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

4 April 2022
196-22

Supporting document 1

Risk and technical assessment – Application A1244

Chymosin from GM *Trichoderma reesei* as a processing aid (enzyme)

Executive summary

The purpose of the application is to amend Schedule 18 – Processing Aids of the Australia New Zealand Food Standards Code (the Code) to include chymosin (EC 3.4.23.4), sourced from a genetically modified (GM) strain of *Trichoderma reesei.* This production organism contains the chymosin gene from the domestic cow, *Bos taurus*. Chymosin is proposed for use as a processing aid in the manufacture of cheese, cheese products and fermented and rennetted milk products. The enzyme preparation will be used according to Good Manufacturing Practice (GMP) conditions.

The evidence assessed by FSANZ provides adequate assurance that the enzyme, in the quantity and form proposed to be used, is technologically justified and achieves its stated purpose. The enzyme meets international purity specifications.

The host organism (*T. reesei)* is neither pathogenic nor toxigenic and has a long history of safe use in food. The gene donor organism (*Bos Taurus*) has a history of safe use for food enzymes and raises no safety concerns. Analysis of the of the GM production strain (*T.reesei* t-AWL31) confirmed the presence and stability of the introduced DNA.

Chymosin produced by alternate GM micoorganisms is already permitted in the Code. The results of bioinformatics searches showed no homology with known toxins or food allergens. The scientific literature includes cases of respiratory allergy to bovine rennet or chymosin, but no cases of allergic reactions in response to oral exposure. There is considerable evidence that people with respiratory allergies can safely consume the allergenic proteins. Wheat is used as a source of glucose for fermentation during production of the enzyme.

No toxicology studies in animals have been conducted with this particular chymosin. Toxicity studies conducted on enzymes produced by related strains of *T.reesei* include a number of studies in rodents, as well as genotoxicity assays. No adverse effects or evidence of pathogenicity were discovered in any of the rodent studies, and no evidence of mutagenicity or clastogenicity was discovered in any of the genotoxicity assays. The most closely related strain is one producing catalase. For that enzyme, a no observed adverse effect level (NOAEL) of 700 mg total organic solids (TOS)/kg bw/day was identified in a 90-day oral toxicity study in rats. The theoretical maximum daily intake (TMDI) of this chymosin was calculated by FSANZ to be 0.125 mg TOS/kg bw. A comparison of the NOAEL and the TMDI results in a large Margin of Exposure (MOE) of approximately 5600. Based on the reviewed data it is concluded that in the absence of any identifiable hazard an Acceptable Daily Intake

(ADI) ‘not specified’ is appropriate. FSANZ concludes that there are no public health and safety concerns.

Table of Contents

1 Introduction

Danisco New Zealand Ltd applied to Food Standards Australia New Zealand (FSANZ) to permit the use of the enzyme chymosin (EC 3.4.23.4) as a processing aid in the manufacture of cheese and cheese products and fermented and rennetted milk products. This enzyme is sourced from a genetically modified (GM) strain of *Trichoderma reesei,* containing the chymosin gene from *Bos taurus.*

Currently, Schedule 18 of the Australia New Zealand Food Standards Code (the Code) includes permission for three chymosin enzymes, produced by *Aspergillus niger, Escherichia coli K-12 strain* GE81 and *Kluyveromyces lactis.* Therefore, this particular chymosin enzyme produced by a GM *T. reesei* needs a pre-market assessment before permission can be given for its use as a processing aid. If permitted, the enzyme will provide an additional option for food and beverage manufacturers that produce dairy products.

1.1 Objectives of the assessment

The objectives of this risk and technical assessment were to:

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

- evaluate potential public health and safety concerns that may arise from the use of this enzyme, produced by a GM microorganism, as a processing aid, specifically by considering the:

  - history of use of the gene donor and production microorganisms
  - characterisation of the genetic modification(s), and
  - safety of the enzyme.

2 Food technology assessment

2.1 Characterisation of the enzyme

2.1.1 Identity and properties of the enzyme

Danisco provided relevant information regarding the identity of the enzyme which has been verified using an appropriate enzyme nomenclature reference.

