A Chymosin Enzyme
from a recombinant strain of *Trichoderma reesei*

PROCESSING AID APPLICATION

Food Standards Australia
New Zealand

Applicant: DANISCO NEW ZEALAND LTD

5th October 2021

CONTENTS

EXECUTIVE SUMMARY:

IFF Health and Biosciences (IFF H&B) is seeking approval for a "Chymosin (EC 3.4.23.4)" enzyme for use as processing aid in dairy application. The enzyme is designated as "Chymosin" throughout the dossier.

The enzyme Chymosin is derived from a selected non-pathogenic, non-toxigenic strain of *Trichoderma reesei* which is genetically modified to overexpress the Chymosin gene from *Bos taurus.*

The enzyme is intended for use in dairy applications primarily in the manufacture of cheese and cheese products, fermented and rennetted milk products. In the applications Chymosin performs the technological function of clotting milk by highly specific cleavage activity of a single bond in κ-chain of casein.

In all these applications, Chymosin will be used as a processing aid where the enzyme is either not present in the final food or present in insignificant quantities having no function or technical effect in the final food.

To assess the safety of the Chymosin for use in these applications, IFF H&B vigorously applied the criteria identified in the guidelines as laid down by Food Standards Australia New Zealand (FSANZ) and U.S. Food and Drug Administration (FDA) utilising enzyme toxicology/safety data, the safe history of use of enzyme preparations from *T. reesei* and of other Chymosin enzymes in food, the history of safe use of the *T. reesei* production organism for the production of enzymes used in food, an allergenicity evaluation, and a comprehensive survey of the scientific literature.

The safety of the food enzyme from *T. reesei* has been assessed using toxicology studies conducted on earlier strains of the IFF H&B *T. reesei* Safe Strain Lineage. The most suitable standard package of toxicological tests from the Safe Strain Lineage was identified to support the safety of the food enzyme object of the current dossier. The toxicological tests showed the following results:

- Ames test: no mutagenic activity under the given test conditions
- Chromosomal aberrations: no clastogenic activity under the given test conditions
- 90-day oral toxicity on rats: Under the conditions of this study, the no-observed-adverse-effect-level (NOAEL) was established at the high dose 700 mg total organic solids (TOS)/kg body weight/day.

Based on a worst-case scenario that a person is consuming Chymosin in dairy based food application, the calculated Theoretical Maximum Daily Intake (TMDI) will be 0.075 mg TOS/kg body weight/day. This still offers a 9,333-fold margin of safety.

Based on the results of safety studies and other evidence, Chymosin has been demonstrated as safe for its intended applications and at the proposed usage levels. Approval of this application would provide manufacturers and/or consumers with benefits of facilitating the coagulation of casein, lowering the manufacturing cost, and improving quality of dairy based foods.

General information

1.1 Applicant details

(a) [redacted]

1.2 Purpose of the application

This application seeks to modify Schedule 18 to Standard 1.3.3 Processing Aids to permit the use of a new *Processing Aid*, subject of this application. The intended use of the processing aid is in dairy processing.

This application is made solely on behalf of IFF Health and Biosciences (IFF H&B), the manufacturer/marketer of the *Processing Aid*. When approved, the *Processing Aid* would be available for use by any food manufacturer in Australia and New Zealand.

Chymosin, the subject of this application, is intended for use in diary processing, for the production primarily of cheese and beverages.

Currently no Chymosin from *B. taurus* expressed in *T. reesei* is permitted as a Processing Aid, however other enzymes including α-amylase, Cellulase, Endo-1,4-beta-xylanase, β-Glucanase, Hemicellulase multicomponent enzyme, Polygalacturonase or Pectinase multicomponent enzyme, from *T. reesei* are listed in Schedule 18 section S18-4(5) as permitted enzymes. Approval of this application would provide food processors with a new enzyme preparation offering the benefits and advantages as discussed in Section 2.3 and Appendix A.

