An Alpha-Amylase Enzyme
from a recombinant strain of Bacillus licheniformis

PROCESSING AID APPLICATION

Food Standards Australia
New Zealand

Applicant: DUPONT NEW ZEALAND LTD

2 November 2020

CONTENTS

General information

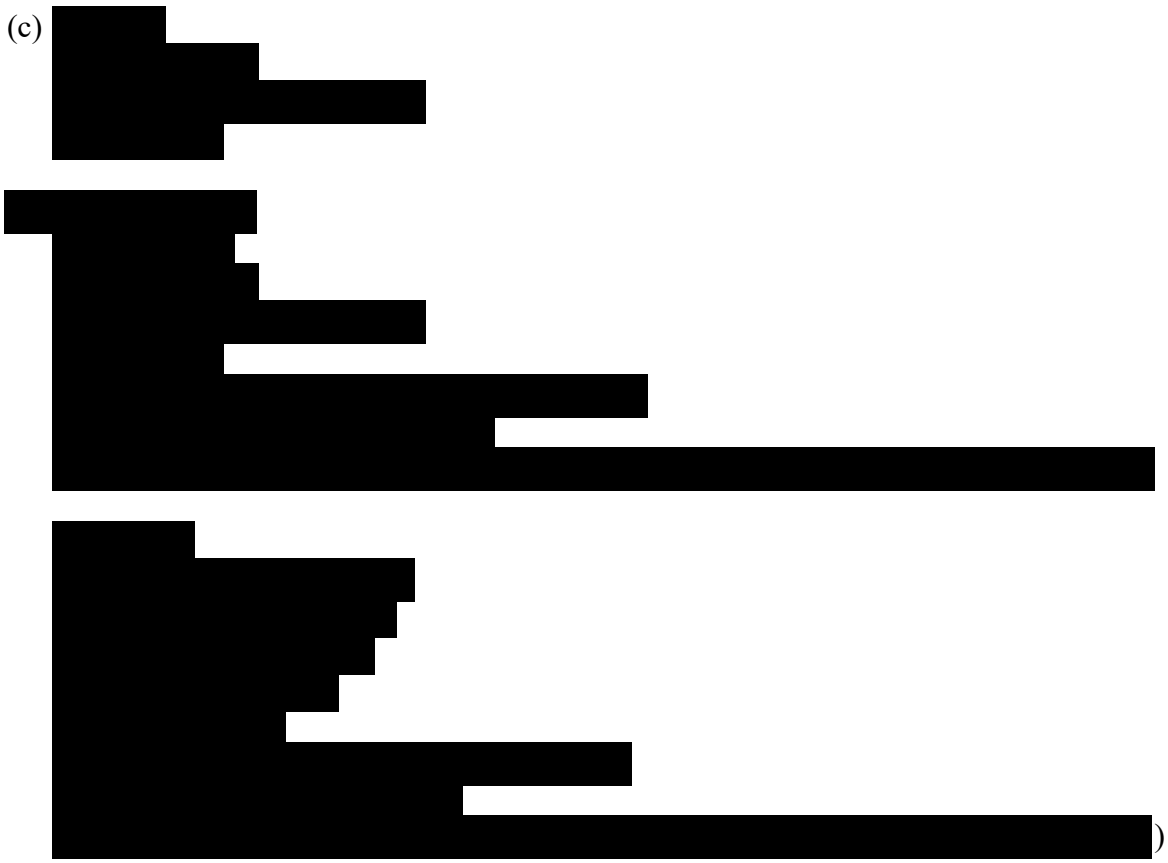
1.1 Applicant details

(a) Applicant:

This application is made by Dansico New Zealand Ltd

(b) Company:

Dansico New Zealand Ltd

(c) 
)

(e) Email Address :

See above

(f) Nature of Applicants Business:
Danisco New Zealand Ltd – A subsidiary of E. I. du Pont de Nemours and Company (DuPont), 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*, subject of this application. The intended use of the processing aid is starch processing, brewing and potable alcohol production.

This application is made solely on behalf of DuPont Nutrition and Biosciences (N&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.

Alpha-amylase, the subject of this application, is intended for the production of sugar syrups, brewing to produce brewed beverages, and in the production of potable alcohol.

Currently no Alpha-amylase from *Cytophaga sp.* expressed in *B. licheniformis* is permitted as a Processing Aid, however Alpha-amylase from *B. licheniformis,* containing the gene for α-Amylase isolated from *G. stearothermophilus* and other enzymes including Chymotrypsin, Endo-1,4-beta-xylanase, β-Galactosidase, Glycerophospholipid cholesterol acyltransferase, and Maltotetraohydrolase from *B. licheniformis* 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

This regulatory measure is porposed to expand the options available to industry for technologically advanced means to carryout the necessary biocatalyst reactions required for the ecomomical production of starch derivatives, brewed beverages and potable alcohol. Like any other enzyme, Alpha-amylase acts as a biocatalyst: with the help of the enzyme, a certain substrate is converted into a certain reaction product. It is not the food enzyme itself, but the result of this conversion that determines the effect in the food or food ingredient. After the conversion has taken place, the enzyme no longer performs a technological function. Depending on the specific food product, the same effect might be achieved by other means as well. However, these methods often involve more expensive or less environmental friendly productions processes, the use of chemicals or recipe changes.

