A β-Galactosidase Enzyme from a recombinant strain of *Bacillus subtilis*

PROCESSING AID APPLICATION

Food Standards Australia New Zealand

Applicant: DANISCO NEW ZEALAND LTD

2 November 2020

CONTENTS

EXECUTIVE SUMMARY:

DuPont Nutrition & Biosciences (DuPont N&B) is seeking approval for a "β-Galactosidase (EC 3.2.1.23)" enzyme for use as processing aid in dairy food applications. The enzyme is designated as "β-Galactosidase or lactase" throughout the dossier, and also referred to as 'lactase' or 'Sweet Dream' in some references. The enzyme β-Galactosidase is derived from a selected non-pathogenic, non-toxigenic strain of *Bacillus subtilis* which is genetically modified to overexpress the β-Galactosidase gene from *Lactobacillus delbrueckii bulgaricus.*

The enzyme is intended for use in dairy processing for production of lactose reduced dairy products including but not limited to milk, yogurt, cheese. In dairy products the β-Galactosidase catalyses the hydrolysis of terminal non-reducing β-D-galactose residues in β-D-galactosides. The lactose in these dairy products is hydrolysed into galactose and glucose.

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

To assess the safety of the β-Galactosidase 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. subtilis* and of other β-Galactosidase enzymes in food, the history of safe use of the *B. subtilis* 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, β-Galactosidase is not mutagenic or clastogenic. Daily oral administration of β-Galactosidase up to and including a dose level of 1000 mg TOS/kg bw/day does not result in any manifestation of systemic, hematologic, or histopathologic adverse effects. This NOAEL is equivalent to 752 mg total protein/kg body weight/day in males and females.

Based on a worst-case scenario that a person is consuming β-Galactosidase from baking application, the calculated Theoretical Maximum Daily Intake (TMDI) will be 11.29 mg TOS/kg body weight/day. This still offers a 125.5-fold margin of safety.

Based on the results of safety studies and other evidence, β-Galactosidase 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 lowering of lactose content in many processed dairy-based food applications.

General information

1.1 Applicant details

(a) Applicant:

This application is made by Dansico 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 (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 for lactose reduction in processed dairy foods.

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.

β-Galactosidase, subject of this application, is intended for use in processed dairy foods, for the reduction of lactose content.

Currently no β-Galactosidase from *L. delbrueckii bulgaricus* expressed in *B. subtilis* is permitted as a Processing Aid, however β-Galactosidase from *A.niger*, *A.oryzae, Kluyveromyces marxianus, Kluyveromyces lactis* and *Papiliotrema terrestris strain AE-BLC* have been approved by FSANZ. Also, β-Galactosidase from *B. bifidum* expressed in *B.subtilis* and *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

β-Galactosidase is an enzyme produced by submerged fermentation of *B.subtilis* carrying the gene encoding the β-Galactosidase gene from *L .delbrueckii bulgaricus.* The enzyme is characterised as a β-D-galactoside galactohydrolase (EC 3.2.1.23). 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 in the milk and dairy product processing (e.g., milk, yogurt, cheese, and whey), and the milk/dairy derived product processing (e.g., sweet condensed milk, evaporated milk, ice cream, sweetened yogurt, and flavored milk) to reduce the lactose in the products

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 β-Galactosidase from *L. delbrueckii bulgaricus* expressed in *B. subtilis* 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 β-Galactosidase from *L. delbrueckii bulgaricus* expressed in *B. subtilis* 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 β-Galactosidase derived from *B. subtilis* containing the gene for β-Galactosidase from *L. delbrueckii bulgaricus* 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, -B7, 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

β-Galactosidase from *L. delbrueckii bulgaricus* produced by *B. subtilis* has not been reviewed by JECFA; there is no specific Codex Standard relevant to this application.

1.8.2 International Legislation

β-Galactosidase derived from *B. subtilis* carrying the gene encoding the β-Galactosidase d gene from *L. delbrueckii bulgaricus* has been determined to be Generally Recognised as Safe (GRAS) in the United States as a food processing aid in production of dairy products by expert opinion.

