An Aminopeptidase Enzyme
from a recombinant strain of *Trichoderma reesei*

PROCESSING AID APPLICATION

Food Standards Australia
New Zealand

Applicant: IFF AUSTRALIA PTY LTD (Trading as Danisco Australia Pty Ltd)

24th February 2025

CONTENTS

General information

1.1 Applicant details

a) Applicant:
This application is made by Danisco Australia (IFF)

b) Company:
Danisco Australia Pty Ltd

c) Address:
IFF Australia Ltd
Ground Floor, 97 Waterloo Rd
Macquarie Park NSW 2113
Australia

d) Contact Details:
[redacted]

e) Email address:
See above

f) Nature of Applicants Business:
Danisco Australia Pty Ltd – A subsidiary of International Flavors and Fragrances Inc (IFF), manufacturer/marketer of specialty food ingredients, food additives and food processing aids. Danisco Australia is also an affliate of Genencor International Ltd, the manufacturer of the prodcut and another subsidiary of International Flavors and Fragrances Inc (IFF).

g) Details of Other Individuals:
No other individuals, companies or organisations are associated with this application.

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 protein and yeast processing, and flavour production.

This application is made solely on behalf of IFF Health & Biosciences (IFF), 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.

Aminopeptidase, subject of this application, is intended for use in protein processing in a variety of foods to facilitate protein hydrolysis.

Currently no Aminopeptidase from *Aspergillus clavatus* expressed in *Trichoderma reesei* is permitted as a Processing Aid, however other enzymes including α- Amylase, α-Arabinofuranosidase, Aspergillopepsin I, Chymosin, Cellulase, Endo-1,4-beta-xylanase, β-Fructofuranosidase , β-Glucanase, Glucoamylase, Hemicellulase multicomponent enzyme, Polygalacturonase or Pectinase multicomponent enzyme, from *Trichoderma reesei* are listed in Schedule 18 section S18-4(5) as permitted enzymes. Approval of this application would provide food processors with a new peptidase enzyme preparation offering the benefits and advantages 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

Aminopeptidase is an enzyme produced by submerged fermentation of *Trichoderma reesei* carrying the gene encoding the aminopeptidase gene from *Aspergillus clavatus.* The enzyme is characterised as a lysyl aminopeptidase (EC 3.2.1.8). 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 intended to be used in protein processing to produce protein hydrolysates of animal plant origin which can be used to provide liquid or powdered ingredients for use in a wide range of food products.

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

Enzyme preparations, including peptides are widely used as processing aids in the manufacture of food products. Currently no Aminopeptidase from *Aspergillus clavatus* expressed in *Trichoderma 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 discussed previously.

B. Impact on international trade

The inclusion Aminopeptidase from *Aspergillus clavatus* expressed in *Trichoderma reesei* in the Australia New Zealand Food Standards Code as a processing aid may promote international trade on products produced with this enzyme product and reduce technical barriers to trade.

1.4. Support for the application

No public health and safety issues related to the proposed change are foreseen. As outlined in Sections 2 through 4 of this submission contain detailed data to support the quality, efficacy

and safety of the Aminopeptidase is produced by submerged fermentation of a genetically modified *Trichoderma reesei* strain. The data pertaining to the Aminopeptidase derived from *Trichoderma reesei* presented in this application is representative of the commercial product for which approval is being sought.

The safety of the production organism and the enzyme product has been thoroughly assessed:

- The production organism has a long history of safe use as production strain for food-grade enzyme preparations and is known not to produce any toxic metabolites.
- The genetic modifications in the production organism are well-characterised and safe and the recombinant DNA is stably integrated into the production organism and unlikely to pose a safety concern.
- The enzyme preparation complies with international specifications ensuring absence of contamination by toxic substances or noxious microorganisms
- Sequence homology assessment to known allergens and toxins shows that oral intake of the Aminopeptidase does not pose food allergenic or toxic concern.
- Mutagenicity studies in vitro showed no evidence of genotoxic potential of the enzyme preparation.
- An oral gavage administration study in rats for 13-weeks showed that all dose levels were generally well tolerated and no evidence of toxicity.