DuPont have identified no public health and safety, nor consumer choice issues related to the proposed change. The enzyme itself is present in neglible amounts in the final food product, is inactivated, and comsumption of the aforementioned food products is not expected to change as a result of this approval, this discussed further in 4.6 of the dossier.

A. Costs and Benefits of the application

Alpha-amylase is an enzyme produced by submerged fermentation of *B. licheniformis* carrying the gene encoding the Alpha-amylase gene from *Cytophaga sp.* 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 the production of sugar syrups, brewing to produce brewed beverages, and in the production of potable alcohol. In brewing, Alpha-amylase performs its technological function in the cooking and mashing phase. When producing potable alcohol, the enzyme is used during slurry mixing, liquefaction and pre-saccharifaction. For starch processing the Alpha-amylase is added pre-liquefaction.

More information on the benefit of this enzyme can be found in Section 2.3 and Appendix A.

Enzyme preparations are widely used as processing aids in the manufacture of food products. Currently no Alpha-amylase from *Cytophaga sp.* 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.

B. Impact on international trade

The inclusion of Alpha-amylase from *Cytophaga sp* 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 Alpha-amylase derived from *Cytophaga sp* expressed in *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.

1.4.1. Data Requirements

No literature search has been conducted in support or constituent of this application. Any data underpinning the application is references as appropriate, identifying any literature referenced in support of this application.

E.1.1 Data related to safety studies
All studies submitted in support of the toxicological assessment for this Alpha-amylase (see toxicological summaries, Appendix B Section 1.3.2) are designed and conducted in accordance with GLP. The study reports are supplied in full as "**Confidential Commercial Information**".

E.1.2 Data related to surveys on chemicals or other substances in food
No data related to surveys on chemical or other substances in food are presented in this application.

E.1.3 Data related to epidemiological /intervention studies in humans
No data epidemiological /intervention studies in humans is are presented in support of this application.

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, DuPont N&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, and toxicological studies are 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.

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

Alpha-amylase from *Cytophaga sp.* expressed in *B. licheniformis* has not been reviewed by JECFA; there is no specific Codex Standard relevant to this application.

1.8.2 International Legislation

Alpha-amylase derived from *B. licheniformis* carrying the gene encoding the Alpha-amylase gene from *Cytophaga sp.* has been determined to be Generally Recognised as Safe (GRAS) in the United States as a food processing aid in production of bakery products by a panel of scientific experts in the USA. The reference for GRAS approval is GRN 664.

1.9. Statutory Declaration

I, Caroline Elizabeth Gray,

of 5 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 9th of November 2020

Before me,

_____

Signature L. C. Holley

Laurene Holley
Barrister
PO Box 25939 St Helliers
AKLD 1740 Ph: 0274578468

1.10. Checklist

	Mandatory Requirements	Check	Page Number	Remarks
General requirements for applications	A. Form of the application	✓		
	Table of contents	✓	2	
	Executive summary	✓		Supplied as separate attachment
	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	✓	5	Section 1.3.1
	E Information to support the application	✓	5	Section 1.4
	E.1 Data requirements	✓	5	Section 1.4.1
	F. Assessment procedure	✓	5	Section 1.5
	G. Confidential commercial information (CCI)	✓	6	Section 1.6
	H. Other confidential information	✓	6	Section 1.6
	I. Exclusive capturable commercial benefit (ECCB)	✓	6	Section 1.7
	J. International and other national standards	✓	6	Section 1.8
	J.1 International Standards	✓	6	Section 1.8.1
	J.2 Other national standards or regulations	✓	6	Section 1.8.2
	K. Statutory declaration	✓	7	Section 1.9
	L. Checklist	✓	8	Section 1.10
3.3.2. Processing aids	A. Technical information on the processing aid			Section 2
	A.1 Information on the type of processing aid	✓	10	Section 2.1
	A.2 Information on the identity of the processing aid	✓	10	Section 2.2
	A.3 Information on the chemical and physical properties of the processing aid	✓	10	Section 2.3
	A.4 Manufacturing process	✓	12	Section 2.4
	A.5 Specification for identity and purity	✓	12	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	✓	14	Section 3
	C.1 General information on the use of the enzyme as a food processing aid in other countries	✓	14	Section 3.1