1.3 Justification for the application

1.3.1. Regulatory Impact Information

A. Costs and Benefits of the application

Chymosin is an enzyme produced by submerged fermentation of *T. reesei* carrying the gene encoding the Chymosin gene from *B. taurus.* The enzyme is assigned the functional classification EC 3.4.23.4. A collection of information detailed in Section 3 supports the safety of the production organism and the enzyme for use in the applications outlined in Section 4.

The enzyme catalyses the hydrolysis of casein and is intended for use to clot milk in the production of cheese and other dairy products.

The enzyme activity is traditionally found in rennet derived from the stomach linings of young ruminant animals. This type of animal rennet has been used in the cheesemaking process for thousands of years. The enzyme in this application is produced by fermentation of the genetically engineered *T. reesei* and doesn't pose issues and concerns associated with animals as the source.

More information on the benefit of this enzyme can be found in Section 2.2 and Appendix A.

Enzyme preparations are widely used as processing aids in the manufacture of food products. Currently no Chymosin from *B. taurus* expressed in *T. reesei* is permitted as a Processing Aid. Approval of this application would provide food processors with a new enzyme preparation offering the benefits and advantages as discussed previously.

B. Impact on international trade

The inclusion of Chymosin from *B. taurus* expressed in *T. reesei* in the Australia New Zealand Food Standards Code as a processing aid may promote international trade on products produced

using this enzyme and reduce technical barriers to trade. In addition, the enzyme doesn't raise concerns at the point of importation that is associated with animal sourced ingredients.

1.4.Support for the application

No marketing or promotional activities have been undertaken for Chymosin derived from *T. reesei* containing the gene for chymosin from *B. taurus* in the Australia/New Zealand market. Hence at this stage, no requests from food manufacturers are provided in support of this application. However, the need and justification for use of the processing aid are discussed in Section 1.3, and it is anticipated that support from the food processing industry will be submitted during the period for public comment on the application Draft Regulatory Measure/Assessment Report.

1.5. Assessment Procedure

This application seeks to modify Schedule 18 to Standard 1.3.3 Processing Aids to permit the use of a Processing aid that is currently not permitted. Based on guidance in the Application Handbook, IFF H&B considers General Procedure Level 1 (up to 350 hours) to be the appropriate procedure for assessment of the application.

1.6.Confidential Commercial Information (CCI)

Certain (identified) technical and manufacturing information included in Appendices B1, B3, -B6, Appendices D1-D3, Appendices E1-E5 and other information including amino acid sequences labelled with Confidential Commercial information is regarded by the applicant as **Confidential Commercial Information** (CCI) and is provided in the application strictly on this basis. This information is the result of a significant research and development effort and investment by the applicant; it is not in the public domain and is considered as either proprietary or commercially sensitive. It would be disadvantageous to the applicant if this information were released into the public domain.

1.7.Exclusive Commercial Capturable Benefit (ECCB)

According to Section 8 of the FSANZ Act, this application is not expected to confer Exclusive Capturable Commercial Benefit (ECCB).

1.8. International and other National Standards

Refer to Appendix D for further details

1.8.1 Codex Standards

Chymosin from *B. taurus* produced by *T. reesei* has not been reviewed by JECFA; there is no specific Codex Standard relevant to this application.

1.8.2 International Legislation

Chymosin derived from *T. reesei* carrying the gene encoding the chymosin gene from *B. taurus* is approved in Denmark, France, and Mexico (See Appendix D).

1.9. Statutory declaration

I, Caroline Elizabeth Gray,

of 7 Te Kare Rd, Wai O Taiki Bay, Auckland 1072, New Zealand, Regulatory Affairs Manager/Director

make the following declaration under the Oaths and Declaration Act 1959:

1. the information provided in this application fully sets out the matters required; and
2. the information is true to the best of my knowledge and belief; and
3. no information has been withheld which might prejudice this application to the best of my knowledge and belief.

I understand that a person who intentionally makes a false statement in a statutory declaration is guilty of an offence under section 11 of the Statutory Declarations Act 1959, and I believe that the statements in this declaration are true in every particular.