Accepted IUBMB[1] name:	Chymosin
Systematic name:	Aspartic protease
Other names:	Rennin
IUBMB enzyme nomenclature:	EC 3.4.23.4
CAS number[2]:	9001-98-3

[1] International Union of Biochemistry and Molecular Biology
[2] Chemical Abstracts Service

Reaction:	Hydrolytic cleavage of 104-Ser-Phe-|-Met-Ala-107 in kappa-casein. It clots milk by cleavage of a single bond in κ-chain of casein.

2.2 Manufacturing process

2.2.1 Production of the enzyme

The enzyme preparation is produced by submerged fermentation of *T. reesei,* carrying the chymosin gene from *B. taurus.* The fermentation processes are consistent with the scientific literature and references provided by Danisco (Aunstrup 1979).

The fermentation process begins with propagation of the culture, seed fermentation and primary fermentation. A new stock culture vial of *T. reesei* is used to start the production of each new batch. Appropriate control measures are in place for temperature, pH, air flow, agitation and oxygen content.

The recovery process separates the biomass then purifies, concentrates and stabilises the enzyme. Either filtration or centrifugation (or a combination of both) is used to separate the cell debris from the liquid of the fermentation broth. The manufacturing site will determine which method is used.

The ultrafiltration step is then used to remove low molecular weight compounds. Diafiltration can also be used to achieve the desired enzyme activity, colour and particle size. Finally, polish filtration is undertaken by microfiltration membranes, fine filtration aids or sterile filtration pads. The concentrate is then dried and agglomerated or stabilised (using glycerol) for a liquid product.

The chymosin is typically sold as a powder or liquid preparation with enzyme activity of 700 IMCU/ml however this will depend on the final product. The commercial enzyme preparation is called Chymostar. A list of raw materials has been provided as Confidential Commercial Information (CCI) and materials conform to the Food Chemical Codex, 6th edition (FCC 2008). This has been confirmed against the Code. For the materials not included in the FCC requirements, in-house limits were established by the manufacturer based on the FCC requirements.

Danisco states that manufacturing is completed in accordance with Good Manufacturing Practice (GMP) and the enzyme meets the general specifications for enzyme preparations used in food processing as established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA 2006) per Table 1 below.

2.2.2 Allergen considerations

Danisco has stated that the materials used in the fermentation process contain wheat which is considered one of the top eight allergens in the United States of America[3] (FALCPA 2004). No other allergenic ingredients were listed. Danisco has provided documentation including a product specification sheet, an allergen declaration and risk assessment considering the VITAL program, the Food Allergy Research and Resource Program at the University of Nebraska, ELISA analysis and recommendations by the Association of Manufacturers and Formulators of Enzyme Products (AMFEP). Danisco concluded that there is no allergenic risk associated with the enzyme however it is worth noting there are documented cases of respiratory allergy to bovine chymosin. Sections 3.3.6 and 3.3.7 provide more information on the allergen risk associated with this enzyme.

[3] Milk, eggs, fish, crustacean shellfish, tree nuts, peanuts, wheat and soybeans

2.2.3 Specifications

The JECFA Combined Compendium of Food Additive Specifications (FAO/WHO 2006) and the Food Chemicals Codex 12th edition (The United States Pharmacopeia 2020) are international specifications for enzymes used in the production of food. These are primary sources of specifications listed in section S3—2 of Schedule 3 of the Code. Enzymes need to meet these specifications. Schedule 3 of the Code also includes specifications for heavy metals (section S3—4) if they are not already detailed within specifications in sections S3—2 or S3—3.

Table 1 provides a comparison of the analysis of a single batch of the chymosin enzyme preparation (Chymostar) with international specifications established by JECFA and Food Chemicals Codex, as well as those in the Code (as applicable). One sample has been tested as it is a new product and only a single batch has been produced. Based on these initial results, the enzyme preparation meets all relevant specifications.

Table 1 *Analysis of enzyme preparation chymosin compared to JECFA, Food Chemicals Codex, and Code specifications for enzymes (single batch)*