	C.2 Information on the potential toxicity of the enzyme processing aid	✓	15	Section 3.2
	C.3 Information on the potential allergenicity of the enzyme processing aid	✓	16	Section 3.3
	C.4 Safety assessment reports prepared by international agencies or other national government agencies, if available	✓	16	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	✓	16	Section 3.5
	D.2 Information on the pathogenicity and toxicity of the source microorganism	✓	16	Section 3.6
	D.3 Information on the genetic stability of the source organism	✓	17	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	✓	17	Section 3.8
	F Information related to the dietary exposure to the processing aid		18	Section 4
	F.1. A list of foods or food groups likely to contain the processing aid or its metabolites	✓	18	Section 4.1
	F.2 The levels of residues of the processing aid or its metabolites for each food or food group	✓	18	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	✓	19	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	✓	19	Section 4.4
	F.5 Information relating to the levels of residues in foods in other countries	✓	19	Section 4.5
	F.6 For foods where consumption has changed in recent years, information on likely current food consumption	✓	19	Section 4.6

2. 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 the Alpha-amylase gene from *Cytophaga sp*. The amount of Alpha-amylase required for efficacy will vary between given applications. Level of use is discussed in detail in Appendix C.

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 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
- CAS number: 9000-90-2

Biological source: The Alpha-amylase enzyme is an enzyme produced by submerged fermentation of *B. licheniformis*, carrying the Alpha-amylase gene from *Cytophaga sp*.

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 Spezyme® SL, or GC 126.

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 function of Alpha-amylase is to catalyse the endohydrolysis of (1→4)-α-D-glucosidic linkages in polysaccharides containing three or more (1→4)-α-linked D-glucose units.

Alpha-amylase is used to maximise the conversion of starchy substrate to fermentable carbohydrate. It will be used in the liquefaction and saccharification of starch (mashing) from malted cereal, cereal and other plant sources (includes barley, maize, wheat, rye, milo, rice, tapioca and potatoes). The resultant process liquors (worts) are fermented, typically by yeast, to produce ethanol.

In potable alcohol production, the hydrolysis of polysaccharides like starch with the use of Alpha-amylase has various benefits. The Alpha-amylase helps the conversion of liquefied starch

into a maltose rich solution. Finally, the hydrolysis of starch with the use of Alpha-amylase increases the percentage of fermentable sugars.

In starch processing Alpha-amylase will be used in combination with other enzymes for the manufacture of glucose from various sources including maize, wheat, milo, tapioca, barley, rice, cassava and potatoes. The resulting substance will be used for manufacture of starch syrups with special saccharide distribution such as maltodextrin or can be further treated as glucose-rich syrups that can be purified to meet various specifications: crystallised to produce dextrose, isomerised to produce high fructose corn syrup, or fermented to produce organic acids or amino acids.

The benefits of using Alpha-amylase in starch processing, brewing and potable alcohol production are detailed in Appendix A.

Substrate specificity:

The function of Alpha-amylase (IUBMB 3.2.1.8) is to catalyse the endohydrolysis of (1→4)-α-D-glucosidic linkages in polysaccharides containing three or more (1→4)-α-linked D-glucose units.

Activity:

The activity of the Alpha-amylase is defined in DLU. The assay uses p-nitrophenyl maltoheptoside substrate with the non-reducing terminal sugar chemically blocked. Alpha glucosidase and a glucoamylase are used as coupling enzymes. The blocked terminal sugar prevents attack by glucoamylase. The rate of p-nitrophenyl release is proportional to alpha-amylase activity and is monitored at 405nm.

Alpha-amylase preparations' enzyme activity will depend on the final product. An example product has the Alpha-amylase activity range of 27150 - 31850 DLU/g. A detailed assay method is present in Appendix A3.

Temperature optimum:

Temperature optimum was determined to lie between 60 and 70°C.

Thermal stability:

The enzyme activity dropped to below 20% after 30 minutes of incubation at 60°C.

pH optimum:

Approximately pH 5.5, with high relative activity at pH interval 4.5-6.

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 are as follows:

Metals:	
Lead	less than 5 mg/kg
Arsenic	less than 1 mg/kg
Cadmium	less than 1 mg/kg
Mercury	less than 1 mg/kg
Microbiological:	
Total viable count	less than 50,000 CFU/ml
Total coliforms	less than 30 CFU/ml
E. coli	absent in 25ml
Salmonella	absent in 25ml
Antibiotic activity	Negative by test
Production strain	Negative by test
Physical properties:	
Form	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.

2.6. Allergenicity of the enzyme:

Bioinformatic analyses based on sequence homology determined that the *Cytophaga sp.* 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.

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 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 the Cytophaga sp. alpha-amylase shows a clear conserved AmyAc_bac_fung_AmyA (cd11318) catalytic domain found in bacterial and fungal alpha-amylases, together with the AmyA (cl33851) superfamily sequence domain found in a large range of alpha-amylases.