Signature _____

Declared at Auckland on 7th of October 2021

Before me, _____ Laurene Catherine Holley . t

Laurene Catherine Holley

Auckland,

_____

Signature

Barrister + Solicitor

Laurene Holley
Barrister
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1.10. Checklist

	Mandatory Requirements	Check	Page Number	Remarks
General requirements for applications	A. Form of the application	✓	N.A.	
	Table of contents	✓	1	
	Executive summary	✓	2	
	B. Applicant details	✓	3	Section 1.1
	C. Purpose of application	✓	4	Section 1.2
	D. Justification for the application	✓	4	Section 1.3
	D.1 Regulatory impact information	✓	4	Section 1.3.1
	D.1.1 Costs and benefits of the application	✓	4	Section 1.3.1
	D.1.2 Impact on international trade	✓	4	Section 1.3.1
	E Information to support the application	✓	4	Section 1.4
	E.1 Data requirements	✓	N.A.	
	F. Assessment procedure	✓	5	Section 1.5
	G. Confidential commercial information (CCI)	✓	5	Section 1.6
	H. Other confidential information	✓		
	I. Exclusive capturable commercial benefit (ECCB)	✓	5	Section 1.7
	J. International and other national standards	✓	5	Section 1.8
	J.1 International Standards	✓	5	Section 1.8.1
	J.2 Other national standards or regulations	✓	5	Section 1.8.2
	K. Statutory declaration	✓	6	Section 1.9
	L. Checklist	✓	7	Section 1.10
3.3.2. Processing aids	A. Technical information on the processing aid	✓	9	Section 2
	A.1 Information on the type of processing aid	✓	9	Section 2.1
	A.2 Information on the identity of the processing aid	✓	9	Section 2.2
	A.3 Information on the chemical and physical properties of the processing aid	✓	9	Section 2.3
	A.4 Manufacturing process	✓	10	Section 2.4
	A.5 Specification for identity and purity	✓	11	Section 2.5
	A.6 Analytical method for detection	✗		Not applicable for enzymes used as processing aids
	C. Information related to the safety of an enzyme processing aid	✓	12	Section 3
	C.1 General information on the use of the enzyme as a food processing aid in other countries	✓	12	Section 3.1
	C.2 Information on the potential toxicity of the enzyme processing aid	✓	13	Section 3.2
	C.3 Information on the potential allergenicity of the enzyme processing aid	✓	14	Section 3.3

	C.4 Safety assessment reports prepared by international agencies or other national government agencies, if available	✓	14	Section 3.4
	D. Additional information related to the safety of an enzyme processing aid derived from a microorganism			Section 3
	D.1 Information on the source microorganism	✓	14	Section 3.5
	D.2 Information on the pathogenicity and toxicity of the source microorganism	✓	15	Section 3.6
	D.3 Information on the genetic stability of the source organism	✓	15	Section 3.7
	E. Additional information related to the safety of an enzyme processing aid derived from a genetically-modified microorganism			Section 3
	E.1 Information on the methods used in the genetic modification of the source organism	✓	16	Section 3.8
	F Information related to the dietary exposure to the processing aid		17	Section 4
	F.1. A list of foods or food groups likely to contain the processing aid or its metabolites	✓	17	Section 4.1
	F.2 The levels of residues of the processing aid or its metabolites for each food or food group	✓	17	Section 4.2
	F.3 For foods or food groups not currently listed in the most recent Australian or New Zealand National Nutrition Surveys (NNSs), information on the likely level of consumption	✓	18	Section 4.3
	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	✓	18	Section 4.4
	F.5 Information relating to the levels of residues in foods in other countries	✓	18	Section 4.5
	F.6 For foods where consumption has changed in recent years, information on likely current food consumption	✓	18	Section 4.6

2. Technical information

Please refer to Appendix A for further details

2.1. Type of processing aid

The Chymosin enzyme is an enzyme produced by submerged fermentation of *T. reesei*, carrying the chymosin gene from *B. taurus*.

