A Maltogenic Alpha-Amylase Enzyme
from a recombinant strain of *Bacillus licheniformis*

PROCESSING AID APPLICATION

Food Standards Australia
New Zealand

Applicant: DANISCO NEW ZEALAND LTD

7 August 2020

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EXECUTIVE SUMMARY:

DuPont Nutrition & Biosciences (DuPont N&B) is seeking approval for a "Maltogenic α-amylase (EC 3.2.1.133)" enzyme for use as processing aid in bakery, potable alcohol, brewing and starch processing applications. The enzyme is designated as "MAA" or "Amylase MAA" throughout the dossier.

The enzyme MAA is derived from a selected non-pathogenic, non-toxigenic strain of *Bacillus licheniformis* which is genetically modified to express the maltogenic α-amylase gene from *G. stearothermophilus.*

The enzyme is intended for use in baking, potable alcohol, brewing and starch processing. In all of these applications the MAA hydrolyses (1 → 4)-alpha-D-glucosidic linkages in polysaccharides, to remove successive alpha-maltose residues from the non-reducing ends of the chains.

In these applications, MAA 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 MAA 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) utilising enzyme toxicology/safety data, the safe history of use of enzyme preparations from *B. licheniformis* and of other MAA 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.

In addition, different endpoints of toxicity were investigated, and the results are evaluated and assessed in this document. In genotoxicity studies, MAA is not mutagenic or clastogenic or aneugenic. Daily oral administration of MAA up to and including a dose level of 55.6 protein/kg bw/day or 80 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 MAA, the calculated Theoretical Maximum Daily Intake (TMDI) will be 0.266mg TOS/kg body weight/day. This still offers a 300-fold margin of safety.

Based on the results of safety studies and other evidence MAA 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 improving the quality of baked foods, higher brewing yields and flexibility in raw material choice, and efficiencies in potable alcohol production.

General information

1.1 Applicant details

(a) Applicant:

This application is made by Danisco New Zealand Ltd

(b) Company:

Danisco New Zealand Ltd

[redacted]

Company)

(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 bakery, brewing, potable alcohol production and starch processing.

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.

MAA, the subject of this application, is intended for use in bakery, brewing, starch processing and potable alcohol production.

Currently no MAA from *G. stearothermophilus* expressed in *B. licheniformis* is permitted as a Processing Aid, however MAA from *Bacillus subtilis* containing the gene for maltogenic α-amylase isolated from *G. stearothermophilus*, and other enzymes including α-amylase, Chymotrypsin, β-Galactosidase Glycerophospholipid cholesterol acyltransferase 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

A. Costs and Benefits of the application

MAA is an enzyme produced by submerged fermentation of *B. licheniformis* carrying the gene encoding the MAA gene from *G. stearothermophilus.* The enzyme is characterised as a Glucan 1,4-alpha-maltohydrolase (EC 3.2.1.133). 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 baking, brewing, starch processing and potable alcohol production. In all these applications the MAA hydrolyses (1→4)-alpha-D-glucosidic linkages in polysaccharides, to remove successive alpha-maltose residues from the non-reducing ends of the chains

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 MAA from *G. stearothermophilus* 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 MAA from *G. stearothermophilus* 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 MAA derived from *B. licheniformis* containing the gene for MAA from *G. stearothermophilus* 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 B1, B3, -B6, Appendices D2, 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. 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

MAA from *G. stearothermophilus* 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

MAA derived from *B. licheniformis* carrying the gene encoding the MAA enzyme from *G. stearothermophilus* has been considered as GRAS in the U.S., approved in Denmark and some other countries for various purposes (See Appendix D).

1.9. Statutory declaration

[redacted]

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.

[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 MAA is an enzyme produced by submerged fermentation of *Bacillus licheniformis*, carrying the maltogenic alpha-amylase gene from *G. stearothermophilus*.

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 Glucan 1,4-alpha-maltohydrolase. Other names used are Maltogenic α-amylase; 1,4- α -D-glucan- α maltohydrolase; 4- α -Dglucan α - maltohydrolase.

- EC number: 3.2.1.133
- CAS number: 160611-47-2

Biological source: The MAA is an enzyme produced by submerged fermentation of *Bacillus licheniformis*, carrying the maltogenic alpha-amylase gene from *G. stearothermophilus*.

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 MAA is MAXLIFE™ P100.

2.2.3 Molecular and Structural Formula:

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

2.3. Chemical and physical properties

The function of MAA is to hydrolyse (1→4)-alpha-D-glucosidic linkages in polysaccharides, to remove successive alpha-maltose residues from the non-reducing ends of the chains.

In principle, the enzymatic conversion of polysaccharides like starch with the help of MAA can be of benefit in the processing of food raw materials, which naturally contain the substrate.

