An Alpha-Amylase Enzyme from a recombinant strain of *Trichoderma reesei*

PROCESSING AID APPLICATION

Food Standards Australia New Zealand

Applicant: DANISCO NZ LTD

12th November, 2019

CONTENTS

EXECUTIVE SUMMARY:

DuPont Nutrition & Biosciences (N&B) is seeking approval for a "Alpha-amylase (EC 3.2.1.1)" enzyme for use as processing aid in brewing application and for use in potable alcohol production. 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 *Trichoderma reesei* which is genetically modified to overexpress the alpha-amylase gene from *Aspergillus kawachii.*

The enzyme is intended for use in brewed beverages and potable alcohol production. In brewing, Alpha-amylase is typically added in to the cereal cooker or in the mashing step and is thus denatured already in the consecutive lautering or mash filtration step. In the potable alcohol production industry, the Alpha-amylase is added in the pre-treatment, liquefaction and/or pre-saccharification step.

In Brewing and Potable alcohol production, Alpha-amylase increase extraction and saccharification of starch maximizing the conversion of starchy substrate to fermentable carbohydrates.

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

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

In addition, different endpoints of toxicity were investigated, and the results are evaluated and assessed in this document. In genotoxicity studies, Alpha-amylase is not mutagenic, clastogenic. Daily oral administration of Alpha-amylase up to and including a dose level of 184 mg total protein/kg bw/day or 229.6 mg TOS/kg bw/day does not result in any manifestation of systemic, hematologic, or histopathologic adverse effects.

Based on a worst-case scenario that a person is consuming Alpha-amylase in a brewed beverage, the calculated Theoretical Maximum Daily Intake (TMDI) will be 0.39 mg TOS/kg body weight/day. This still offers a 589 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 facilitating the brewing and potable alcohol production processes by imparting processing efficiencies and/or more consistent product quality. Moreover, the applications lead to more effective production processes, resulting in better production economy and environmental benefits such as the use of less raw materials and the production of less waste. process, lowering the manufacturing cost, and improving quality of final foods.

General information

1.1 Applicant details

(a) Applicant:

This application is made by Danisco New Zealand Ltd

(b) Company:

Dansico New Zealand Ltd

(c) Address:

[redacted]

(d) Contact Details:

[redacted]

(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, 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 brewing and potable alcohol production.

This application is made solely on behalf of DuPont Industrial Biosciences (IB), 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 brewing to produce brewed beverages, and in the production of potable alcohol.

Currently no Alpha-amylase from *Aspergillus kawachii* expressed in *T. reesei* is permitted as a Processing Aid, however other enzymes including Cellulase, Alpha-1,4-beta-xylanase, β-Glucanase, Hemicellulase multicomponent enzyme, and Polygalacturonase or Pectinase multicomponent enzyme, from *T. reesei* are all 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 *T. reesei* carrying the gene encoding the Alpha-amylase gene from *A. kawachii.* The enzyme is characterized 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 the brewing industry 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.

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 Alpha-amylase from *Aspergillus kawachii* expressed in *T. reesei* is permitted as a Processing Aid. Approval of this application would provide food processors with a new enzyme preparation offering the benefits and advantages as discussed previously.

B. Impact on international trade

The inclusion of Alpha-amylase from *Aspergillus kawachii* expressed in *T. 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 marketing or promotional activities have been undertaken for Alpha-amylase derived from *T. reesei* containing the gene for Alpha-amylase from *Aspergillus kawachii* 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, 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 B3-B6, Appendices D1-D3, Appendices E1-E5 and other information including amino acid sequences labelled with Confidential Commercial information is regarded by the applicant as **Confidential Commercial Information** and is provided in the application strictly on this basis. In addition, all toxicological studies submitted in support of this application are also considered **Confidential.** 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 *A. kawachii* expressed in *T. reesei* has not been reviewed by JECFA; there is no specific Codex Standard relevant to this application.

1.8.2 International Legislation

Alpha-amylase from *T. reesei* carrying the gene encoding the Alpha-amylase gene from *A. kawachii* has been determined to be Generally Recognized as Safe (GRAS) in the United States as a food processing aid in production of brewed goods and potable alcohol production by a panel of scientific experts in the USA (Refer Appendix D1, GRAS Expert Panel letter).

1.9. Statutory declaration

I, Caroline Elizabeth Gray,

of 5 Te Kare Rd, Wai O Taiki Bay, Auckland 1072, New Zealand, regulatory affairs manager, solemnly and sincerely declare that:

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.

And I make this solemn declaration conscientiously believing the same to be true and by virtue of the Oaths and Declarations Act 1957.

Declared at [location] this [date]

[redacted] 13th of November 2019

Before me,

[redacted]

1.10. Checklist

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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 *T. reesei*, carrying the Alpha-amylase gene from *A. kawachii*.

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: Alpha-amylase enzyme is an enzyme produced by submerged fermentation of *T. reesei*, carrying the Alpha-amylase gene from *A. kawachii*.

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 Distillase® CS.

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 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 maximize 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.

The benefits of the conversion of starchy substrates with the help of Alpha-amylase in Brewing are:

- Liquefaction of starchy substrates;
- Maximized conversion of starchy substrates to fermentable carbohydrate;

- Increased productivity due to higher conversion rate from starch to fermentable
- carbohydrates;
- Potential for higher alcohol yield;
- Potential for use of less raw material;
- Increased flexibility in the choice of raw materials;
- Decrease mash viscosity and hereby fouling during cereal cooker operations;
- Removal of beer haze from starch.

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.