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 _____ on _____ of _____

Before me,

_____

Signature

1.10. Checklist

	Mandatory Requirements	Check	Page Number	Remarks
General requirements for applications	A. Form of the application	✓	N.A.	
	Table of contents	✓	1	
	Executive summary	✓	2	
	B. Applicant details	✓	3	Section 1.1
	C. Purpose of application	✓	4	Section 1.2
	D. Justification for the application	✓	4	Section 1.3
	D.1 Regulatory impact information	✓	4	Section 1.3.1
	D.1.1 Costs and benefits of the application	✓	4	Section 1.3.1
	D.1.2 Impact on international trade	✓	4	Section 1.3.1
	E Information to support the application	✓	5	Section 1.4
	E.1 Data requirements	✓	N.A.	
	F. Assessment procedure	✓	5	Section 1.5
	G. Confidential commercial information (CCI)	✓	5	Section 1.6
	H. Other confidential information	✓		
	I. Exclusive capturable commercial benefit (ECCB)	✓	5	Section 1.7
	J. International and other national standards	✓	5	Section 1.8
	J.1 International Standards	✓	5	Section 1.8.1
	J.2 Other national standards or regulations	✓	5	Section 1.8.2
	K. Statutory declaration	✓	6	Section 1.9
	L. Checklist	✓	7	Section 1.10
3.3.2. Processing aids	A. Technical information on the processing aid	✓	9	Section 2
	A.1 Information on the type of processing aid	✓	9	Section 2.1
	A.2 Information on the identity of the processing aid	✓	9	Section 2.2
	A.3 Information on the chemical and physical properties of the processing aid	✓	9	Section 2.3
	A.4 Manufacturing process	✓	10	Section 2.4
	A.5 Specification for identity and purity	✓	10	Section 2.5
	A.6 Analytical method for detection	×		Not applicable for enzymes used as processing aids
	C. Information related to the safety of an enzyme processing aid	✓	12	Section 3
	C.1 General information on the use of the enzyme as a food processing aid in other countries	✓	12	Section 3.1
	C.2 Information on the potential toxicity of	✓	13	Section 3.2

	the enzyme processing aid			
	C.3 Information on the potential allergenicity of the enzyme processing aid	✓	14	Section 3.3
	C.4 Safety assessment reports prepared by international agencies or other national government agencies, if available	✓	14	Section 3.4
	D. Additional information related to the safety of an enzyme processing aid derived from a microorganism			Section 3
	D.1 Information on the source microorganism	✓	14	Section 3.5
	D.2 Information on the pathogenicity and toxicity of the source microorganism	✓	14	Section 3.6
	D.3 Information on the genetic stability of the source organism	✓	15	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	✓	15	Section 3.8
	F Information related to the dietary exposure to the processing aid		16	Section 4
	F.1. A list of foods or food groups likely to contain the processing aid or its metabolites	✓	16	Section 4.1
	F.2 The levels of residues of the processing aid or its metabolites for each food or food group	✓	16	Section 4.2
	F.3 For foods or food groups not currently listed in the most recent Australian or New Zealand National Nutrition Surveys (NNSs), information on the likely level of consumption	✓	17	Section 4.3
	F.4 The percentage of the food group in which the processing aid is likely to be found or the percentage of the market likely to use the processing aid	✓	17	Section 4.4
	F.5 Information relating to the levels of residues in foods in other countries	✓	17	Section 4.5
	F.6 For foods where consumption has changed in recent years, information on likely current food consumption	✓	17	Section 4.6

2. Technical information

Please refer to Appendix A for further details

2.1. Type of processing aid

The β-Galactosidase enzyme is an enzyme produced by submerged fermentation of *B. subtilis*, carrying the β-Galactosidase gene from *L. delbrueckii bulgaricus*.

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 β-D-galactoside galactohydrolase. Other names used are lactase; β-lactosidase; β-D-lactosidase; lactozym; trilactase; and β-D-galactanase.

- EC number: 3.2.1.23
- CAS number: 9031-11-2

Biological source: The β-Galactosidase enzyme is an enzyme produced by submerged fermentation of *B. subtilis*, carrying the β-Galactosidase gene from *L. delbrueckii bulgaricus*.

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 β-Galactosidase is Bonlacta™.

2.2.3 Molecular and Structural Formula:

β-Galactosidase is a protein. The amino acid sequence is known. Please refer to Appendix E.

2.3. Chemical and physical properties

When added to dairy products the β-Galactosidase hydrolyses lactose to glucose and galactose thereby reducing the lactose content and providing a product more suited to those with an intolerance for lactose in their diet. The depletion of lactose in dairy products is also reducing the total amount of calories in the food product.

Depending on the application, the conversion of lactose with help of β-Galactosidase in dairy processing can result in the following benefits:

- Depletion of lactose for production of low lactose/lactose free dairy products

Substrate specificity:
The function of β-Galactosidase is to catalyse the reaction of Hydrolysis of terminal non-reducing β-D-galactose residues in β-D-galactosides.

