An Alpha-Amylase Enzyme
from a recombinant strain of *B. licheniformis*

PROCESSING AID APPLICATION

Food Standards Australia
New Zealand

Applicant: IFF Australia Pty Ltd (Trading as Danisco Australia Ltd)

7 June 2024

CONTENTS

EXECUTIVE SUMMARY:

IFF Health and Biosciences (IFF) is seeking approval for an "Alpha-amylase (EC 3.2.1.1)" enzyme to be used as processing aid in carbohydrate processing. The enzyme is designated as "Alpha-amylase" throughout the dossier.

The enzyme Alpha-amylase is derived from a selected non-pathogenic, non-toxigenic strain of *Bacillus licheniformis* which is genetically modified to express a protein engineered alpha-amylase gene.

The enzyme is intended for use in carbohydrate processing to produce glucose syrups and other starch hydrolysates, and in potable alcohol production. The technological benefits of using Alpha-amylase in the production of glucose syrups and other starch hydrolysates (carbohydrate processing) include more efficient liquefaction, reducing the viscosity of gelatinised starch and increasing in soluble dextrins and oligosaccharides. The technological benefits of using Alpha-amylase to produce distilled alcohol are more efficient liquefaction, energy efficiency, viscosity reduction, and generally improved processing.

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

To assess the safety of the Alpha-amylase for use in these applications, IFF 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 *B. licheniformis* and of other Alpha-amylase enzymes in food, the history of safe use of the *B. licheniformis* 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 *B. licheniformis* has been assessed using toxicology studies conducted on earlier strains of the IFF *B. licheniformis* Safe Strain Lineage. The most suitable standard collection 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:

1. Negative as a mutagen, clastogen, and aneugen in genotoxicity studies; and
2. 90-day oral toxicity on rats: Under the conditions of the study, the no-observed adverse-effect-level (NOAEL) was established at the high dose 500 mg total organic solids (TOS)/kg body weight/day (corresponding to 272 mg TP/kg bw/day).

Based on a worst-case scenario consumption estimates for Alpha-amylase in the designated applications, the calculated Theoretical Maximum Daily Intake (TMDI) will be 1.14 mg TOS/kg body weight/day. This offers a 439-fold margin of safety.

Based on the results of safety studies and other evidence, Alpha-amylase 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 mentioned above.

General information

1.1 Applicant details

(a) Applicant:

This application is made by Danisco Australia Pty Ltd

(b) Company:

Danisco Australia Pty Ltd

(c) Address:

[redacted]

(d) Contact Details:

[redacted]
Regulatory Affairs Manager

[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.

(g) Details of Other Individuals etc.:

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,* Alpha-amlyase.

This application is made solely on behalf of 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.

Alpha-amylase, subject of this application, is intended for use in carbohydrate processing and potable alcohol production.

Currently no protein engineered Alpha-amylase expressed in *Bacillus licheniformis* is permitted as a processing aid, however, other enzymes including chymotrypsin, endo-1,4-beta-xylanase, beta-galactosidase, glycerophospholipid cholesterol acyltransferase, maltotetraohydrolase, pullulanase, and protease from *Bacillus licheniformis* are permitted and 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

Alpha-amylase is an enzyme produced by submerged fermentation of *B. licheniformis* carrying the gene encoding a protein engineered alpha-amylase. The enzyme is characterised as an Alpha-amylase (EC 3.2.1.1). 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 is intended for use in to be used as a processing aid in production of distilled alcohol and for the production of glucose syrups and other starch hydrolysates. 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 protein engineered Alpha-amylase expressed in *B. licheniformis* 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 and throughout the dossier.

