http://www.efsa.europa.eu/sites/default/files/scientific_output/files/main_documents/3938.pdf

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 were established by a repeated mutation process from the prior strain. (Mutagen used: NTG (N-methyl-N'-nitro-N-nitrosoguanidine), UV (Ultraviolet), EMS (Ethyl methanesulfonate) and CI(Cell isolation) (Appendix D - 3 and Appendix D - 4)

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).

In brief, 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 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 food enzyme catalyses the lipids into fatty acids and mono-, di-glycerides or glycerols. Triacylglycerol lipase will be used in the following food manufacturing process:

- Baking
- Milk and dairy processing
- Fats and oil processing

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.
The table below provides recommended dose ranges in the various food processes:

Table F - 1: Recommended Dose Ranges

Application	Raw material (RM)	Recommended use levels (mg TOS/kg RM)
Baking	Flour	1 – 4
Milk and dairy processing	Dairy products	14 – 144
Fats and oil processing	Edible fats and oils of animal, fish or vegetable	72 – 719

Doses are expressed in Total Organic Solids (TOS).

The maximum proposed level of the enzyme preparation including diluent (dextrin) to food products is 1.17%. (As indicated above, the maximum use level of triacylglycerol lipase is 719 mgTOS/kg RM. TOS of triacylglycerol lipase enzyme powder is 88.0% (see the section A.5.2).) 1.17% is calculated by the following:

719/ (0.88×1000) = 0.817 g/kg

0.817/0.07 = 11.67 g/kg = 1.17%

0.88: TOS (88%)

0.07: 7% lipase

F.2 Anticipated Residue Levels of Triacylglycerol lipase

The recommended use levels of the enzyme triacylglycerol lipase are given, based on the raw materials used in the various food processes.

(i) The case of application other than fats and oil processing (Baking and Milk and dairy processing)

Table F - 2: Maximal level in final food (Baking and Milk and dairy processing)

Application		Raw material (RM)	Maximal recommended use level (mg TOS/kg RM)	Final food	Ratio RM/final food	Maximal level in final food (mg TOS/kg food)
Solid food	Baking	Flour	4	Bread, Pastries	0.55	**2.2**
	Mi k and dairy processing	Dairy products	144	Processed cheese, cream cheese, sauces, soups, dressings, desserts, ice cream, baking goods, snack foods, convenience food, fats	0.05	**7.2**

(ii) The case of application of Fats and oil processing

The application range is narrow since the final product of this application is dietary supplemets only. And dietary supplements are commonly consumed at constant amount. Therefore TMDI in this application can estimate from the use level of the enzyme in the raw material and the daily consumption of the final product.

Table F - 3: The recommended use levels of the enzyme (fats and oil processing)

Application		Raw material (RM)	Maximal recommended use level (mg TOS/ kg RM)	Final food
Solid food	Fats and oils processing	Ed ble fats and oils of animal, fish or vegetable	719	Dietary supplements containing DHA/EPA

F.3 Information on the Likely Level of Consumption of Triacylglycerol lipase

The food enzyme is used in the manufacture of a wide variety of foods and food ingredients. 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 Ref (Hansen, 1966; Douglass et al., 1997). This method enables to calculate 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

The recommended use levels of the enzyme 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.

(i) The case of application other than fats and oil processing (Baking and Milk and dairy processing)

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:

Table F - 4: The Total TMDI (Baking and Milk and dairy processing)

TMDI in food (mg TOS/kg body weight/day)	**TMDI in beverage (mg TOS/kg body weight/day)**	**TMDI (mg TOS/kg body weight/day)**
7.2x0.0125=0.090	–	**0.090**

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 triacylglycerol lipase was calculated to be:

0.090 mg TOS/kg body weight/day.

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 triacylglycerol lipase from *Candida cylindracea*;
- 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).

(ii) The case of application of fats and oil processing

As described above, the maximal recommended use levels of the enzyme (fats and oil processing) is

719 mgTOS/kg RM.

Paramaters are as follows;

- The maximal recommended use level of the enzyme in the raw material (DHA/EHA concentrate):
  719 mgTOS/kg RM
- Concentration of DHA/EPA in the DHA/EPA concentrate (Tanaka et al., 1993):
  Approximatelly 50%.
- Surveyed recommended daily consumption of DHA/EPA by the producers:
  500 mg/person/day

From these parameters;

- 500mg of DHA/EPA corresponds to approximately 1,000mg DHA/EPA concentrate.
  500mg / 50% = 1000mg
- The amount of the enzyme in the 1,000mg of DHA/EPA concentrate corresponds to 0.719mgTOS
  (719 mgTOS / 1000000 mg) x 1000 mg = 0.719 mgTOS

- Consequently, the Total TMDI in this application will be **0.012 mgTOS/kg body weight/day**.
  (0.719 mgTOS/person/day) / 60kg body weight = 0.012

Also in this case it should be stressed that this TMDI is based on conservative assumptions and represents a highly exaggerated value because of the same reasons descrived above.

Total of TMDI

The above mentioned two kinds of the case of application are independent so that it should be add up the TMDI of each application.

Total TMDI = 0.090 + 0.012 = 0.102 mgTOS/kg body weight/day

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

This enzyme is approved in Japan and China. The approved food uses and maximum use levels are identical to those proposed for use in Australia. As a result it is anticipated that the levels of residues in foods imported from these jurisdictions would be identical to those manufactured in Australia.

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

Not applicable.

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