Activity:
The activity of the β-Galactosidase is defined in SDLU. The principle of this assay method is that lactase hydrolyses o-nitrophenyl-β-D-galactopyranoside (ONPG) into o-nitophenol (ONP) and

galactose. The reaction is stopped after ten minutes at 30°C with sodium carbonate and the liberated ONP is measured in a spectrophotometer.

β-Galactosidase preparations' enzyme activity will depend on the final product. A detailed assay method is present in Appendix A3.

Temperature optimum:
The optimum temperature range lies between 50 and 60°C. The relative activity reduced significantly at 60°C.

Thermal stability:
β-Galactosidase retained over 80% of activity after 15 seconds of incubation over the temperature range 60 to 75°C. A significant reduction was observed when the temperature is 70°C and above.

pH optimum:
Maximum activity is observed at pH 7.0. Enzyme demonstrated activity in the range from pH 6-7.4.

pH stability:
Optimal stability is seen at the pH interval 3.5-4.5 and the enzyme activity is observed in the pH range 3.5-5.5.

Interaction of the enzyme with different foods:
The β-Galactosidase enzyme preparation will be used as a processing aid where the enzyme is not present or active in the final food or is present in negligible amounts with no technical function in the final food.

Nutritional implication:
β-Galactosidase is a protein and any residual amounts remaining in food consumed would accordingly have the same nutritional value. However, the use levels of β-Galactosidase 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 β-Galactosidase 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 β-Galactosidase 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
Antibiotic activity	Negative by test
Production strain	Negative by test

Physical properties:

Appearance	clear to dark brown liquid

Standard for identity:
β-Galactosidase 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 this enzyme shows a clear conserved LacZ superfamily (Beta-galactosidase/beta-glucuronidase) and Bgal_small_N (beta-galactosidase small chain) domains, characteristic for lactase (= β-Galactosidase, IUBMB EC 3.2.1.23) activities.

This enzyme, the subject of this dossier, is not one of the approved β-Galactosidase enzymes on Schedule 18 of the ANZ Food Standards Code. In our case the enzyme protein originates from *L. delbrueckii subsp. bulgaricus* and is expressed in *B. subtilis*. The identity between the five FSANZ approved β-Galactosidase enzymes *(Aspergillus niger, Aspergillus oryzae, Bacillus circulans ATCC31382, Kluyveromyces marxianus, Kluyveromyces lactis*) ranges from 9.2 - 98.8%. The identity between this β-Galactosidase enzyme and the five FSANZ approved β-Galactosidase enzymes ranges from 9.3 – 27.0%. It is good to realise that the β-Galactosidase

within one species can show strain dependent amino acid sequence variability. Also, microorganism species may contain more than one β-Galactosidase encoding genes with different sequences.

β-Galactosidase enzyme derived from *B. subtilis*, carrying the β-Galactosidase gene from *L. delbrueckii subsp. bulgaricus* has been determined to be GRAS in the United States by expert opinion, and been used in dairy applications in Europe since 2019. There have not been any adverse events reported since β-Galactosidase has been in commercial use in these countries.

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 β-Galactosidase sequence against the complete Uniprot database was performed, with a threshold E-value of 0.1. The majority of matches were beta-galactosidases (lactases), with none of the top 1000 database matches being annotated as either toxin or venom.

Therefore, the β-Galactosidase 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 *B. subtilis* 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. subtilis* DH617 are thus one of the pillars supporting the DuPont N&B *B. subtilis* Safe Strain Lineage. The position of the food enzyme in the DuPont N&B *B. subtilis* Safe Strain Lineage is presented in Appendix B2.

Toxicological testing

To assess the safety of β-Galactosidase, 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, 1000 mg TOS/kg bw/day, equivalent to 752 mg total protein/kg body weight/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 (Refer to Appendix B).

3.3. Allergenicity of the enzyme

Bioinformatic analyses based on sequence homology determined that the *L. delbrueckii bulgaricus* β-Galactosidase 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. Safety assessment reports prepared by international agencies or other national government agencies, if available

As discussed in section 1.8, β-Galactosidase from *L. delbrueckii bulgaricus* expressed in *B. subtilis* has not been reviewed by JECFA; there is no specific Codex Standard relevant to this application. It has, however, been determined to be GRAS by expert opinion in the United States and approved by both France and Denmark for various purposes. Refer Appendix D for safety reports/approval letters. 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 DH 617 is a strain of *B. subtilis* which has been genetically modified by DuPont N&B to express a β-Galactosidase gene from *L. delbrueckii bulgaricus*.