B. Impact on international trade

The inclusion of protein engineered Alpha-amylase expressed in *B. licheniformis* 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 marketing or promotional activities have been undertaken for the protein engineered Alpha-amylase derived from *B. licheniformis* 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 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 A3, A11, Appendices B1, B3-B5, Appendix D1, and Appendices E1-E7 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 in order to avoid identification or disclosure of proprietary strain, product or other details considered commercially sensitive. Both redacted and non-redacted version of all documentation has 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

The protein engineered Alpha-amylase produced by *B. licheniformis* has not been reviewed by JECFA; there is no specific Codex Standard relevant to this application.

1.8.2 International Legislation

The protein engineered Alpha-amylase produced by *B. licheniformis* has been determined to be Generally Recognized as Safe (GRAS) in the United States as a food processing aid in carbohydrate processing and the manufacture of potable alcohol by a panel of scientific experts in the USA.

1.9. Statutory declaration

I, [redacted],

of [redacted] 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 [redacted]

Declared at Auckland on 10th of June 2024

A.J

Before me, [redacted]

[redacted]

Signature [redacted]

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	✓	5	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	✓	10	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	✓	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	✓	14	Section 3.4
	D. Additional information related to the safety of an enzyme processing aid derived from a microorganism		16	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	✓	14	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		16	Section 4
	F.1. A list of foods or food groups likely to contain the processing aid or its metabolites	✓	16	Section 4.1
	F.2 The levels of residues of the processing aid or its metabolites for each food or food group	✓	16	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	✓	17	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	✓	17	Section 4.4
	F.5 Information relating to the levels of residues in foods in other countries	✓	17	Section 4.5
	F.6 For foods where consumption has changed in recent years, information on likely current food consumption	✓	17	Section 4.6

Technical information

Please refer to Appendix A for further details

2.1. Type of processing aid

The Alpha-amylase enzyme is an enzyme produced by submerged fermentation of *B. licheniformis* carrying a protein engineered alpha-amylase gene.

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 4- α -D-glucan glucanohydrolase. Other names used are glycogenase; α amylase, α-amylase; endoamylase; Taka-amylase A; 1,4- α -D-glucan glucanohydrolase.

- EC number: 3.2.1.1 (Appendix A1)
- CAS number: 9000-90-2 (Appendix A2)

Biological source: The Alpha-amylase enzyme is an enzyme produced by submerged fermentation of *B. licheniformis*, carrying an alpha-amylase gene developed using protein engineering.

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 Alpha-amylase is [redacted].

2.2.3 Molecular and Structural Formula:

Alpha-amylase is a protein. The amino acid sequence is known. Please refer to Appendix E.

2.3. Chemical and physical properties

The alpha-amylase food enzyme is intended to be used as a processing aid in production of distilled alcohol and in production of glucose syrups and other starch hydrolysates. Alpha-amylase catalyses endohydrolysis of (1→4)-alpha-D-glucosidic linkages in polysaccharides containing three or more (1→4)-alpha-linked D-glucose units. The substrates for alpha-amylase are starch, glycogen and related polysaccharides and oligosaccharides. The reaction products of the hydrolysis of starch, glycogen and related polysaccharides and oligosaccharides with the help of alpha-amylase are maltodextrins, maltooligosacharides and glucose. Like the substrates and the enzyme, the reaction product(s) also naturally occur(s) in various organisms, including germinating cereal grains.

When included in starch processing or potable alcohol production, Alpha-amylase can increase liquefaction efficiency and beneficially impact viscosity reduction. Additionally, in potable alcohol production, addition of Alpha-amylase can impact energy efficiency, process flexibility (pH and temperature) and excessive raw material consumption.

Substrate specificity:

The function of Alpha-amylase is to catalyse the hydrolysis of the (1→4)- α -D-glycosidic linkages in polysaccharides containing three or more (1→4)- α -D-glycosidic units. The substrates for Alpha-amylase are starch, glycogen and related polysaccharides and oligosaccharides.

Activity:

The activity of the Alpha-amylase is defined in LPAU/g. The substrate employed in the assay is pnitrophenyl maltoheptoside substrate with the non-reducing terminal sugar chemically blocked. The rate of p-nitrophenyl release is proportional to amylase activity and is monitored at 405nm.