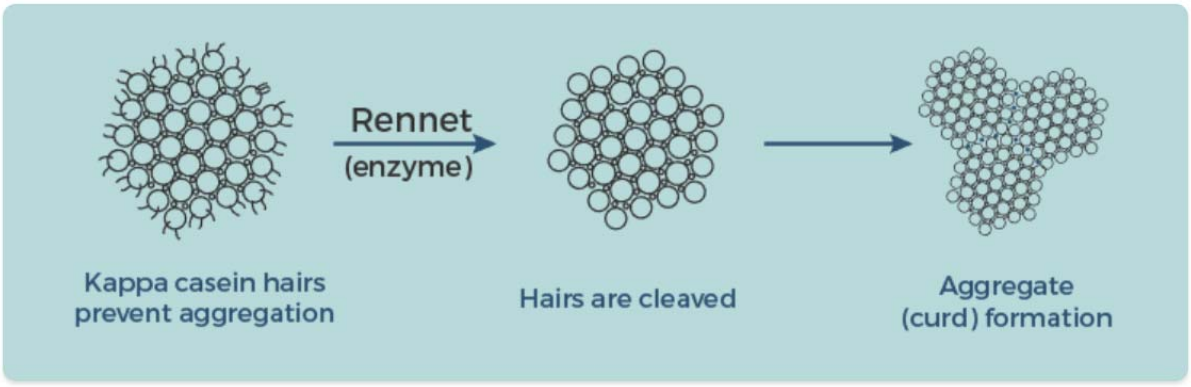
Analysis	Danisco results	JECFA	The Code (section S3-4)
Lead (mg/kg)	0.06	≤ 5	≤2
Arsenic (mg/kg)	<0.1	-	≤1
Cadmium (mg/kg)	<0.01	< 0.5	≤1
Mercury (mg/kg)	<0.01	< 0.5	≤1
Coliforms (cfu/g)	< 1	≤30	-
Salmonella (in 25 g)	Absent	Absent	-
E. coli (in 25 g)	Absent	Absent	-
Antimicrobial activity	Absent	Absent	-
Listeria monocytogenes	Absent		
Staphylococcus aureus	Absent		
Mycotoxins	Absent	No toxicologically significant levels	No toxicologically significant levels

2.3 Technological purpose of the enzyme

The enzyme preparation is intended to be used in the manufacture of cheese, cheese products and fermented and rennetted milk products. Casein forms hydrophilic micelles in milk. The kappa casein on the outside of the micelles allows the casein to remain separate until chymosin is used to cleave them. Once the reaction has occurred and the kappa casein

removed, the micelles become hydrophobic and clot. This clot is the formation of cheese and the separation of whey from the curds.

The function of chymosin is to hydrolyse 104-Ser-Phe-|-Met-Ala-107 in kappa casein. The image below illustrates the action that chymosin has on the kappa casein bond to produce milk curd. Rennet, the enzyme pictured, is sourced from the stomachs of cows. The chymosin assessed in the application is a non-animal version of this enzyme.



Chymosin clips off the hairy layer and allows the casein micelles to attach

Figure 1 Chymosin (rennet pictured) cleaves the kappa casein to produce curd[4].

The stated technological purpose of chymosin in cheese, cheese products and fermented and rennetted milk products is consistent with the scientific literature (Jensen et al. 2015). No studies were provided as evidence of its use and benefits in production.

Table 2 *Chymosin enzyme preparation physical/chemical properties*

Physical/chemical properties	
Enzyme activity	700 International Milk Coagulation units IMCU/ml
Appearance	Clear amber liquid or powder
Temperature optimum	25 to 45 degrees for activity
Temperature stability/storage	Stable below 10 degrees for 40 weeks
pH optimum	~3.8
pH stability	Stable between 5.3 - 6.3

Danisco provided the results of a characterisation study on the enzyme, indicating that it is stable up to 40 weeks at below 10° C, with close to 100% activity remaining. The enzyme has an optimum pH of around 3.8 but clots milk at 6.7, which is the pH of milk (Szecsi and Harboe 2013), making the enzyme an appropriate choice for dairy processing.

The chymosin preparation will be used as a processing aid where the enzyme is present in negligible amounts with little to no technical function in the final food. Additional evidence has

[4] Cheese Science Toolkit, Chymosin. Accessed on 20 January 2022 https://www.cheesescience.org/chymosin.html

been provided by Danisco to confirm this. Chymosin is a protein and any residual amounts remaining in food consumed would accordingly have the same nutritional value as any other dietary protein. However, the use levels of chymosin are very low and as with other enzymes that are currently approved and used as processing aids, use of this preparation would not have any nutritional significance.

Danisco claims and FSANZ agrees that chymosin performs its technological function during food processing and, as such, meets the definition of a processing aid.

