21 March 2019
[75-19]

Supporting document 1

Risk and technical assessment report – Application A1167

Lactase from *Bacillus subtilis* as a PA (Enzyme)

Executive summary

DuPont Australia Pty Ltd submitted an application to Food Standards Australia New Zealand (FSANZ) seeking to permit the use of the enzyme β-galactosidase (EC 3.2.1.23) as a processing aid. The enzyme is derived from a genetically modified strain of *Bacillus subtilis* containing the β-galactosidase gene from *Bifidobacterium bifidum.* It is used in the production of low lactose and lactose free dairy products and the production of galacto-oligosaccharides (GOS).

The food technology assessment concluded that the use of this enzyme is technologically justified and has been demonstrated to be effective in achieving its stated purpose. The enzyme performs its technological purpose during production and manufacture of foods and is therefore appropriately categorised as a processing aid. The enzyme preparation meets international purity specifications.

FSANZ concluded there are no public health or safety concerns for the general population associated with the use of β-galactosidase from *Bacillus subtilis* as a processing aid. The novel β-galactosidase gene was confirmed to be present in the production strain and was inherited over several generations.

A β-galactosidase with an identical amino acid sequence showed no evidence of genotoxicity in a bacterial reverse mutation assay or a chromosomal aberration assay. In a 90-day oral gavage study in rats, the NOAEL was the highest dose tested, 1000 mg/kg bw/day total protein, which is equivalent to 1416.4 mg/kg bw/day TOS. The TMDI is calculated to be 2.25 mg/kg bw/day TOS. From these values, the Margin of Exposure (MoE) is approximately 630.

Bioinformatic data indicated a lack of homology with known toxins or allergens. Batch analyses showed that levels of wheat and soy residues from the fermentation medium were below the limit of detection in the enzyme preparation. The risk of allergic reaction to wheat or soy in the enzyme preparation is considered low.

Based on the reviewed data it is concluded that in the absence of any identifiable hazard an Acceptable Daily Intake (ADI) ‘not specified’ is appropriate. A dietary exposure assessment was therefore not required.

Table of contents

1. Introduction

FSANZ received an application from DuPont Australia Pty Ltd, seeking permission for a new microbial source for the already permitted enzyme, β-galactosidase (EC 3.2.1.23), as a processing aid.

The enzyme designated "CB108 Lactase" in this report, is produced by submerged fermentation of a genetically modified (GM) strain of *Bacillus subtilis* carrying the lactase gene from *Bifidobacterium bifidum* encoding the wild-type truncated lactase enzyme.

If approved, CB108 Lactase will be used in lactose-reducing enzyme preparations for certain dairy foods (e.g. milk, yogurt, cheese) at a level consistent with Good Manufacturing Practice (GMP). When added to dairy foods, CB108 Lactase reduces the levels of lactose and produces galacto-oligosaccharides (GOS), a dietary fibre, *in situ* within those foods. This β-galactosidase will also be used in the production of GOS, which is primarily used in infant formula products.

CB108 Lactase will be used as a processing aid at low levels and is either not present in the final food or present in insignificant quantities having no technical function in the final food. It has been determined GRAS in the US and is approved in Denmark and France. It is in the process of being assessed in Canada.

1.1 Objectives of the assessment

The objectives of this risk assessment were to:

- determine whether the proposed purpose is clearly stated and that the enzyme achieves its technological function in the quantity and form proposed to be used as a food processing aid
- evaluate any potential public health and safety concerns that may arise from the use of CB108 Lactase sourced from a genetically modified strain of *Bacillus subtilis*.

2 Food technology assessment

2.1 Identity

Information regarding the identity of the enzyme provided in the application has been verified using the appropriate internationally accepted reference for enzyme nomenclature, the International Union of Biology and Molecular Biology (IUBMB[1]).

***Accepted name*:**	β-galactosidase
***Systematic name*:**	β-D-galactoside galactohydrolase
EC[2] number:	3.2.1.23
CAS registry number:	9031-11-2
Commercial name:	Saphera