The benefits of the hydrolysis of polysaccharides like starch with the help of an Alpha-amylase in potable alcohol production are:

- Formation of fermentable sugars due to the conversion of liquefied starch into maltose rich solution;
- Higher solid concentration during mashing (energy efficiency);
- Process flexibility (lower pH and temperature);
- Potential higher alcohol yield due to the improved processing, and thereby less use of raw materials.

Substrate specificity:

Alpha-amylase hydrolyses (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 SSU. The substrate employed in the assay is 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 410nm.

Alpha-amylase preparations' enzyme activity will depend on the final product. An example product has the Alpha-amylase activity range of 10000-13000 SSU/g. A detailed assay method is present in Appendix A3.

Temperature optimum:

The optimum temperature range lies between 60 and 70°C with activities above 8,000 SSU/ml enzyme. When the temperature goes above 80°C, the activity decreases significantly, to below 2000 SSU.

Thermal stability:

A significant reduction was observed when the temperature is above 70°C. Less than 10%2000 SSU/mg enzyme of remaining activity was observed after incubation at 85°C for 20 minutes in all the buffer tested.

pH optimum:

The optimum pH range lies between pH 2.0 and pH 4. 0.

pH stability:

The enzyme activity decreased dramatically at pH above 6.5, and became undetectable above pH 7.25.

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
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:

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 catalyze 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 catalyze 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 A. kawachii alpha-amylase shows a clear conserved AmyAc superfamily sequence domain characteristic for eukaryotic alpha-amylase activities, together with a DUF1966 superfamily sequence domain found in several fungal alpha-amylases, and a CBM20 family starch binding domain.

A selection of alpha-amylase sequences of the species listed on Schedule 18 of the ANZ Food Standards Code were retrieved from the UniProtKB database and analysed for homology. The alpha-amylase enzyme, subject of this dossier, shows high homology with several analyzed alpha-amylase enzymes on Schedule 18 of the ANZ Food Standards Code, e.g. 95.15% identity with the A. niger one. The identity between the FSANZ approved alpha-amylases (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, G. stearothermophilus) ranges from 20.37 (G. stearothermophilus to B. subtilis) to 99.60% (A. niger amyB to A. oryzae amy3). 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 was performed, with a threshold E-value of 0.1. The majority of matches were Alpha-amylase, 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 (Appendix B2).

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 utilized 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 *T. reesei* 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 *T. reesei* LOH4AkAApaA are thus one of the pillars supporting the DuPont N&B *T. reesei* Safe Strain Lineage. The position of the food enzyme in the DuPont N&B *Trichoderma reesei* 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, equivalent to 229.6 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 *A. kawachii* 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.

3.4 Safey assessment reports prepared by international agenicies or other national government agencies, if available

As discussed in section 1.8 Alpha-amylase from *A. kawachii* expressed in *T. 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 in the United States and approved by both France and Denmark for various purposes. Refer Appendix D for safety reports/approval letters.

3.5 Information on the source micro-organism

The production organism strain LOH4AkAApaA is a strain of *T. reesei* which has been genetically modified by DuPont N&B to overexpress an Alpha-amylase gene from *A. kawachii*.

T. reesei has a long history of safe use in industrial scale enzyme production. The safety of this species as an industrial enzyme producer has been reviewed by Nevalainen *et al*. (1994), Blumenthal (2004) and Olempska-Beer et al. (2006). The organism is considered non-pathogenic for humans and does not produce fungal toxins or antibiotics under conditions used for enzyme production. It is generally recognized 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 Alpha-amylase gene was placed under the *T. reesei cbh1* promoter, and *T. reesei cbh1 terminator.* 6-8copies of the expression cassette were integrated into the recipient genome, using either the *A. nidulans* acetamidase (*amdS*) gene or the endogenous *T. reesei* orotate phosphoribosyl transferase (*pyr2*) gene as a selectable marker.

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

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

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

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

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

3.7 Genetic stability of the source organism

The parental strain of the production strain *Trichoderma reesei* LOH4AkAApaA 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 B2 for list of example enzyme preparations produced using LOH4AkAApaA and its derivatives. Furthermore, the production strain has demonstrated to be 100% stable as confirmed by genome sequencing. Refer also section 3.6.

3.8 Method used in the genetic modification of the source organism

The production organism of the Alpha-amylase preparation, the subject of this submission, is *T. reesei* strain LOH4AkAApaA. It is derived by recombinant DNA methods from strain RL-P37. The purpose of this genetic modification is to enhance Alpha-amylase production levels. RL-P37, a commercial production strain, is derived, as a result of several classical mutagenesis steps, from the well-known wild-type strain QM6a. Virtually all strains used all over the world for industrial cellulase production today are derived from QM6a. The donor organism is *A. kawachii*. Amylase expression cassette was integrated into the host genome. 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 Alpha-amylase expression site was determined, and

no change was observed between samples prior and after fermentation. The results demonstrate that the insertion cassettes have 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.

4.0 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:

- 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)	Recommended use levels (mg TOS/kg RM)	Maximal recommended use levels (mg TOS/kg RM)
Brewing	Cereal	37-371	371
Potable alcohol production	Cereal	12-116	116

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

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.

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: 0.39 mg TOS/kg body weight/day. The NOAEL has been determined for Alpha-amylase to be at 184 mg total protein/kg bw/day (equivalent to 229.6 mg TOS/kg bw/day). Based on a worst-case scenario of daily food consumption, the NOAEL would offer a 589-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:

- 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 (alcoholic drinks) produced with Alpha-amylase is not expected to have a significant change.

5.0 References

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