This Processing Aid falls into the category "Enzymes of microbial origin" from the Food Standard Code section 1.3.3-6 Enzymes.

2.2. Identity

2.2.1 Chemical/Common Name:

The systematic name of the principle enzyme activity is aspartic protease. The other names used are chymosin or rennin (but this should be avoided since it leads to confusion with renin). Chymosin is an enzyme with broad specificity similar to that of pepsin A. It clots milk by cleavage of a single bond in κ-chain of casein.

- EC number: 3.4.23.4 (Appendix A1)
- CAS number: 9001-98-3 (Appendix A2)

Biological source: The Chymosin enzyme is an enzyme produced by submerged fermentation of *T. reesei*, carrying the Prochymosin gene from *B. taurus*.

2.2.2 Marketing Name of the Processing Aid:

The marketing name of this enzyme preparation will depend on the application. An example marketing name of Chymosin is Chymostar™.

2.2.3 Molecular and Structural Formula:

Chymosin is a protein. The amino acid sequence is known. Please refer to Appendix E.

2.3. Chemical and physical properties

The function of Chymosin is to catalyse the reaction of hydrolytic cleavage of 104-Ser-Phe-|-Met-Ala-107 in kappa-casein.

In principle, the enzymatic conversion of casein with the help of chymosin may be of benefit in the processing of all foods and food ingredients which naturally contain the substrate casein. The clearest function of chymosin is to clot milk by highly specific cleavage activity of a single bond in κ-chain of casein.

The beneficial effects mentioned in the food applications below are of value to the food chain because they lead to better processability and/or more consistent product quality. Moreover, the applications lead to more effective production processes, resulting in better production economy and environmental benefits such as the use of less raw materials and the production of less waste.

Therefore, the benefits of the conversion of casein with the help of chymosin in Dairy processing (cheese production) are:

- Coagulation of milk prior to syneresis (& curd/whey separation) resulting in cheese
- Cheese production for (ovo-)lacto-vegetarians by using fermentation-produced chymosin (FPC) instead of animal rennet
- Improved processes - better, quicker and more stable processes
- Improved yield

Activity:

Chymosin preparations' enzyme activity will depend on the final product. An example product has min 700 IMCU/ml. A detailed assay method is present in Appendix A2.

Information on the activity of the food enzyme under various reaction conditions:

The pH and temperature characteristics of the food enzyme chymosin from *T. reesei* were measured. In addition, the characteristics are also well known from animal rennet. (Note that the expressed gene originates from *B. taurus* (cow), and thus the enzyme protein is the same as present in animal rennet, which has been used since antiquity for cheese production.)

Further details on the characteristics of the enzyme under various conditions are detailed in Appendix A.

Interaction of the enzyme with different foods:

The Chymosin enzyme preparation will be used as a processing aid where the enzyme is not present or active in the final food or present in negligible amounts with no technical function in the final food.

Nutritional implication:

Chymosin is a protein and any residual amounts remaining in food consumed would accordingly have the same nutritional value. 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.

2.4. Manufacturing process

The enzyme is produced by a submerged fermentation process using appropriate substrate and nutrients. When fermentation is complete, the biomass is removed by centrifugation/filtration. The remaining fermentation broth containing the enzyme is filtered and concentrated. The concentrated enzyme solution is then standardised and stabilised with diluents. Finally, a polish filtration is applied.

Full details on the raw materials used for the production are provided in Appendix E. Note that this information is proprietary and "**Confidential Commercial Information**" status is requested.

The production of Chymosin is monitored and controlled by analytical and quality assurance procedures that ensure that the finished preparation complies with the specifications and is of the appropriate quality for use as a processing aid in food processing applications.