2.4 Technological justification of the enzyme

The enzyme is intended for use in dairy applications in the manufacture of cheese, cheese products and fermented and rennetted milk products.

There is already an established history of use for chymosin in dairy processing (Garg and Johri 1995) and three other chymosin enzymes are approved for use in the Code. Considering this, Danisco has highlighted that approval would provide manufacturers with an additional choice of enzyme to facilitate the coagulation of casein, support effective production processes and reduce the use of raw materials.

In-house testing by Danisco compared chymosin (as Chymostar™) against a similar fermentation derived coagulant and an animal rennet. These tests focus on pH and temperature and demonstrate the similarities between animal and non-animal rennet. Chymostar™ was found to have a higher function of relative clotting speed compared to the other enzymes. It was also found to have similar clotting speed when measured at various temperatures. The results provide evidence for the benefits of non-animal rennet enzyme, Chymostar™.

2.5 Food technology conclusion

FSANZ concludes that the use of chymosin from GM *Trichoderma reesei* in dairy processing is clearly described in the application and is consistent with its known technological functions in milk clotting. Analysis of the evidence provides adequate assurance that the use of this enzyme, in the quantity and form proposed to be used, produced under GMP controls and processes, is technologically justified. The enzyme meets international purity specifications.

3 Safety assessment

Some information relevant to this section is CCI, so full details cannot be provided in this public report.

3.1 History of use

3.1.1 Host organism

Trichoderma reesei is a filamentous fungus that was first isolated in 1944 from cotton canvas. The original isolate QM6a is the type strain for *T. reesei* (Olempska-Beer et al. 2006), and has been registered with the American Type Culture Collection as ATCC 13631. Due to the secretion of a range of cellulolytic enzymes, this fungus has been used since the 1980s for the industrial production of enzymes for a range of industries including food (Nevalainen and Peterson 2014; Paloheimo et al. 2016).

In this application the production strain *T. reesei* t-AWL31 was derived from the *T. reesei* strain RL-P37, which was originally derived from QM6a. Danisco has provided information to

confirm the identity of strain RL-P37 as *T. reesei.* Using the safe strain lineage concept[5], the information provided by Danisco showed that the production strain t-AWL31 was non-toxigenic. FSANZ has previously assessed the safety of *T. reesei* as the source organism for a number of enzymes used as processing aids, including triacylglycerol lipase (A1159; 2019), alpha-glucosidase (A1169; 2019), xylanase (A1174; 2020), glucoamylase (A1194; 2020) and alpha-amylase (A1195; 2020).

T. reesei is classified as a biosafety level 1 organism, based on the United States Public Health Service Guidelines[6], and is considered non-pathogenic to humans. Although some *T. reesei* strains can produce mycotoxins, most industrial production strains do not produce mycotoxin or antibiotics under conditions used for enzyme production (Nevalainen et al. 1994; Blumenthal 2004).

3.1.2 Gene donor organism

The gene that encodes the chymosin enzyme was synthesised *in vitro* based on the sequence from *B. taurus* available in public databases. As the donor organism (*B. taurus*) is cattle and the gene was chemically synthesised so there is no potential for carryover of other factors from the donor organism, there are no public health and safety issues with the gene donor.

3.2 Characterisation of the genetic modification(s)

3.2.1 Description of DNA to be introduced and method of transformation

The chymosin enzyme is encoded by the prochymosin gene (refer to section 3.1.2). Data provided by Danisco and analysed by FSANZ confirmed the expected chymosin amino acid sequence.

The prochymosin gene was inserted into the genome of *T. reesei* and placed under the control of the native cellobiohydrolase 1 (*cbh 1*) gene regulatory sequences, using the native orotate phosphoribosyl transferase (*pyr2*) gene as a selectable marker. The *pyr2* gene allows for selection of positive transformants by growth on minimal media devoid of uridine (Jørgensen et al. 2014).

3.2.2 Characterisation of inserted DNA

Data provided by Danisco confirmed the presence of the inserted DNA in the production strain *T. reesei* t-AWL31. No bacterial vector DNA was introduced during the genetic modification, hence antibiotic resistance genes are not found in the *T. reesei* t-AWL31 production strain.

3.2.3 Genetic stability of the inserted gene

The stability of the introduced DNA in the production strain was examined by genome sequencing. DNA extracted from cultures after prolonged fermentation and stock culture prior to fermentation as a control were analysed. This data substantiates the stability of the prochymosin gene in the *T. reesei* t-AWL31 genome.