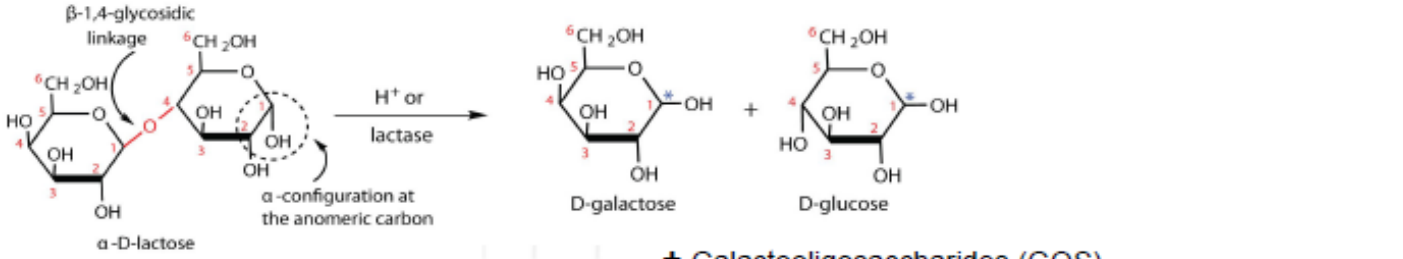
[1] IUBMB enzyme database
[2] EC: Enzyme Commission, internationally recognised number that provides a unique identifier for the enzyme

Other names:	β-galactosidase, Exo-(1->4)-beta-D-galactanase, beta-galactosidase, lactase (ambiguous), beta-lactosidase, maxilact, hydrolact, beta-D-lactosidase, S 2107, lactozyme, trilactase, beta-D-galactanase, oryzatym, sumiklat, Milky Whey.
Host microorganism:	*Bacillus subtilis*
Gene donor microorganism:	*Bifidobacterium bifidus*
Reaction catalysed:	Hydrolysis of terminal non-reducing β-D-galactose residues in β-D-galactosides

2.2 Technological purpose

β-Galactosidase catalyses the hydrolysis of terminal non-reducing β-D-galactose residues in β-D-galactosides. It can use galactosides such as lactose as a substrate. Lactose is hydrolysed into galactose and glucose. GOS are made up of galactose and glucose units.

Figure 1 *Lactase reaction to produce GOS from lactose*



Y (galactosyl acceptor)	Z (product)
Glucose	Lactose
Galactose	Gal-Gal
Lactose	Galactosyl-lactose β-(1,X); (X= 2, 3, 4 or 6)
GOS	$[Gal]_n$-Glc (1=n=6)

2.2.1 Activity

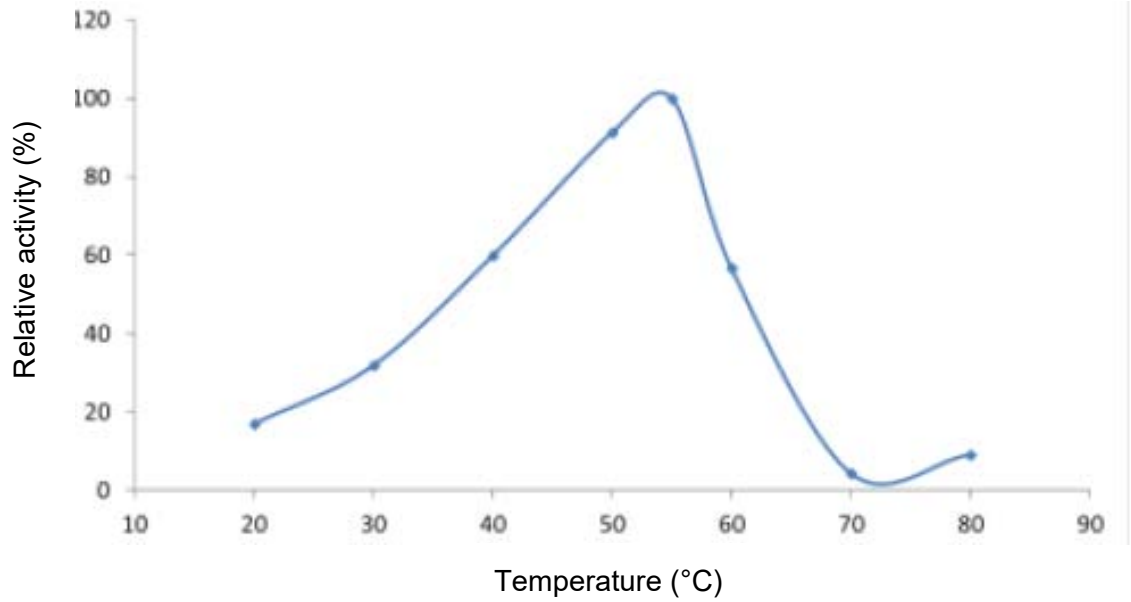
The activity of CB108 Lactase is defined in BLU[3] units/g. The principle of this assay method is that lactase hydrolyses 2-nitrophenyl-β-D-galactopyranoside into 2-nitophenol and galactose. The reaction is terminated after fifteen minutes, after which sodium carbonate and liberated 2-nitophenol are measured by spectrophotometry. The activity of CB108 Lactase is approximately 540-760 BLU/g enzyme. A detailed assay method was provided with the application.