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 issued 1st July 2024, IFF considers General Procedure Level 1 (up to 240 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 A3, A10, Appendices B1, B3, B7-B8, B10, Appendix D1-D4, and Appendices E1-E4 labelled with '**Confidential Commercial Information**', is regarded by the applicant as Confidential Commercial Information 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.

Certain redactions throughout the dossier have also been made to avoid identification or disclosure of proprietary strain, product or other details considered commercially sensitive. Both redacted and non-redacted versions of all documentation have been supplied to FSANZ. The applicant requests that only redacted versions are provided for public consultation purposes.

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

Aminopeptidase from *Aspergillus clavatus* expressed in *Trichoderma reesei* has not been reviewed by JECFA; there is no specific Codex Standard relevant to this application.

1.8.2 International Legislation

Aminopeptidase from *Aspergillus clavatus* expressed in *Trichoderma reesei* has been determined to be Generally Recognized as Safe (GRAS) in the United States as a food processing aid in protein processing, yeast processing and flavour production by a panel of scientific experts in the USA. [redacted]. Other international approvals are discussed in Appendix D.

1.9. Statutory declaration

I, [redacted]

of [redacted] Regulatory Affairs Manager/Director

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

the information provided in this application fully sets out the matters required; and
the information is true to the best of my knowledge and belief; and
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 [redacted]

Declared at [redacted] on 25th day of February 2025

Before me, [redacted] Solicitor of High Court of New Zealand

[redacted]

Signature

[redacted]
Director
Kemps Weir Lawyers Limited
Auckland

1.10. Checklist

	Mandatory Requirements	Check	Page Number	Remarks
General requirements for applications	A. Form of the application	✓	N.A.	English as specified
	Table of contents	✓	1	
	Executive summary	✓	N/A	Supplied separately
	B. Applicant details	✓	2	Section 1.1
	C. Purpose of application	✓	3	Section 1.2
	D. Justification for the application	✓	3	Section 1.3
	D.1 Regulatory impact information	✓	3	Section 1.3.1
	D.1.1 Costs and benefits of the application	✓	3	Section 1.3.1
	D.1.2 Impact on international trade	✓	3	Section 1.3.1
	E Information to support the application	✓	3	Section 1.4
	E.1 Data requirements		3	
	F. Assessment procedure	✓	4	Section 1.5
	G. Confidential commercial information (CCI)	✓	4	Section 1.6
	H. Other confidential information	✓	4	
	I. Exclusive capturable commercial benefit (ECCB)	✓	4	Section 1.7
	J. International and other national standards	✓	4	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	✓	10	Section 2.5
	A.6 Analytical method for detection	×		Not applicable for enzymes used as processing aids
	B. Information related to the safety of a chemical processing aid		N.A.	Processing aid is an enzyme, Section C is therefore relevant
	C. Information related to the safety of an enzyme processing aid	✓	11	Section 3
	C.1 General information on the use of the enzyme as a food processing aid in other countries	✓	11	Section 3.1
	C.2 Information on the potential toxicity of the enzyme processing aid	✓	12	Section 3.2
	C.3 Information on the potential allergenicity of the enzyme processing aid	✓	13	Section 3.3
	C.4 Safety assessment reports prepared by international agencies or other national government agencies, if available	✓	13	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	✓	13	Section 3.5
	D.2 Information on the pathogenicity and toxicity of the source microorganism	✓	13	Section 3.6
	D.3 Information on the genetic stability of the source organism	✓	14	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	✓	14	Section 3.8
	F Information related to the dietary exposure to the processing aid		15	Section 4
	F.1. A list of foods or food groups likely to contain the processing aid or its metabolites	✓	15	Section 4.1
	F.2 The levels of residues of the processing aid or its metabolites for each food or food group	✓	15	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	✓	16	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	✓	16	Section 4.4
	F.5 Information relating to the levels of residues in foods in other countries	✓	16	Section 4.5
	F.6 For foods where consumption has changed in recent years, information on likely current food consumption	✓	16	Section 4.6

2. Technical information

Please refer to Appendix A for further details

2.1. Type of processing aid

The Aminopeptidase enzyme is an enzyme produced by submerged fermentation of *Trichoderma reesei* , carrying the aminopeptidase gene from *Aspergillus clavatus*.