2.5. Specification for identity and purity

Impurity profile:

Appropriate GMP controls and processes are used in the manufacture of Chymosin to ensure that the finished preparation does not contain any impurities of a hazardous or toxic nature. The specification for impurities and microbial limits are as follows:

Metals:	
Lead	less than 5 mg/kg
Microbiological:	
Total viable count	less than 10,000 CFU/ml
Total coliforms	less than 10 CFU/ml
E. coli	absent in 25 ml
Salmonella	absent in 25 ml
Listeria monocytogenes	absent in 25 ml
Antibiotic activity	Negative by test
Production strain	Negative by test
Physical properties:	
Appearance	clear amber liquid

Standard for identity:

Chymosin meets the specifications laid down by the Joint FAO/WHO Expert Committee on Food Additives and the Food Chemicals Codex.

3. Safety

Refer to Appendix B for further details

3.1. Use of the enzyme as a food processing aid in other countries

Enzyme products are developed for a specific function, i.e. to catalyse a specific chemical reaction. That reaction determines the IUBMB classification[1]. Enzyme variants may be selected to have a better performance of that function under the specific conditions of the application (e.g. temperature or pH). Enzymes of a certain IUBMB classification share conserved structural elements, called domains, which are needed for their specific function. As such the enzymes of our approval procedures do resemble those already permitted by FSANZ both in function and in structure.

Figure 1 below shows an example of natural variation of alpha-amylases. The same holds for any other enzyme type. While significant differences in sequence amongst the various species exist, they all catalyse the same reaction and therefore fit under the same IUBMB entry. There will also be natural variation within one species. All this also applies to the enzymes under the current approval procedures by FSANZ:

% amino acid sequence identity	*B. amyloliquefaciens*	*B. licheniformis*	*G. stearothermophilus*	*A. niger*	*A. oryzae*	*Z. mays*	*O. sativa*	*H. vulgare*	*P. vulgaris*	*H. sapiens*
Bacillus amyloliquefaciens	100									
Bacillus licheniformis	80	100								
Geobacillus stearothermophilus	65	65	100							
Aspergillus niger	21	21	22	100						
Aspergillus oryzae	23	24	24	66	100					
Zea mays (corn)	24	26	25	28	27	100				
Oryza sativa (rice)	25	27	25	27	26	89	100			
Hordeum vulgare (barley)	25	23	24	25	28	70	69	100		
Phaseolus vulgaris (bean)	26	27	25	24	27	67	65	64	100	
Homo sapiens (human)	25	33	29	22	28	23	22	23	24	100

α-amylases in nature have divergent amino acid sequences but have the same catalytic activity and IUBMB number

Figure 1. Variation of enzymes in nature.

The expressed mature enzyme amino acid sequence of Chymostar chymosin shows clear conserved cd05478 (Pepsin A, aspartic protease produced in gastric mucosa of mammals) and pfam00026 (Eukaryotic aspartyl protease; Aspartyl (acid) proteases include pepsins, cathepsins, and rennin domains, characteristic for rennin (chymosins).

Chymostar enzyme, the subject of this dossier, is not one of the three approved chymosin enzymes (from *A. niger*, *E. coli* K-12 strain GE81, *K. lactis*) on Schedule 18 of the ANZ Food Standards Code. In our case the bovine chymosin B enzyme protein is expressed from *Trichoderma reesei*.

[1] The nomenclature of enzymes is primarily based on the recommendations of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB) and it describes each type of characterized enzyme for which an EC (Enzyme Commission) number has been provided (Citation Swissprot Expase Web Site at https://enzyme.expasy.org/, cited 10 September, 2021).

The US FDA affirmed that chymosin was "generally recognized as safe" (GRAS), meaning that it is exempt from the premarket approval requirements that apply to new food additives. The source of that enzyme was *E. coli* K-12 (FDA, 1992). Subsequently, chymosin preparations produced from *Kluyveromyces marxianus var. lactis* and *Aspergillus niger var. awamori* were also affirmed as GRAS(FDA, 1993). JECFA also published monographs on chymosine preparations from the three sources mentioned above, and the Committee established an ADI "not specified" for the recombinant chymosin B preparations (JECFA). In 2008 the US FDA issued a 'no questions' letter to a GRAS Notice submitted on chymosin enzyme preparation from *Trichoderma reesei* expressing the bovine prochymosin B gene[2] (Appendix D).