[3] Bifido Lactase unit (BLU), where enzyme activity is defined as that which causes the conversion of 1µmol of substrate conversion per second under defined conditions.

2.2.2 Optimum temperature

The optimum temperature for enzyme activity is 55˚C. Enzyme activity rapidly decreases at temperatures greater than 55°C whilst activity at 70°C is less than 10% of optimum activity.

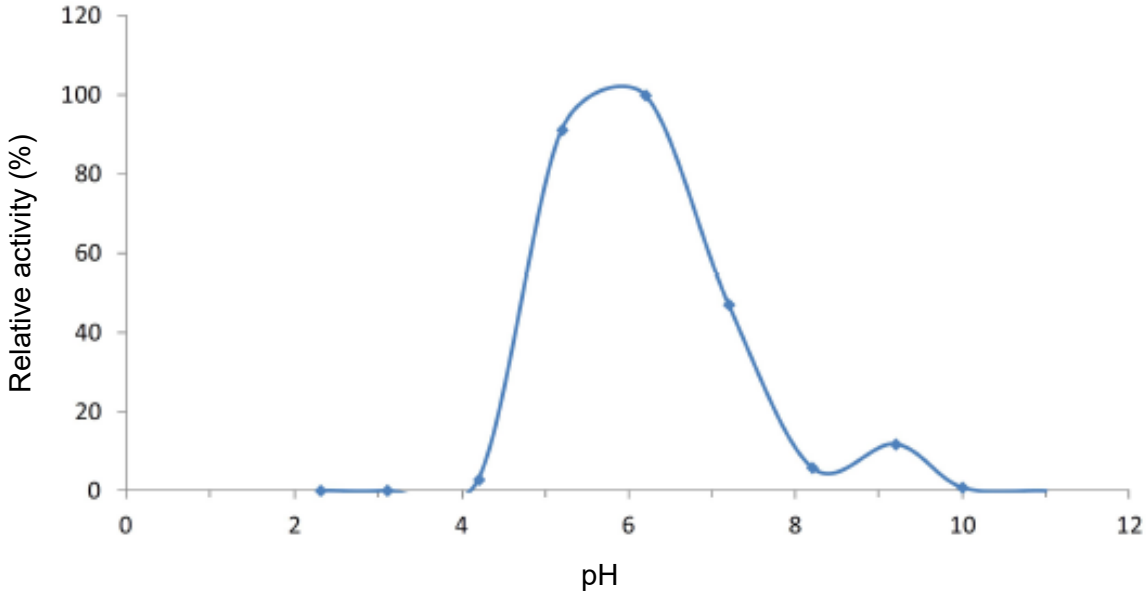
Figure 2 *Optimum temperature for CB108 Lactase activity*



2.2.3 Optimum pH

The optimum pH for enzyme activity is 6.2 and it is active in the range pH 4 - 8.

Figure 3 *Optimum pH for CB108 Lactase activity*



CB108 Lactase enables food manufacturers to produce low lactose/lactose-free, reduced-energy and reduced sugar dairy products. It also allows the production of dairy products

containing GOS and the production of GOS. In the production of low lactose dairy products or GOS (Figures 4 and 5), the enzyme is inactivated during the production process.