Activity of enzyme under various temperatures and pH

Alpha-amylase exhibits activity from pH 3.5 to pH 9.2, and from 30 to 100°C. The optimum pH is pH 4-7.5, whereas the optimum temperature range lies between 60-90˚C. The data show that the alpha-amylase is inactivated at temperatures above 83˚C when incubated for 30 minutes. See Appendix A for further information.

Alpha-amylase preparations' enzyme activity will depend on the final product. An example product has the Alpha-amylase activity range of 52515-64185 LPAU/g.

Interaction of the enzyme with different foods:

The Alpha-amylase 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:

Alpha-amylase is a protein and any residual amounts remaining in food consumed would accordingly have the same nutritional value. However, the use levels of Alpha-amylase 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 Alpha-amylase 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 Alpha-amylase to ensure that the finished preparation does not contain any impurities of a hazardous or toxic nature. The specification for impurities and microbial limits is as follows:

Metals:	
Lead	less than 5 mg/kg
Microbiological:	
Total viable count	less than 10,000 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	Brown liquid

Standard for identity:

Alpha-amylase 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 natural variation of alpha-amylases. The same holds for any other enzyme types. 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.

Our Alpha-amylase enzyme derived from *B. licheniformis*, carrying the protein engineered alpha-amylase gene has been determined to be GRAS in the United States. There have not been any adverse events reported since related to the use of this Alpha-amylase.

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 Alpha-amylase 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 alpha amylases, 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 Alpha-amylase sequence was performed against the Uniprot animal toxin database. This yielded no matches.

Therefore, the Alpha-amylase 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.

The position of the food enzyme in the IFF *B. licheniformis* Safe Strain Lineage is presented in Appendix B3.

Toxicological testing
Toxicology studies with Alpha-amylase produced by *B. licheniformis* have not been conducted. Instead, the safety of Alpha-amylase from *B. licheniformis* has been assessed using toxicology studies conducted on earlier strains of the IFF *B. licheniformis* Safe Strain Lineage. A review of toxicology studies conducted with enzyme preparations produced by *B. licheniformis* strains indicates that, regardless of the *B. licheniformis* production strain, all enzyme preparations are not mutagenic, clastogenic or aneugenic in genotoxicity assays and do not adversely affect any specific target organ (Appendix B3 and Appendix B4). Due to the consistency of the findings from enzyme preparations derived from different *B. licheniformis* strains, it is expected that any new enzyme preparation produced from *B. licheniformis* strains would have a similar toxicological profile.

For the determination of the safety of Alpha-amylase from *B. licheniformis*, we use the results of toxicology studies conducted on the production strain most closely related to the Alpha-amylase production strain, JML1584. JML1584 is a *B. licheniformis* strain producing alpha-amylase from *Cytophaga sp.* The results of the studies can be found in Appendix B to FSANZ application A1219.

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. licheniformis* Alpha-amylase 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 Safey assessment reports prepared by international agenicies or other national government agencies, if available

As discussed in section 1.8 Alpha-amylase from the protein engineered Alpha-amylase expressed in *B. licheniformis* has not been reviewed by JECFA; there is no specific Codex Standard relevant to this application. It has, however, been determined to be GRAS in the United States by an expert panel. To date, no other approvals have been finalised. Refer Appendix D for further information.

3.5 Information on the production organism

The production organism strain [redacted] is a strain of *B. licheniformis* which has been genetically modified by IFF to express an Alpha-amylase gene designed by protein engineering. It is derived by recombinant DNA methods from strain Bra7 by inactivation of the genes encoding genes of undesirable traits. Bra7 is a classical industrial strain used for α-amylase production by IFF and its parent companies since 1989.