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 principal enzyme activity is aminopeptidase. Other names used are lysyl aminopeptidase, aminopolypeptidase, and peptidase, amino-.

- EC number: 3.4.11.15
- CAS number: 114796-97-3

Biological source: The Aminopeptidase enzyme is an enzyme produced by submerged fermentation of *Trichoderma reesei*, carrying the aminopeptidase gene from *Aspergillus clavatus*.

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 Aminopeptidase is [redacted].

2.2.3 Molecular and Structural Formula:

Aminopeptidase is a protein. The amino acid sequence is known. Please refer to Appendix E4 '**Confidential Commercial Information**'.

2.3. Chemical and physical properties

The function of Aminopeptidase is to catalyse the cleavage of the N-terminal peptide bond in proteins and peptides with preferential release of N-terminal lysine.

When Aminopeptidase is added to the various food applications for the purpose of protein hydrolysis it will degrade the component proteins of the food into peptides resulting in protein hydrolysates with improved and desirable properties. Aside from providing desirable flavouring attributes, the transformation of the substrate proteins and peptides can facilitate the production of peptides with better functional properties such as solubility (Cheng and Medina, 2014), emulsification, gelling and foaming (Whitehurst and Law, 2010).

Substrate specificity:

The function of Aminopeptidases is to use protein and peptide substrates to catalyse the cleavage of the N-terminal peptide bond in proteins and peptides with preferential release of N-terminal lysine.

Activity:

The activity of the Aminopeptidase is defined in KAPU/g. The assay is colorimetric and monitors the rate of degradation of H-Lys-pNA·2HBr substrate. The release of the substrate's p-nitroanalide(pNA) is measured at 405nm using a Konelab analyser. The linear range of the assay is ~0.2–0.4 Abs/min or ~ 5 – 10 KAPU/mL. Refer to Appendix A for further details.

Interaction of the enzyme with different foods:

The Aminopeptidase 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:
Aminopeptidase is a protein and any residual amounts remaining in food consumed would accordingly have the same nutritional value. However, the use levels of Aminopeptidase 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. Additional detail on the manufacturing process is provided in Appendix A.

Full details on the raw materials used for the production are provided in Appendix E5. Note that this information is proprietary and "**Confidential Commercial Information**" status is requested.

The production of Aminopeptidase is monitored and controlled by analytical and quality assurance procedures that ensure that the finished preparation complies within 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 Aminopeptidase 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 50,000 CFU/g
Total coliforms	less than 30 CFU/g
E. coli	absent in 25g
Salmonella	absent in 25g
Antibiotic activity	Negative by test
Production strain	Negative by test
Physical properties:	
Appearance	Clear, light brown or golden liquid

Standard for identity:
Aminopeptidase 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. 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 the 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. This also applies to the enzymes currently approved 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 Aminopeptidase shows a clear conserved zinc-peptidase-like superfamily domain, which includes aminopeptidases.

Aminopeptidase enzyme, the subject of this dossier, is one of the permitted processing aids on Schedule 18 of the ANZ Food Standards Code, i.e. from *Aspergillus oryzae*. In our case the enzyme protein is expressed from *Trichoderma reesei*. The identity between the FSANZ approved *Aspergillus oryzae* aminopeptidase and the *Aspergillus clavatus* aminopeptidase, subject of this dossier, is approximately 71%. Aminopeptidase sequences within one species can show strain dependent amino acid sequence variability. Also, several microorganism species contain more than one aminopeptidase encoding genes with different sequences.

The aminopeptidase enzyme derived from *Trichoderma reesei* carrying the aminopeptidase gene from *Aspergillus clavatus* [redacted] and has been

approved by several countries such as [redacted]. There have not been any adverse events reported since this aminopeptidase has been in commercial use in these countries.

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

3.2. Toxicity of the enzyme

Toxin homology study

A BLAST search for homology of the Aminopeptidase sequence against the complete Uniprot database (http://www.uniprot.org/), was performed, with a threshold E-value of 0.1. The majority of matches were peptide hydrolases, with none being annotated as either toxin or venom.