[5] The term *safe strain lineage* refers to related strains that have all been derived by genetic modification from a single precursor that has been thoroughly characterized and shown to be non-toxigenic and non-pathogenic (Pariza and Cook 2010; EFSA 2018).
[6] For more information please see the following CDC webpage: https://www.cdc.gov/labs/BMBL.html

3.3 Safety of chymosin

3.3.1 History of safe use of the enzyme

Chymosin (rennin) is an enzyme found in rennet, a substance produced by the epithelium of the abomasum (fourth stomach, true gastric stomach) of young ruminants. Rennet has been used for thousands of years for the purpose of curdling milk in order to make cheese. The chymosin that is the subject of this application is that of *Bos taurus*, the domestic European cow, and is intended for the same uses for which chymosin from abomasums of calves was traditionally used.

Bovine chymosin produced by genetically modified microorganisms (strains of *Aspergillus niger, Escherichia coli* K-12 strain GE81*,* and *Kluyveromyces lactis.*) were approved by JECFA at the 37th meeting in 1990, with monographs published online in 2006[7][8][9], and are also approved in the Code.

3.3.2 Bioinformatics concerning potential for toxicity

Results of a BLAST search for homology of the chymosin sequence against the complete Uniprot database were provided. The threshold E-value[10] was 0.1. None of the 1000 database matches was annotated as a toxin or venom. The majority of matches were chymosins or pepsins.

Additionally, a specific BLAST search was conducted for homology of the mature chymosin sequence against the Uniprot animal toxin database. This search did not identify any matches.

3.3.3 Toxicology data

No toxicology studies in animals have been conducted with the chymosin that is the subject of this application, which is based on the principle of safe strain lineage. Danisco provided a confidential summary of toxicity studies conducted on enzymes produced by related strains of *T.reesei*. The toxicity studies include a number of studies in rodents, including 90-day toxicity studies in rats, as well as genotoxicity assays including bacterial reverse mutation assays and *in vitro* chromosomal aberration assays. No adverse effects or evidence of pathogenicity were discovered in any of the *in vivo* studies, and no evidence of mutagenicity or clastogenicity was discovered in any of the genotoxicity assays.

Of the *T. reesei* strains for which toxicity data are available for the enzymes they produce, Danisco identified that strain most closely related to t-AWL31 is a strain producing catalase. For that enzyme, a no observed adverse effect level (NOAEL) of 700 mg total organic solids (TOS)/kg bw/day was identified in a 90-day oral toxicity study in rats. The study was conducted in compliance with OECD test guideline 408, and U.S. FDA (21 CFR part 58) Good Laboratory Practice Standards, with minor identified deviations that did not affect the scientific validity of the study. FSANZ has reviewed the study report and is satisfied with the integrity of the study and the conclusions relating to the test article.

[7] https://www.fao.org/fileadmin/user_upload/jecfa_additives/docs/Monograph1/Additive-132.pdf
[8] https://www.fao.org/fileadmin/user_upload/jecfa_additives/docs/Monograph1/Additive-131.pdf
[9] https://www.fao.org/fileadmin/user_upload/jecfa_additives/docs/Monograph1/Additive-133.pdf
[10] The E value (or Expect value) indicates the significance of a match found when searching a sequence database. The closer an E value is to zero, the less likely an alignment could have been produced by chance.

3.3.4 Potential for allergenicity

Cases of respiratory allergy due to bovine rennet or chymosin were identified by literature search. A case of asthma due to exposure to powdered rennet, which was prevented by the use of liquid rennet, was described by Niinimäki and Saari (1978). Jensen et al. (2006) concluded that rennet, and particularly rennet powder, is a potent respiratory allergen as a result of an assessment of employees at a rennet-producing plant. Sixty percent (21/35) of employees had symptoms consistent with hay fever (allergic rhinitis) and 10 of those individuals reported that their symptoms principally occurred in the workplace. Nine individuals had mild to moderate symptoms of asthma, and six of those reported that their symptoms were related to the workplace. Fourteen individuals had a positive response to skin prick test with one or more rennets, and sensitisation rate was highest among those who regularly worked with rennet powder. Neither Niinimäki and Saari (1978) or Jensen et al. (2006) identified which component of rennet was the allergen. In contrast, a case of asthma in a cheese factory worker that was due specifically to bovine chymosin was reported by Gómez Torrijos et al. (2018). The proteins in bovine rennet, which principally comprise chymosin and pepsin, were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein bands with the ability to bind to IgE from the affected patient were identified from bovine chymosin but not bovine pepsin.