The species *B. subtilis* is an accepted source of enzymes in the literature and pathogenic strains are not described in the Bergey Manual or in the ATCC and other catalogs. The species *B. subtilis* does not appear on the Proposal for a Council Directive amending the "Directive 90/679/EEC on the protection of workers from risks related to exposure to biological agent at work" and is also not present on the European Guideline 93/88/EEG, the list of pathogens from the Dutch Guidelines for Genetically Modified Organisms (COGEM), the German “Berufsgenossenschaft der chemischen Industrie”, or the Belgian “VLAREM II”. B. subtilis isaccepted as a safe host for the construction of Risk Group I GMMs in several countries, like Germany, The Netherlands, and accepted as a host of certified host-vector systems under the NIH Guidelines in the USA.

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

B. subtilis occurs ubiquitously in the environment (soil, water, plants and animals) and as a result can be also found in food. The bacterium has already been used for decades for the production of food enzymes with no known reports of adverse effects to human health or the environment (de Boer and Diderichsen, 1991). For example, alpha-amylase enzyme preparations from *B. subtilis*

have been used commercially since 1929, when they were used in the manufacture of chocolate syrup to reduce its viscosity.

Recently, scientists with the US Food and Drug Administration (FDA) reviewed the safe use of food-processing enzymes from recombinant microorganisms, including *B. subtilis* (Olempska-Beer et al. 2006). An extensive risk assessment of B. subtilis, including its history of commercial use has been published by the US Environmental Protection Agency (1997). It was concluded that B. subtilis strains used for enzyme manufacture are neither pathogenic nor toxigenic to humans. It is, however, prudent to ascertain the safety of the production strain as certain food-borne illness related strains may produce surfactin, a membrane spanning lipopeptide and amylolysin, a heat-stable toxin regarded to be a virulence factor (Apetroaie-Constantin et al., 2009)

3.7. Genetic stability of the source organism

The parental strain of the production strain *B. subtilis* BG125 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 QM6a 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 β-Galactosidase preparation, the subject of this submission, is *B. subtilis* strain DH617. It is derived by recombinant DNA methods from strain BG125. The purpose of this genetic modification is to enhance β-Galactosidase production levels. DH 617, a commercial production strain, developed using techniques to introduce a gene encoding the wild type *L. delbrueckii* subsp. *bulgaricus* lactase gene. The donor organism is *L. delbrueckii* subsp. *bulgaricus*. The β-Galactosidase 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 β-Galactosidase expression site was determined, and no change was observed between samples prior and after fermentation. The results demonstrate that the insertion cassette has been stably maintained through generations during the fermentation process.

Full details of the genetic modifications and stability of the inserted genes are provided in Appendix E1-E3. Note that this information is proprietary and "**Confidential Commercial Information**" status is requested.

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), this β-Galactosidase will be used in:

- 1 Dairy products (excluding butter and fats)

4.2. Levels of residues in food

The proposed application rate of β-Galactosidase in its intended application is listed below.

Application		Raw material (RM)	Maximal recommended use level (mg TOS/L RM)	Example Final food (FF)	Ratio RM/FF	Maximal level in FF (mg TOS/kg food)
Liquid food	Dairy Processing	Milk	1290	Whey drinks	1	**1290**
Solid food	Cheese	Milk	112.91	Cheese	10	**1129.1**
	Dairy Processing	Milk	1290	Yogurt, Yogurt drinks, milk drinks, plain milk, Milk shake, ice cream	1	**1290**

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

In the production of dairy products, the lactose will convert lactose into galactose and glucose. The use of β-Galactosidases to deplete lactose from milk is extensive in the dairy industry as a response to the commonly occurring lactose intolerance. The depletion of lactose in dairy products is also reducing the total amount of calories in the final dairy product. The β-Galactosidases is denatured by heat during the pasteurisation step.

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 β-Galactosidases, 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: 11.29 mg TOS/kg body weight/day. The NOAEL has been determined for β-Galactosidases to be at 752 mg total protein/kg bw/day (equivalent to 1000 mg TOS/kg bw/day). Based on a worst-case scenario of daily food consumption, the NOAEL would offer a 125.5-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. β-Galactosidase is not expected to be used in production of any foods or food groups that are currently not listed in NNSs. If such usage arises, an application would be made to inform FSANZ.

4.4. Percentage of the food group in which the processing aid is likely to be found or the percentage of the market likely to use the processing aid

The enzyme would be used as a processing aid in about:

- 5% of the tonnage of dairy products sold in Australia and New Zealand

4.5. Levels of residues in food in other countries

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

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

Not applicable. Consumption of foods (alcoholic drinks) produced with Alpha-amylase is not expected to have a significant change.

5. References

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