There have not been any adverse events reported since microbially produced chymosin has been in commercial use.

Please refer to section 1.8 and Appendix D for details on the different approval procedures in the countries listed above.

3.5. Toxicity of the enzyme

Toxin homology study

A BLAST search for homology of the chymosin sequence against the complete Uniprot database was performed, with a threshold E-value of 0.1. The majority of matches were chymosins and pepsins, with none of the top 1000 database matches being annotated as either toxin or venom.

In addition, a specific BLAST search for homology of the mature chymosin sequence was performed against the Uniprot animal toxin database. This yielded no matches.

Therefore, the chymosin sequence does not share homology with a known toxin or venom sequence.

Safe Strain Lineage concept

The Safe Strain Lineage concept has been discussed by Pariza and Johnson (2001) in their publication on the safety of food enzymes and is commonly utilised by enzyme companies in the determination of the safety of their products for specific uses, as appropriate.

The primary issue in evaluating the safety of a production strain is its toxigenic potential, specifically the possible synthesis by the production strain of toxins that are active via the oral route. The toxigenic potential of the production organism is confined to the Total Organic Solid (TOS) originating from the fermentation.

As the toxicological evaluation is based on the TOS originating from fermentation of the production organism, studies conducted on strains from the Safe Strain Lineage can support other production strains pertaining to this same Safe Strain Lineage.

Toxicological testing

[2] GRN No. 230 Chymosin enzyme preparation from *Trichoderma reesei* expressing the bovine prochymosin B gene, The applicant for GRN No. 230 affirmation is Genencor International Inc., that is now merged by IFF which is this applicant (http://wayback.archive-it.org/7993/20171031055428/https://www.fda.gov/downloads/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/UCM264067.pdf, cited on 10 September, 2021)

Toxicology studies with Chymosin produced by *T. reesei* have not been conducted. Instead, the safety of Chymosin from *T. reesei* has been assessed using toxicology studies conducted on earlier strains of the IFF *T. reesei* Safe Strain Lineage. A review of toxicology studies conducted with enzyme preparations produced by *T. reesei* strains indicates that, regardless of the *T. reesei* production strain, all enzyme preparations are not mutagenic, clastogenic or aneugenic in genotoxicity assays and do not adversely affect any specific target organ (Appendix B2 and Appendix B3). Due to the consistency of the findings from enzyme preparations derived from different *T. reesei* strains, it is expected that any new enzyme preparation produced from *T. reesei* strains would have a similar toxicological profile.

For the determination of the safety of Chymosin from *T. reesei*, we use the results of toxicology studies conducted on the production strain most closely related to the chymosin production strain. It is a *T. reesei* strain producing Catalase, an enzyme produced by a strain with the same low-viscosity mutations as the Chymosin producing strain. (Appendix B2 and Appendix B3).

A summary of the results of the studies can be found in Appendix B.

In addition, safety was further assessed according to the decision tree in the Pariza-Johnson guidelines (2001) for assuring the safety of a new enzyme preparation.

3.3 Allergenicity of the enzyme

Bioinformatic analyses based on sequence homology determined that the *B. taurus* Chymosin is unlikely to pose a risk of food allergenicity. Refer to Appendix B for additional information on the safety of the enzyme as to its allergenicity potential.

An allergen statement is given in Appendix A9.

3.4 Safety assessment reports prepared by international agencies or other national government agencies, if avaiable

As discussed in section 1.8 Chymosin from *B. taurus* expressed in *T. reesei* has not been reviewed by JECFA; there is no specific Codex Standard relevant to this application. It has, however, been approved in Denmark, France and Mexico for various purposes. Refer Appendix D for safety reports/approval letters.