The benefits of the use of MAA in certain food processes may include:

Baking

- Lowering the rate of amylopectin retrogradation and thereby reduce staling
- Maintain the crumb softness and resilience of baked bread longer

- Ensure a uniform volume and an improved crumb structure of the bakery product, which might otherwise be impaired by processing of the dough

Brewing

- Conversion of liquefied starch into maltose rich solution
- More uniform formation of fermentable sugars, leading to less product variations (e.g. taste profile) caused by batch to batch variations of different raw materials, e.g. different malt batches
- Increased flexibility in the choice of raw materials (raw grain/malt ratio)
- Higher brewing yield due to the improved processing and thereby less use of raw materials

Starch processing

- Conversion of liquefied starch into maltose rich solution
- Compared to acid catalysed hydrolysis the reaction is more specific and there is less formation of side products

Potable alcohol production

- Formation of fermentable sugars due to the conversion of liquefied starch into maltose rich solution
- More uniform formation of fermentable sugars, leading to less product variations (e.g. taste profile) caused by batch to batch variations of different raw materials
- Potential higher alcohol yields due to the improved processing, and thereby less use of raw materials.

Substrate specificity:
Glucan 1,4-alpha-maltohydrolase (IUBMB 3.2.1.133) hydrolyses (1 → 4)-alpha-D-glucosidic linkages in polysaccharides, to remove successive alpha-maltose residues from the non-reducing ends of the chains.

Activity:
The activity of the MAA is defined in BU. This method is used to determine beta amylase activity on fermentation, recovery and liquid concentrate samples. The assay is colorimetric and monitors the rate of degradation of p-nitrophenyl-maltotrioside. The rate of p-nitrophenyl release is proportional to amylase activity and is monitored at 405nm. Note that this procedure involves a stop reaction at alkaline pH. The alkaline pH is necessary to stop the reaction and to provide optimal color development for the endpoint reaction.

An MAA preparations' enzyme activity will depend on the final product. An example product has the amylase activity range of 20000-32500 units/g. A detailed assay method is present in Appendix A3.

Temperature optimum:
Temperature optimum was determined to be 50°C with high relative activity between 40- 50°C. The relative activity reduced significantly at 60°C and above.

Thermal stability:

MAA retained activity after 30 minutes of incubation from 40°C -70°C. A significant reduction was observed when the temperature is 80°C and above. No enzyme activity is observed at temperatures above 90°C.

pH optimum:

Enzyme demonstrated activity in the range from pH 4.0 to 7.2. The optimum pH range lies between pH 4.4 and 6.8. Enzyme activity was barely detectable at pH 3.6.

Interaction of the enzyme with different foods:

The MAA 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:

MAA is a protein and any residual amounts remaining in food consumed would accordingly have the same nutritional value. However, the use levels of MAA 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 MAA 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 MAA 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 10,000 CFU/g
Total coliforms	less than 30 CFU/g
E. coli	absent in 25g
Salmonella	absent in 25g
Antibacterial activity	Negative by test
Production strain	Negative by test
Physical properties:	
Appearance	Off white powder

Standard for identity:

MAA 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 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 Geobacillus stearothermophilus maltogenic alpha-amylase MAA shows a clear conserved AmyAc_AmyMalt_CGT-ase_like Alpha amylase catalytic domain found in maltogenic amylases, cyclodextrin glycosyltransferase, and related proteins, together with the AmyA (cl33851) superfamily sequence domain found in a large range of alpha-amylases, and a CBM20 family starch binding domain.

The only approved maltogenic alpha-amylase on Schedule 18 of the ANZ Food Standards Code is the one obtained from Bacillus subtilis containing the gene for maltogenic α-amylase isolated from Geobacillus stearothermophilus. This alpha-amylase sequences was retrieved from the

UniProtKB database and analysed for homology. The identity between this FSANZ approved mature maltogenic alpha-amylase sequence and the enzyme subject of this dossier is 100%.

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

DuPont N&B has determined by scientific procedures that production organism *B. licheniformis* MDT 06-221 is safe as a production organism as it pertains to the DuPont *B. licheniformis* Safe Strain Lineage (see Appendix B).

Toxin homology study

A BLAST search for homology of the Amylase MAA sequence against the complete Uniprot database was performed, with a threshold E-value of 0.1. The majority of matches were Maltogenic alpha-amylases, Alpha-amylases and Cyclomaltodextrin glucanotransferases, with none of the top 1000 database matches being annotated as either toxin or venom. Please refer to Appendix B1 Toxin Homology Search Results submitted separately as in the excel file for detailed analysis results (**Confidential Commercial Information**).

In addition, a specific BLAST search for homology of the Amylase MAA sequence was performed against the Uniprot animal toxin database. This yielded no matches (Appendix B2). Therefore, the Amylase MAA 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* Bra7 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 MAA, 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, 55.6 mg total protein/kg bw/day, equivalent to 80 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 *G. stearothermophilus* MAA 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. Wheat may be present in the final preparation. As this enzyme is used for bakery purpose, the presence of wheat in the enzyme preparation is not expected to introduce any additional food allergen risk to consumers.