Figure 4 *Dairy Processing using CB108 Lactase*

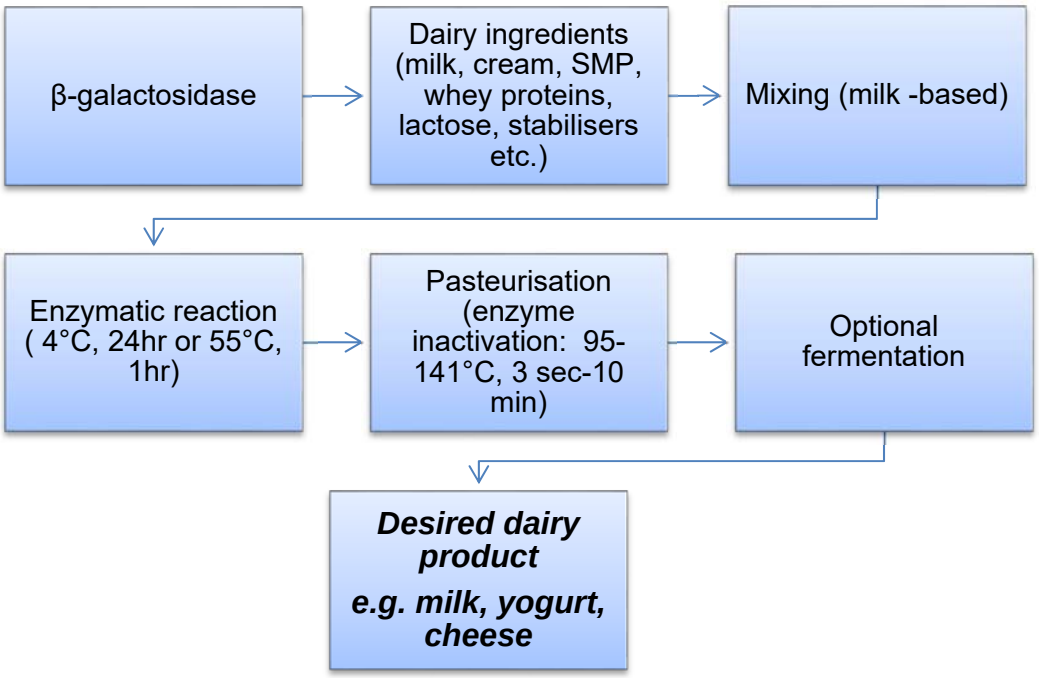
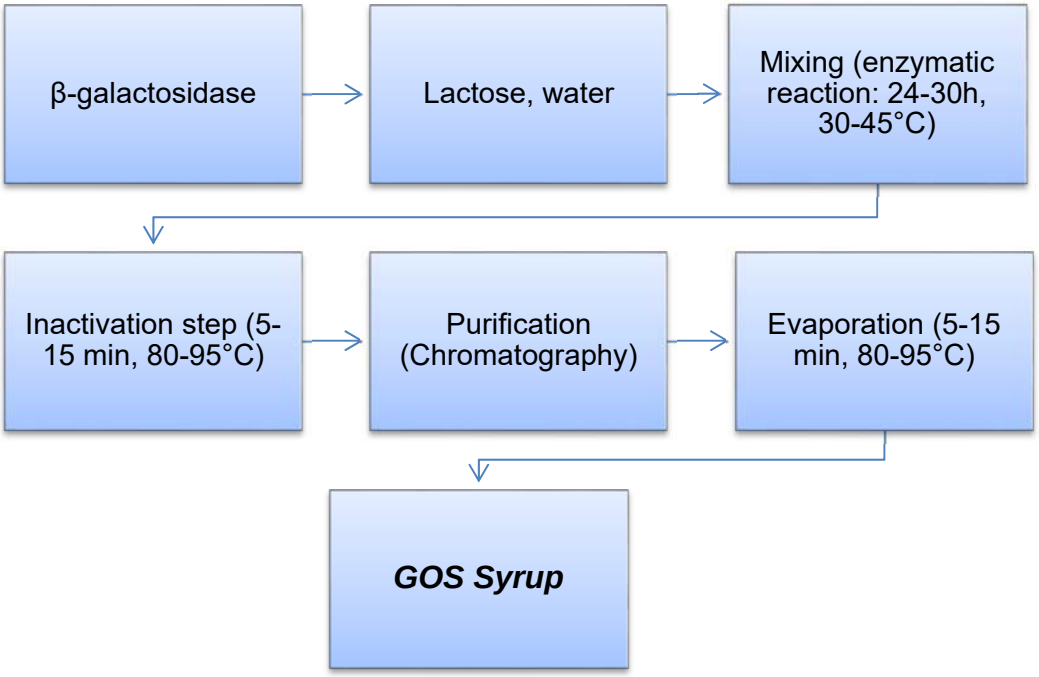


Figure 5 *GOS production using CB108 Lactase*



For both dairy processing and GOS production applications, the enzyme is used in raw milk, whey, or lactose, where, upon heating, the enzyme is denatured and therefore not active in the final food.

2.3 Technological justification

Enzyme preparations are widely used as processing aids in the manufacture of food products, with β-galactosidases used extensively in the dairy industry to deplete lactose in milk.

β-Galactosidases are used primarily for the production of low lactose and lactose free dairy products for consumers with lactose intolerance and for dairy products with reduced sugars and calorie content. They have not however, been widely used for GOS production *in situ* in dairy products mainly because a higher lactose content is needed to generate GOS. Currently no β-galactosidase from *B. subtilis* is permitted as a processing aid in the Code.