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

Although *Trichoderma reesei* is scientifically determined by IFF as a Safe Strain Lineage, the food enzyme object of the current dossier is supported by toxicological studies on the specific food enzyme object of this dossier. The toxicological studies on the Aminopeptidase from production strain [redacted] are thus one of the pillars supporting the IFF *Trichoderma reesei* Safe Strain Lineage. The position of the food enzyme in the IFF *Trichoderma reesei* Safe Strain Lineage is presented in Appendix B2.

Toxicological testing

To assess the safety of Aminopeptidase, different endpoints of toxicity were investigated and are evaluated and assessed in this document:

- Negative as a dermal irritant;
- Negative as an ocular irritant;
- Negative as a mutagen, clastogen, and aneugen in genotoxicity studies; and
- Not observed to adversely affect any specific target organs.

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 *Trichoderma reesei* Aminopetidase 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. Liquid glucose dervied from wheat is used as a fermantion ingredients, however, according to Clause S9-3 to Standard 1.2.3, this substance would be exempt from mandatory allergen declaration.

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

As discussed in section 1.8, Aminopeptidase from *Aspergillus clavatus* expressed in *Trichoderma reesei* has not been reviewed by JECFA; there is no specific Codex Standard relevant to this application. It has, however, been determined to be GRAS by expert opinion in the United States. Refer Appendix D for safety reports/approval letters.

3.5. Information on the production microorganism

The production organism strain [redacted] is a strain of *Trichoderma reesei* which has been genetically modified by IFF to overexpress an aminopeptidase gene from *Aspergillus clavatus*.

Trichoderma 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 strain contains copies of a synthetic [redacted] gene encoding an aminopeptidase placed under the control of the highly efficient promoter and terminator obtained from endogenous *Trichoderma reesei* [redacted] encoding gene. Copies of the expression cassette were integrated into the recipient chromosome by using the native *Trichoderma reesei* [redacted] gene and the *Aspergillus* [redacted] gene as selectable markers.

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

3.6. Pathogenicity and toxicity of the source organism

The safety of the production organism must be the prime consideration in assessing the safety of an enzyme preparation intended for use in food (Pariza and Foster, 1983). If the organism is nontoxigenic and non-pathogenic, then it is assumed that foods or food ingredients produced from the organism, using current Good Manufacturing Practices, are safe to consume (IFBC 1990). Pariza and Foster (1983) define a non-toxigenic organism as "one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure" and a non-pathogenic organism as "one that is very unlikely to produce disease under ordinary circumstances." *Trichoderma reesei* strains used in enzyme manufacture meet these criteria for non-toxigenicity and non-pathogenicity.

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 review of the literature search on the organism uncovered no reports that implicate *Trichoderma reesei* in any way with a disease situation, intoxication, or allergenicity among healthy adult humans and animals. The species is not present on the list of pathogens used by the EU (Directive Council Directive 90/679/EEC, as amended) and major culture collections worldwide. It is classified as a Biosafety Level 1 (BSL1) microorganism by the American Type Culture Collection (ATCC) based on assessment of the potential risk using U.S. Department of Public Health guidelines with assistance provided by ATCC scientific advisory committees. BSL1 microorganisms are not known to cause diseases in healthy adult humans. In the USA, *Trichoderma reesei* is not listed as a Class 2 or higher containment agent under the National Institute of Health (NIH) Guidelines for Recombinant or synthetic nucleic acid molecules (NIH, 2019).

Strain [redacted] 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). Further details are discussed in Appendix B.

3.7. Genetic stability of the source organism

The parental strain of the production strain *Trichoderma reesei* [redacted] and its derivatives have been used for industry scale enzyme manufacturing for decades by IFF and its parental companies, and has demonstrated stable enzyme expression even at large scale fermentation. Please also refer to Appendix B7 for list of example enzyme preparations produced using [redacted] and its derivatives. Furthermore, the production strain has demonstrated to be 100% stable as confirmed by genome sequencing.