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

As discussed in section 1.8 MAA from *G. stearothermophilus* 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 for various purposes. Refer Appendix D for safety reports/approval letters.

3.5 Information on the source micro-organism

The production organism strain MDT 06-221 is a strain of *B. licheniformis* which has been genetically modified by DuPont N&B to overexpress a MAA gene from G. stearothermophilus.

Industrial strains belonging to the *B. licheniformis* species have a long history of safe use in food enzyme manufacturing (Recombinant DNA Safety Considerations, OECD, Paris, 1986). They have been used for decades in the production of enzymes, and in almost two decades as recombinant organisms for the production of a variety of bio-industrial products like food grade enzymes, vitamins, antibiotics, and additives and references therein (Schallmey et al., 2004). The role of Bacillus licheniformis in the fermentation of traditional African fermented locust bean condiment was described by Olajuyigbe and Ajele, 2008.

Bacillus licheniformis is among the most widely used bacteria for the production of enzymes and specialty chemicals. Industrial applications include production of amylase, protease, inosine, ribosides, and amino acids. Uses of proteases include use in detergent products and for dehairing and batting in the leather industry. Uses of amylases include e.g. desizing of textiles and starch modification for sizing of paper (Erikson, 1976; Ferrari et al., 1993).

The parent strain *B. licheniformis* and strains derived from it have been in use for a long time for industrial scale production of alpha-amylase and other enzymes for food processing applications.

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.

The MAA gene from the donor strain *G. stearothermophilus* was integrated into the host chromosome. After integration all vector sequences of the plasmid were deleted by recombination between direct repeated *cat* sequences. The result is a strain which only the *G. stearothermophilus* (formerly known as *B. stearothermophilus*) MAA and the native *cat* gene were introduced into the host strain. This cassette was amplified using several rounds of growth at increasing concentrations of chloramphenicol to obtain the final production strain.

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 Information on Pathogenicity and toxicity of the source micro-organism

The host organism is *B. licheniformis* Bra7. *B. licheniformis* Bra7 is a classical industrial strain used for α-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 α-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 stability 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 3.6.

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

The production organism of the MAA preparation, the subject of this submission, is *B. licheniformis* strain MDT 06-221. It is derived by recombinant DNA methods from strain Bra7. The purpose of this genetic modification is to enhance MAA 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 *G. stearothermophilus*. The MAA 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. Flanking DNA sequence for MAA 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 (15-5), MAA will be used in:

- 7. Breads and bakery products
- 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 MAA 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)
Baking	Flour	0.5 – 5.07	5.07
Brewing	Cereal	5.22 – 52.2	52.2
Potable alcohol production	Cereal	5.22 – 52.2	52.2
Starch processing	Starch	6.15 – 61.5	61.5

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

In Baking, MAA typically performs its technological function during the dough or batter handling contributing to an improved and consistent product. MAA is denatured by heat during the baking step.

In Brewing, MAA will be used in the mashing process where the enzyme will hydrolyse the starchy content of the mash into fermentable sugars, i.e maltose. After mashing the liquid (wort) is separated from the spent grains by filtration and the wort is boiled for 1-2 hours for sterilisation.

In potable alcohol production, MAA, along with other enzymes such as alpha-amylase and xylanase, will be used in the pre-treatment, liquefaction and/or pre-saccharification step, where the enzyme will hydrolyse the starchy content of the mash into fermentable sugars, especially maltose.

In potable alcohol production, solids are separated from the fermentation slurry at the end of fermentation and any enzyme protein precipitate will be removed with the solids. The liquids are then distilled. The distilled alcohol is subsequently filtered through a molecular sieve at temperatures well over boiling to adsorb further traces of water- and water-soluble protein. Therefore, the enzymes will not be present/active in the end product due to distillation in the case of alcohol production.

In Starch processing, MAA performs its technological function during the saccharification step for production of maltose by hydrolysis of the liquefied starch. The MAA is denatured by heat during a dedicated inactivation step or removed during subsequent carbon or ion exchange treatments.

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 MAA, 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: 0.266 mg TOS/kg body weight/day. The NOAEL has been determined for MAA to be at 80 mg TOS/kg bw/day (equivalent to 55.6 mg total protein /kg bw/day). Based on a worst-case scenario of daily food consumption, the NOAEL would offer a 300-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:

- 0.05-2% of the tonnage of bread and bakery, alcoholic beverages (including alcoholic beverages that have had the alcohol reduced or removed), and sugars, honey and related products sold in Australia and New Zealand

4.5. Levels of residues in food in other countries

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

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

Not applicable. Consumption of foods (alcoholic drinks, bakery products and sugar products) produced with MAA are not expected to have a significant change.

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