CB108 Lactase enables production of lactose reduced dairy products including but not limited to milk, yogurt, cheese, and the production of GOS. CB108 Lactase is also considered a β-galactosidase since it is involved in the hydrolysis of lactose to galactose and glucose. In dairy processing, the enzyme converts lactose into GOS and glucose. CB108 Lactase will be able to generate GOS in raw milk, whey and even low lactose milks, with a lactose content as low as 5%. This provides low lactose or lactose free dairy products with reduced sugars and caloric content in dairy product whilst enabling dairy products to contain GOS.

The enzyme will also be used in the production of GOS which is suitable for addition to infant formula products. Because CB108 Lactase shows a low degree of hydrolysis and a high degree of transgalactosylation that is not dependent on the initial lactose concentration, the GOS yield in dairy products is directly increased in comparison to other β-galactosidases currently used.

Food enzymes are mostly used by manufacturers according to GMP, which limits the amount of substance that is added to food to the lowest possible level necessary to accomplish its desired effect. Food manufacturers adjust the use levels depending on the food application and the enzyme suppliers suggested usage levels. The recommended usage levels are shown according to the proportion of total organic solids (TOS), calculated as follows:
% TOS = 100 - (A + W + D) where:
A = % ash, W = % water and D = % diluents and/or other formulation ingredients.

Table 1 *Recommended use levels for CB108 Lactase*

Application	***Raw material (RM)***	***Recommended use levels (mg TOS/kg RM)***
Dairy processing	Milk, Whey	151-601
GOS production	Lactose	317-932

2.4 Manufacturing process

The manufacturing process for the production of CB108 is a three-part process consisting of fermentation, recovery and formulation of the final commercial enzyme preparation (see Figure 6). These are summarised below with full details provided in the application.

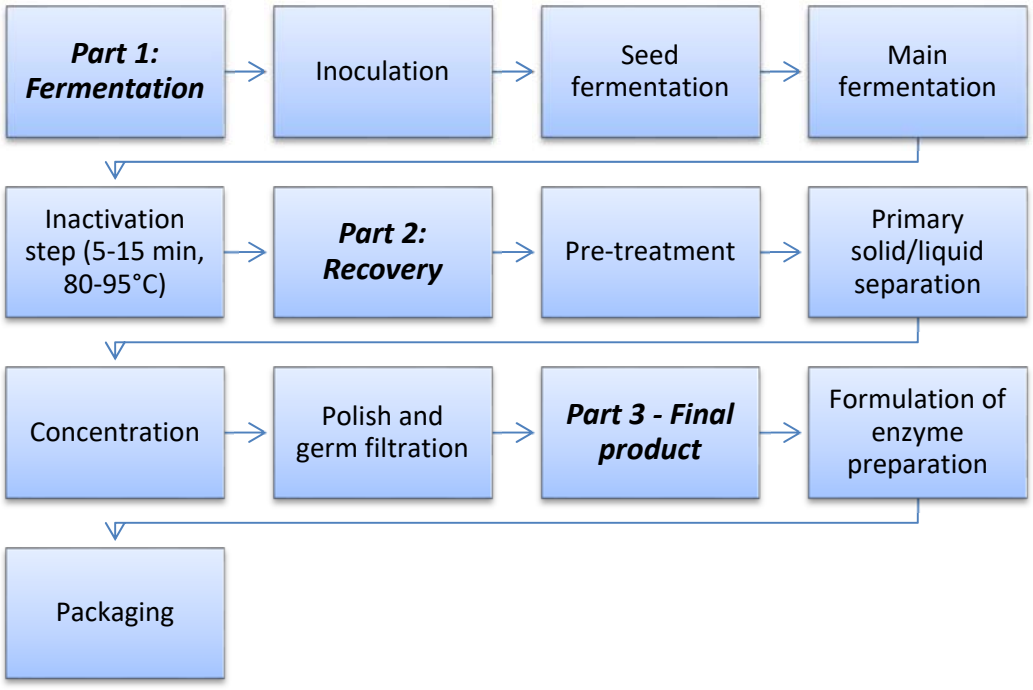


***Figure 6**: Manufacturing process for CB108 Lactase*

2.4.1 Fermentation

CB108 Lactase is manufactured by submerged fermentation of a genetically modified strain of *B. subtilis*, a process commonly used for the production of food-grade enzymes. The fermentation process involves three steps, laboratory propagation (inoculation) of the culture, seed fermentation and main fermentation. At all stages of the fermentation, microbial growth is checked for correct morphological development of the microorganism and for the presence of any contamination.