3.8. Method used in the genentic modification of the source organism

The production organism of the Aminopeptidase preparation, the subject of this submission, is *Trichoderma reesei* strain [redacted]. It is derived by recombinant DNA methods from strain [redacted]. The purpose of this genetic modification is to enhance aminopeptidase production levels. [redacted], a commercial production strain, is derived, as a result of several classical mutagenesis steps, from the well-known wild-type strain [redacted]. Virtually all strains used all over the world for industrial cellulase production today are derived from [redacted]. There was no actual strain used as donor. The published amino acid sequence of aminopeptidase Y from *Aspergillus clavatus* was used to synthesise a gene [redacted] for expression in *Trichoderma reesei*. 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. Samples were then used for genomic DNA extraction and next generation sequencing. A complex integration site for aminopeptidase expression site was determined, and no change was observed between samples prior and after fermentation. The results demonstrate that the insertion cassette has been stably maintained through generations during the fermentation process.

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.

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), Aminopeptidase will be used in:

- Protein processing
- Yeast processing
- Flavouring production

The food categories Aminopeptidase could be used in include, but are not exclusively, dairy, egg, meat, fish, vegetable protein, gravies and bouillon, and processed yeast

4.2. Levels of residues in food

The proposed application rate of Aminopeptidase 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 RM)
Protein processing	Proteins	140-2,125	2,125
Yeast processing	Yeast culture or yeast extract, cell walls and autolysed yeast	723-4,340	4,340
Flavouring production	Material of vegetable, animal or microbial origin	140-7,042	7,024

IFF expects the Aminopeptidase to be inactivated or removed during the subsequent production and refining processes for all applications.

In all of the proposed applications, Aminopeptidase performs its technological function in the degradation of the substrate proteins present, resulting in protein hydrolysates with improved properties.

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 Aminopeptidase, 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: 2.26 mg TOS/kg body weight/day. The NOAEL has been determined for Aminopeptidase to be at 1000 mg TOS/kg bw/day. Based on a worst-case scenario of daily food consumption, the NOAEL would offer a 442 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. Aminopeptidase 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:

- <5% of the tonnage of yeast and flavouring products manufactured in Australia or New Zealand.
- <10% of processed protein products

4.5. Levels of residues in food in other countries

Applications and levels of use of the Aminopeptidase 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 produced with Aminopeptidase is not expected to have a significant change.

3. References

Blumenthal CZ (2004). Production of toxic metabolites in *Aspergillus niger, Aspergillus oryzae, and Trichoderma reesei*: justification of mycotoxin testing in food grade enzyme preparations derived from the three fungi. Regulatory Toxicology and Pharmacology, 39(2), 214-228

Cheng & Medina, Griffith Laboratories International, Inc. Method for preparing a proteinaceous vegetale flavor enhancer. United States Patent, US 8,828,462 B2, Sept. 9, 2014.

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

Hansen, S.C. (1996). Acceptable daily intake of food additives and ceiling on levels of use. Food Cosmet. Toxicol., 4, 427-432.

JECFA (Joint FAO/WHO Expert Committee on Food Additives) 2006. General Specifications and Considerations for Enzyme Preparations Used in Food Processing.

Mandels M and Reese ET (1957). Induction of cellulase in Trichoderma viride as influenced by carbon sources and metals. J Bacteriol. 73, 269-278

Nevalainen H, Suominen P, Taimisto K (1994). On the safety of *Trichoderma reesei*, J. Biotechnol. 37, 193-200

NIH (2019) Guidelines for research involving recombinant of synthetic nucleic acid molecules, National Institutes of Health

Olempska-Beer ZS, Merker RI, Ditto MD, DiNovi MJ (2006). Food-processing enzymes from recombinant microorganisms—a review. Regul Toxicol Pharmacol 45, 144-158

Pariza,MW, Johnson EA (2001). Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing – Update for a New Century. Regul Toxicol Pharmacol, 33(2), 173-86,

Pariza, M.W. and E.M. Foster. 1983. Determining the Safety of Enzymes Used in Food Processing. J. Food Prot., 46: 453-468.

Whitehurst RJ and Law B (2010). Enzymes in Food Technology, Sheffield academy press, 117-137