No cases of allergic reaction in response to oral exposure to bovine rennet or chymosin were located by literature search, and there is considerable evidence that people with respiratory allergies can safely consume the allergenic proteins (Cullinan et al. 1997; Brisman 2002; Poulsen 2004; Armentia et al. 2009). For example, respiratory allergy to wheat flour is common in bakers, but affected bakers can eat bread without adverse effects.

Results of recent (<2 years) bioinformatics searches of the AllergenOnline[11] database for homology with the bovine chymosin were provided. The searches included full-length alignment, 80mer sliding window search, and 8 amino acid exact match search. No significant sequence similarity with known food allergens was identified.

Wheat is used as a source of glucose for fermentation during production of the enzyme.

3.3.5 Assessments by other regulatory agencies

Documents were provided by Danisco to show that bovine chymosin synthesized by genetically modified *T. reesei* is approved for use in the USA, Denmark, France and Mexico. These approvals were not accompanied by written assessments.

Bovine chymosin synthesized by genetically modified *T. reesei* has not been assessed by JECFA.

The EFSA Panel on Food Contact Materials, Enzymes and Processing Aids conducted a safety evaluation of bovine rennet ('the food enzyme containing chymosin and pepsin from the abomasum of calves and cows') (EFSA 2021). Based on the history of safe use, the EFSA Panel considered that toxicological data and a dietary exposure assessment were not required. The Panel considered that the risk of allergic sensitisation by dietary exposure could not be excluded, but the likelihood of this occurring was low. Overall, the Panel concluded that this food enzyme does not give rise to safety concerns under the intended conditions of use.

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

3.4 Dietary exposure assessment

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

The budget method is a valid screening tool for estimating the theoretical maximum daily intake (TMDI) of a food additive (Douglass et al. 1997). The calculation is based on physiological food and liquid requirements, the processing aid concentration in foods and beverages, and the proportion of foods and beverages that may contain the processing aid. The TMDI can then be compared to an ADI or a NOAEL to estimate a margin of exposure for risk characterisation purposes.

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

- the maximum physiological requirement for solid food (including milk) is 25 g/kg body weight/day
- 50% of solid food is processed
- the maximum physiological requirement for non-milk beverages is 100 mL/kg body weight/day (the standard level used in a budget method calculation)
- 25% of non-milk beverages are processed
- 10 litres of milk produces 1 kg cheese and 1 litre of milk contains 7 grams of whey protein
- soft drinks can contain up to 17.5 g of whey protein per litre, and bakery products can contain up to 40 g of whey protein per kg
- the highest of all proposed uses in final foods for all uses in solid foods was used in the TMAL calculation (there was only one use level proposed for non-milk beverages)
- all of the enzyme remains in the final food
- all foods contain the highest use level of 0.4 mg TOS/kg raw material (milk).

Based on these assumptions, Danisco calculated the TMDI of the enzyme to be 0.075 mg TOS/kg body weight/day.

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

- The maximum physiological requirement for solid food (including milk) is 50 g/kg body weight/day (the standard level used in a budget method calculation where there is potential for the enzyme to be in baby foods or general purpose foods that would be consumed by infants).
- FSANZ would generally assume 12.5% of solid foods contain the enzyme based on commonly used default proportions noted in the FAO/WHO Environmental Health Criteria (EHC) 240 Chapter 6 on dietary exposure assessment (FAO/WHO 2009). However, Danisco has assumed a higher proportion of 50% based on the nature and extent of use of the enzyme and therefore FSANZ has also used this proportion for solid foods as a worst case scenario.

All other inputs and assumptions used by FSANZ remained as per those used by Danisco. The TMDI based on FSANZ's calculations for solid food and non-milk beverages is 0.125 mg TOS/kg body weight/day.

Both the FSANZ and applicant's estimates of the TMDI will be overestimates of the dietary exposure given the conservatisms in the budget method. This includes that it was assumed that the enzyme remains in the final foods and beverages whereas Danisco has stated that it