The approved alpha-amylases on Schedule 18 of the ANZ Food Standards Code are the ones obtained from A. niger, A. oryzae, B. amyloliquefaciens, B. licheniformis, B. licheniformis containing the gene for α-amylase isolated from G. stearothermophilus, B. subtilis, B. subtilis containing the gene for α-amylase isolated from G. stearothermophilus, and G. stearothermophilus. These alpha-amylase sequences were retrieved from the UniProtKB database and analysed for homology. The identity between these FSANZ approved mature alpha-amylase

sequences ranges from 13% (G. stearothermophilus to B. subtilis) to 99% (A. niger to A. oryzae).

The identity between the mature Cytophaga sp. alpha-amylase sequence, subject of this dossier, and the FSANZ approved mature alpha-amylase sequences ranges from 18% (B. subtilis vs G. stearothermophilus) to 74% (B. amyloliquefaciens). It is good to realize that the alpha-amylase sequences within one species can show strain dependent amino acid sequence variability. Also, several microorganism species contain more than one alpha-amylase encoding genes with different sequences (e.g. A. niger amyA and amyB).

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

3.2. 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 endo-1,4-beta xylanases, 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 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.

Although *B. licheniformis* is scientifically determined by DuPont N&B 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 *B. licheniformis* JML 1584 are thus one of the pillars supporting the DuPont N&B *B. licheniformis* Safe Strain Lineage. The position of the food enzyme in the DuPont N&B *B. licheniformis* Safe Strain Lineage is presented in Appendix B2.

Toxicological testing

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

- 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: the NOAEL (no observed adverse effect level) is established at the highest dose tested, 500 mg total protein/kg bw/day, equivalent to 272 mg total organic solid (TOS)/kg bw/day in male and female rats.

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 *Cytophaga sp*. 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 *Cytophaga sp*. expressed in *B. licheniformis* has not been reviewed by JECFA; there is no specific Codex Standard relevant to this application. It has, however, been assessed by a GRAS panel and approved by Denmark and France for various purposes. Refer Appendix D for safety reports/approval letters.

3.5 Information on the source micro-organism

The production organism of the Alpha-amylase preparation, the subject of this submission, is *B. licheniformis* strain JML 1584. It is derived by recombinant DNA methods from strain Bra7. The purpose of this genetic modification is to express the gene encoding an Alpha-amylase from *Cytophaga sp*. Bra7 is a classical industrial strain used for α-amylase production by DuPont N&B and its parent companies since 1989. 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 B.

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 micro-organism

The host/source organism is *B. licheniformis* Bra7. *B. licheniformis* Bra7 is a classical industrial strain used for Alpha-amylase production by DuPont N&B 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. Further information is provided in Appendix B.

3.7. Genetic stablity of the source 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 DuPont N&B and its parental companies and has demonstrated stable enzyme expression even at large scale fermentation. Please also refer to Appendix B4 for list of example enzyme preparations produced using Bra7 and its derivatives. Furthermore, the production strain has demonstrated to be 100% stable as confirmed by genome sequencing. Refer also section 3.8.

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

The production organism of the Alpha-amylase preparation, the subject of this submission, is *B. licheniformis* strain JML 1584. It is derived by recombinant DNA methods from strain Bra7. The purpose of this genetic modification is to enhance Alpha-amylase production levels. Bra7, a commercial production strain is derived, as a result of several classical mutagenesis steps, from the well-known wild-type *B. licheniformis*. Many strains used by Dupont N&B and its associated companies globally for industrial enzyme production today are derived from Bra7. The donor organism is *Cytophaga sp*. The Alpha-amylase expression cassette was integrated into the host genome.

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 Alpha-amylase 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 60 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.

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, Alpha-amylase will be used in:

- 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	Recommended Use Level (mg TOS/kg RM)	Max. recommended levels (mg TOS/kg RM)
Potable alcohol production	Cereals	4.66-11.6	11.66
Starch processing	Starch	1.40-14.46	14.46
Brewing	Cereals	9.1-181.8	181.8

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

Alpha-amylase is used to liquefy starch from various sources. In starch processing, brewing and potable alcohol production Alpha-amylase increases extraction and saccharification of starch maximising the conversion of starchy substrate to fermentable carbohydrates.

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: 0.79 mg TOS/kg body weight/day. The NOAEL has been determined for Alpha-amylase to be at 272 mg total protein/kg bw/day (equivalent to 500 mg TOS/kg bw/day). Based on a worst-case scenario of daily food consumption, the NOAEL would offer a 633-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 (NNS)

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:

- 50% of the tonnage of potable alcohol products sold in Australia and New Zealand
- 20 % of the tonnage of brewed products 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 (potable alcohol and brewed beverages) produced with Alpha-amylase are not expected to have a significant change.

References

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