3.5 Information on the source micro-organism

The production organism strain *Trichoderma reesei* t-AWL31 (also referred to as DP-Nyj88 in the Danish approval) is a strain of *T. reesei* which has been genetically modified by IFF H&B to over-express a Chymosin gene from *B. taurus.*

T. reesei has a long history of safe use in industrial scale enzyme production. The safety of this species as an industrial enzyme producer has been reviewed by Nevalainen *et al*. (1994), Blumenthal (2004) and Olempska-Beer et al. (2006). The organism is considered non-pathogenic for humans and does not produce fungal toxins or antibiotics under conditions used for enzyme production. It is generally recognised as a safe production organism and is the source organism of a range of enzyme preparations that are used as processing aids in the international food and feed industries. It is also considered as suitable for Good Industrial Large-Scale Practice (GILSP) worldwide and meets the criteria for a safe production microorganism as described by Pariza and

Johnson (2001). The production organism is a strain of *T. reesei* t-AWL31, which has been genetically engineered to express the *B. taurus* prochymosin gene, into the chromosome of the *T. reesei* host. The prochymosin gene was placed under the control of the native *T. reesei cbh1* gene promoter and terminator, using the *T. reesei pyr2* gene (orotate phosphoribosyl transferase) as a selectable marker. This yielded the final production strain *T. reesei* t-AWL31.

Full details of the gene and recombinant microorganism are provided in Appendix E1-E2. Note that this information is proprietary and "**Confidential Commercial Information**" status is requested.

3.6 Pathogenicity and toxicity of the source micro-organism

Trichoderma reesei was first isolated from nature in 1944. The original isolate, QM6a (Mandels and Reese, 1957), and its subsequent derivatives have been the subject of intense research due to their usefulness in the production of cellulases.

A literature search was conducted on August 28, 2017 using the searching term "*Trichoderma reesei*" and "food safety OR toxin OR toxicology OR pathogen" on PubMED resulting in 43 records. A review of the literature search uncovered no reports that implicate *Trichoderma reesei* in any way with a disease situation, intoxication, or allergenicity among healthy adult human and animals.

Strain QM6a and its derivatives have been safe producers of commercial cellulase enzyme preparations for food applications. The industrial enzyme preparations are still confirmed by the enzyme manufacturers not to have antibiotic activity according to the specifications recommended by JECFA (2006).

T. reesei has a long history of safe use in industrial scale enzyme production. The safety of this species as an industrial enzyme producer has been reviewed by Nevalainen et al. (1994) and Blumenthal (2004). The organism is considered non-pathogenic for humans and does not produce fungal toxins or antibiotics under conditions used for enzyme production. It is generally considered a safe production organism and is the source organism of a range of enzyme preparations that are used as processing aids in the international food and feed industries. It is listed as a safe production organism for cellulases by Pariza and Johnson (2001) and Olempska-Beer et al. (2006), and various strains have been approved for the manufacture of commercial enzyme preparations by Food Standards Australia New Zealand, and internationally, for example, in Canada (Food and Drugs Act Division 16, Table V), the United States (21CFR § 184.1250), Mexico, Brazil, France, Denmark, China, and Japan. Further details are discussed in Appendix B.

More specifically, enzymes produced by IFF's *T. reesei* strains (formerly DuPont) have been approved and included in the FSANZ Food Standards Code under Applications A1159, A1169, A1174 A1194, and A1195.

3.7 Genetic Stability of the source organsim

The parental strain of the production strain *Trichoderma reesei* QM6a and its derivatives have been used for industry scale enzyme manufacturing for decades by IFF H&B and its parental companies and has demonstrated stable enzyme expression even at large scale fermentation. Please also refer to Appendix B2 for list of example enzyme preparations produced using QM6a

and its derivatives. Furthermore, the production strain has demonstrated to be 100% stable as confirmed by genome sequencing. Refer also section 3.6.