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 host micro-organism

The host/source organism, *B. licheniformis* Bra7 is a classical industrial strain used for Alpha-amylase production by IFF and its parent companies since 1989. The strain was developed from its wild-type parent, by classical strain improvement only, for optimal Alpha-amylase production and lowered protease production. The host strain Bra7 is a stable strain, which can easily be maintained as a homogeneous population under the usual laboratory and production conditions. For an extensive overview of countries that accepted *B. licheniformis* as a safe production organism for a broad range of food enzymes, please refer to Appendix D.

Further information is provided in Appendix B

3.7 Genetic stability of the host and production organism

The parental strain of the production strain *B. licheniformis* Bra7 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 B2 for list of example enzyme preparations produced using Bra7 and its derivatives. Furthermore, the production strain has demonstrated to stable over the course of fermentaion, as confirmed by genome sequencing. Refer also Appendix E3, noting that this information is proprietary and "**Confidential Commercial Information**" status is requested.

3.8 Method used in the genetic modification of the production organism

The production organism of the Alpha-amylase preparation, the subject of this submission, is *Bacillus licheniformis* [redacted]. It is derived by recombinant DNA methods from strain Bra 7. The purpose of this genetic modification is to enhance alpha-amylase production levels. Strain *B. licheniformis* Bra7 was developed from its wild-type parent, by classical strain improvement only, for optimal alpha-amylase production and lowered protease production. The alpha-amylase [redacted] coding gene inserted into the host organism was not isolated from any donor strain, but instead the gene was designed by extensive protein engineering using family shuffling starting from a plurality of bacterial alpha-amylase sequences and encodes an enzyme protein with a sequence most similar to a [redacted] (synonym: [redacted]) alpha-amylase 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. NGS sequencing was used to characterise the production strain for the insertion site at generation 0 and compared to the end of fermentation. Any DNA rearrangement of the inserted expression cassettes was measured as to change of flanking DNA sequence in the analysis. No change was observed between the genomic DNA samples extracted from generation 0 to those extracted at the end of fermentation. This indicates there had been no insertions of the expression cassette at new sites in the B. licheniformis, indicating stability of the strain over the course of fermentation.

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), Alpha-amylase will be used in:

- 6.2 Flours, meals and starches
- 11.2 Sugars and sugar syrups
- 14.2 Alcoholic beverages (including alcoholic beverages that have had the alcohol reduced or removed)

4.2. Levels of residues in food

The proposed application rate of Alpha-amylase in its intended application is listed below.

	Application	Raw Material (RM)	Maximal recommended use level (mg TOS/kg RM)	Example Final food (FF)	Rate RM/FF	Maximal recommended use level in FF (mg TOS/kg food)
Liquid Food	Carbohydrate processing	Starch	15.84	Sugar syrups in Soft Drinks	0.12	1.90
	Potable Alcohol	Cereal	15.84	Potable alcohol	1/0.35 =2.86	45.30
Solid Food	Carbohydrate processing	Starch	15.84	Modified starch in Bakery, Dairy	0.05	0.79

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

In Alpha-amylase performs its technological function at various points in during starch processing or potable alcohol production. The Alpha-amylase is denatured by heat during the process after its technological function has been carried out. Please refer Appendix A for process details.

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 Alpha-amylase, 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: 1.14 mg TOS/kg body weight/day. The NOAEL has been determined for alpha-amylase to be at 500 mg total protein/kg bw/day (equivalent to 272 mg TOS/kg bw/day). Based on a worst-case scenario of daily food consumption, the NOAEL would offer a 439-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. Alpha-amylase 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:

- 20% of the tonnage of potable alcohol products sold in Australia and New Zealand
- 15 % of the tonnage of Flours, meals and starch product sold in Australia and New Zealand
- 20 % of the tonnage of Sugars and sugar syrups sold in Australia and New Zealand

4.5. Levels of residues in food in other countries

Applications and levels of use of the Alpha-amylase 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 (alcoholic drinks) produced with Alpha-amylase is not expected to have a significant change.

5. References

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