2.4.2 Recovery

Recovery separates the fermentation broth into biomass and liquid containing the CB108 Lactase enzyme. This is achieved by either filtration, centrifugation, or a combination of both.

The liquid containing the enzyme is then concentrated via ultra-filtration. Diafiltration[4] may follow ultra-filtration and carbon treatment may additionally be used.

The final recovery step is purification of the enzyme by polish filtration using either microfiltration membranes, fine filtration aids such as diatomaceous earth or sterile filtration pads. The ultra-filtered concentrate is then dried and agglomerated.

2.4.3 Formulation

The ultra-filtered concentrate is formulated and analysed in accordance with the general specifications for enzyme preparations used in food processing as established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2006) and the FCC.

[4] Diafiltration is a technique that uses ultrafiltration membranes to completely remove, replace, or lower the concentration of salts or solvents from solutions containing proteins, peptides, nucleic acids, and other biomolecules.

The production process follows standard industry practices and is conducted in accordance with GMP.

2.5 Product specification

There are international specifications for enzyme preparations used in food production. These have been established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA; Food Safety and Quality[5]) and the Food Chemicals Codex (FCC, 2014). Both of these specifications are primary sources listed in Schedule 3 (Identity and Purity), specifically section S3—2. Enzyme preparations must meet these purity specifications.

The production of CB108 Lactase is monitored and controlled by analytical and quality assurance procedures that ensure that the finished preparation complies with the specifications of the Food Chemical Codex, 6th edition (FCC 2008). The applicant provided analysis for three separate β-galactosidase batches confirming the enzyme preparation meets the required international specifications (Table 2).

Table 2: *Specification for impurities and microbial limits compared to the JECFA and Food Chemicals Codex (FCC) enzyme specifications*

Analysis	***Specifications***		
	CB108 Lactase	***JECFA***	***FCC***
Lead (mg/kg)	< 5	≤ 5	≤ 5
Cadmium (mg/kg)	< 0.5	< 0.5	
Arsenic (mg/kg)	< 3	< 3	
Mercury (mg/kg)	< 0.5	< 0.5	
Coliforms (cfu/g)	< 30	≤ 30	≤ 30
E.coli (in 25g)	absent	absent	absent
Salmonella (in 25g)	absent	absent	absent

2.5.1 Physical properties

The CB108 Lactase enzyme preparation is available as a white to brown powder or brown liquid, depending on the application. The carriers used for the enzyme preparation are non-allergenic, consisting of either potato starch or maize based maltodextrin.

2.5.2 Product Stability

Thermally, the CB108 Lactase enzyme is relatively stable for 10 minutes at 50°C, while it is inactivated after 10 minutes of incubation at 70°C.

Upon storage, CB108 Lactase enzyme is stable for 18 months at 4°C or ambient room temperature, without significant loss of activity.

[5] JECFA food safety and scientific advice

2.6 Food Technology Conclusion

CB108 Lactase provides consistency and efficiencies during the processing and manufacturing of dairy foods. It efficiently produces GOS via hydrolysis of lactose to galactose and glucose in raw milk or whey, including those with low lactose levels. CB108 Lactase benefits manufacturers and consumers by providing low lactose and lactose-free dairy foods with reduced sugars and caloric content. FSANZ concluded the use of CB108 Lactase is technologically justified in the form and quantity proposed for use as a processing aid in the manufacture of dairy food and to produce ingredients for infant formula products. It has been demonstrated to be effective in achieving its stated purpose. The enzyme also meets international purity specifications.

3 Safety assessment

3.1 History of use

3.1.1 Host organism

B. subtilis, is widely distributed in the environment by virtue of its natural occurrence in soil and is also detectable in water, air and decaying plant material (US EPA 1997). The bacterium is not pathogenic to humans or toxigenic (de Boer and Diderichsen 1991; US EPA 1997) and has been recommended for a qualified presumption of safety (QPS) by the Scientific Committee of the European Food Safety Authority (EFSA 2007).

FSANZ has previously assessed the safety of *B. subtilis* as the host organism for a number of enzymes used as processing aids. Standard 1.3.3 of the Code permits the use of the following enzymes derived from *B. subtilis*: α–acetolactate decarboxylase, α- and β–amylase, β–glucanase, hemicellulase endo-1,4- β-xylanase, hemicellulase multicomponent enzyme, maltogenic α –amylase, metalloproteinase, pullulanase and serine proteinase.

3.1.2 Gene donor organism(s)

The gene sequence for β-galactosidase was sourced from *B. bifidum*. *B. bifidum* is commonly isolated from human faeces and is generally regarded as non-pathogenic (Biavati and Mattarelli 2012).