3.8 Method used in the genetic modification of the source organism

Method used in the genetic modification of the source organism

The modification employed a method by which a synthetic gene encoding a prochymosin wild type enzyme was inserted into the genomic DNA. This prochymosin gene was placed under the control of the native *T. reesei cbh1* gene promoter and terminator, using the *T. reesei pyr2* gene (orotate phosphoribosyl transferase) as a selectable marker for uridine prototrophy. This yielded the final production strain *T. reesei* t-AWL31.

The prochymosin B protein sequence was from *B. taurus*. The DNA encoding prochymosin B was obtained by *in vitro* synthesis according to the published amino acid sequence.

Full details of the genetic modifications are provided in Appendix E2 (Confidential Commercial Information).

The genetic stability of the inserted gene has been demonstrated by genome sequencing. Broth samples were taken prior and after prolonged fermentation mimicking commercial fermentation conditions. Results indicated that *T. reesei* t-AWL31 maintains the inserted DNA stably during fermentations that last for the intended period of commercial-scale production.

Full details of the genetic modifications and stability of the inserted genes are provided in Appendix E1-E3. Note that this information is proprietary and "**Confidential Commercial Information**" status is requested.

4. Dietary exposure

Refer to Appendix C for further details

4.1. List of food or food groups likely to contain the enzyme or its metabolites

According to the food group classification system used in Standard 1.3.1-Food Additives Schedule 15 (15-5), Chymosin will be used in:

- 1.2 Fermented and rennetted milk products
- 1.6 Cheese and cheese products

4.2. Levels of residues in food

The proposed application rate of Chymosin in its intended application is listed below.

Application	Raw material (RM)	Recommended use levels (mg TOS/kg RM)	Maximal recommended use levels (mg TOS/kg food)
Dairy Processing	Milk or milk derivatives	<0.1-0.4	0.4

IFF H&B expects the Chymosin to be inactivated or removed during the subsequent production and refining processes for all applications.

Chymosin performs its technological function usually after pasteurisation of casein containing milk products. Chymosin performs its technological function during food processing and does not perform any technological function in the final food. The reasons why the enzyme does not exert any (unintentional) enzymatic activity in the final food can be due to a combination of various factors, depending on the application and the process conditions used by the individual food producer. These factors include depletion of the substrate, denaturation of the enzyme during processing, lack of water activity, wrong pH, etc. When making cheese, all the casein is hydrolysed, i.e. there is no substrate left.

The most appropriate way to estimate the human consumption in the case of food enzymes is using the Budget Method (Hansen, 1966; Douglass *et al.*, 1997). This method enables one 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.

Based on the raw materials used in the various food processes, the recommended use levels of the enzyme Chymosin, for the calculation of the TMDI, the maximum use levels are chosen. The TMDI is calculated on basis of the maximal values found in food and beverages multiplied by the average consumption of food and beverages per kg body weight/day. Consequently, the TMDI will be: 0.075 mg TOS/kg body weight/day. The NOAEL has been determined for Chymosin to be at 700 mg TOS/kg bw/day). Based on a worst-case scenario of daily food consumption, the NOAEL would offer a 9333-fold margin of safety. It should be stressed that this Total TMDI is based on conservative assumptions and represents a highly exaggerated value. Please refer to Appendix C for details.

4.3. Likely level of consumption of foods or food groups not currently listed in the most recent Australian or New Zealand National Nutrition Surveys (NNSs)

Not applicable. Chymosin is not expected to be used in production of any foods or food groups that are currently not listed in NNSs. If such usage arises, an application would be made to inform FSANZ.

4.4. 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

The enzyme would be used as a processing aid in about:

- 25% of the tonnage of Fermented and rennetted milk products, or cheese and cheese products sold in Australia and New Zealand

4.5. Levels of residues in food in other countries

Applications and levels of use of the Chymosin preparation in other countries is the same as presented in section 4.2.

4.6. Likely current food consumption for foods where consumption has changed in recent years

Not applicable. Consumption of foods (fermented dairy and cheese products) produced with Chymosin is not expected to have a significant change.

5. References

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