3.2 Characterisation of the genetic modification(s)

Full details of the genetic modification to the production organism were provided to FSANZ for assessment and cannot be disclosed as some of the material is confidential commercial information. This information covered detailed information for the construction of the gene expression system, transformation, presence and expression of the novel gene in the production strain and genetic stability.

3.2.1 Description of DNA to be introduced and method of transformation

The gene for β-galactosidase was introduced into an expression plasmid and transformed into the host using standard methodologies. The expression plasmid contains appropriate regulatory elements to facilitate transcription and translation of the enzyme in the production strain. The plasmid also contains a metabolic selection marker.

3.2.2 Characterisation of inserted DNA

The presence of the plasmid was confirmed using plasmid copy number analysis and polymerase chain reaction (PCR) to detect the presence of the inserted enzyme gene.

3.2.3 Genetic stability of the inserted gene

A genotypic analysis was performed using PCR. The data showed no change in plasmid copy number over 60 generations. This data indicates the plasmid DNA was maintained across the generations, and no change in flanking DNA sequence was observed.

3.3 Safety of CB108 lactase

3.3.1 History of safe use of the enzyme

CB108 Lactase has been on the market in Europe since 2017.

The US FDA advised in November 2015 that they had no questions on a GRAS notification (GRN 579) for a lactase with an identical amino acid sequence, produced by a different but closely related strain of *Bacillus subtilis*, JL47, genetically modified to express lactase from *Bifidobacterium bifidum*. The GRAS notification was for use in the production of galacto-oligosaccharide for infant formula and in the production of fresh dairy products, which are the same proposed uses as for CB108 Lactase.

No adverse events associated with the use of CB108 Lactase have been reported in any of these markets.

The applicant claims that the amino acid sequence of the mature CB108 Lactase is identical to a large part of the sequence of a β-galactosidase approved by FSANZ (A1135), which is produced in a strain of *Bacillus licheniformis* genetically modified to express β-galactosidase from *Bifidobacterium bifidum*. However, FSANZ note that the CB108 Lactase is significantly truncated compared to this previously assessed enzyme in A1135. Both enzymes are significantly truncated compared to the native protein.

3.3.2 Toxicology studies in animals

No toxicology studies have been performed using CB108 lactase as the test article. Safety of CB108 lactase may be inferred from rat studies performed using a lactase with an identical amino acid sequence, identified as lactase BIF917. The lactase is produced by a different but closely related strain of *Bacillus subtilis*, JL47, genetically modified to express lactase from *Bifidobacterium bifidum.*

Acute oral toxicity study in rats (DuPont 2014) Regulatory status: GLP

The test article for this study was an aqueous solution of lactase enzyme with a protein concentration of 133.13 mg/mL and consisting of 17.83% Total Organic Solids (TOS). The enzyme was obtained from *Bacillus subtilis* JL47. The test subjects were female Crl:CD(SD) rats, approximately 10 weeks old on the day of dosing. Rats were housed individually in solid-bottom cages under standard laboratory environmental conditions. Water was provided *ad libitum.* Certified rodent diet was provided *ad libitum* except in the period 17 to 17.75 hours prior to dosing, when rats were fasted, and for 3 to 3.5 hours after dose administration. The design of the study was an up-and-down procedure according to OECD guideline 425. The first rat was dosed by oral gavage with 5000 mg/kg bw enzyme solution. The rat was observed for clinical signs prior to dosing, within 30 minutes after dosing, twice more on the

day of dosing and daily thereafter. The bodyweight of the rat was recorded on Days -1, 1, 8 and 15. No abnormal clinical signs or effects on bodyweight were observed in the first rat within 48 hours after dosing, and therefore no reduction in dosage was indicated. Accordingly, two more rats were gavaged with at the same dosage of lactase. These rats were subject to the same observations, and determinations of bodyweight, as the first rat. No clinical abnormalities were observed in any rat and all rats gained bodyweight normally. On Day 15 following dosing, rats were anaesthetized, killed and gross necropsies were performed. No gross abnormalities were found in any rat. It was concluded that the acute oral LD50 for the enzyme is >5000 mg/kg bw, or 5 g/kg bw, in female rats.

90-day oral gavage toxicity study in rats (DuPont 2014) Regulatory status: GLP; compliant with OECD Section 4 (Part 408) Repeated Dose 90-day Oral Toxicity Study in Rodents.

The test article for this study was the same as that used in the acute oral toxicity study described above; that is, an aqueous solution of lactase enzyme from Bacillus subtilis JL47 with a protein concentration of 133.13 mg/mL and consisting of 17.83% Total Organic Solids (TOS). The test article was referred to as H-30869. Test subjects were male and female Crl:CD(SD) rats. Rats were pair-housed by sex in solid-bottomed cages under standard laboratory environmental conditions. Water was provided *ad libitum*, and certified rodent diet was provided *ad libitum* except during scheduled fasting periods. Rats were acclimatised for 7 days prior to study start, during which period they were subject to clinical observation, measurement of bodyweight gain, and given prestudy ophthalmic examinations and neurobehavioral evaluations. Rats were randomly assigned to study groups (10/sex/group) and gavaged daily, at a dose volume of 10 mL/kg bw, with 0, 100, 300 or 1000 mg/kg bw/day total protein. The control article and vehicle was deionised water. Samples of dose formulations collected during weeks 1, 6 and 12 of the study were analysed by the sponsor and confirmed to be within 10% of intended dose concentrations and suitable for use on the study.

During the in-life phase of the study, rats were observed for mortality/moribundity twice daily, for general clinical observations once daily, and detailed clinical observations on Day 1 and weekly thereafter. Bodyweights were recorded on Day 1 and weekly thereafter, and food consumption was measured weekly. Ophthalmologic examinations were conducted on study day 90, and neurobehavioral evaluation was conducted on days 88 or 89. Neurobehavioural evaluation included functional observational battery (FOB) assessments and motor activity assessments. The FOB included righting reflex, approach and touch response, auditory response, tail pinch response, forelimb grip strength, and hindlimb grip strength. Motor activity was measured by interruption of photobeams in an automated activity monitor placed in a darkened room. At the end of motor activity assessment, rats were examined for pupillary constriction and the activity monitor was examined for evidence of polyuria or diarrhoea. Rats were fasted overnight in metabolism cages prior to scheduled termination, and urine was collected for analysis. On the day of scheduled termination rats were anaesthetised with isoflurane and blood was collected for haematology, coagulation parameters and clinical chemistry. Animals were then killed and subject to detailed necropsy and collection of a comprehensive range of tissues. Fresh weights were recorded at necropsy for liver, kidneys, heart, spleen, thymus, adrenal glands, brain, ovaries and uterus of females, and testes, epididymides, prostate and seminal vesicles of males. All collected tissues were preserved for histopathology.

Treatment with lactase had no effect on survival. Three rats died prior to scheduled kill, but their deaths were not related to the test article. One male in the 300 mg/kg bw/day group was found dead on Day 85, although the cause of death could not be determined on gross necropsy. Two males, one in the control group and one in the 300 mg/kg bw/day group, died as a result of blood collection on the day of scheduled kill. There were no treatment-related

effects on bodyweight, bodyweight gain, food consumption, food conversion efficiency, clinical observations, ophthalmological findings, or findings on either FOB or motor activity evaluations. There were no treatment-related effects on group mean haematology values, or group mean urinalysis values. The only change in a group mean value among those for coagulation parameters was a minimal (2.3%) increase in prothrombin time in 1000 mg/kg bw/day females when compared to female controls. There was no corresponding change in the group mean value for males treated with 1000 mg/kg bw/day. The minimal increase was not considered to be treatment-related. There were no changes in group mean values for clinical chemistry parameters that were considered to be adverse. Group mean aspartate aminotransferase (AST) was 23% higher in 1000 mg/kg bw/day males, and 5% higher in 1000 mg/kg bw/day females than in their sex-matched controls. However there were no corresponding changes in alanine aminotransferase (ALT) or sorbitol dehydrogenase (SDH), and no histopathological correlates. The elevations in AST were therefore considered to be spurious findings. Group mean SDH was 34% lower in 1000 mg/kg bw/day males than in male controls but remained within historical range, and decreased SDH is not considered to be an adverse finding. Group mean alkaline phosphatase (ALP) values were minimally higher in 1000 mg/kg bw/day males and females (34% and 72% increase respectively) compared to sex-matched controls, but there were no corresponding changes in total bile acids or bilirubin, or microscopic changes in the liver. The increases in ALP were therefore not considered adverse. Group mean total bile acid values were lower in treated males than in control males, although there was no dose-response relationship and no similar effect in females. The findings were attributed to high values for total bile acid in two control males. A decrease in serum bile acid is not considered to be an adverse effect. Changes in organ weight values were limited to an increase in group mean kidney weight, relative to bodyweight, in 1000 mg/kg bw/day females.

No test article-related gross lesions were observed in either sex during necropsy. There was no statistically significant increase in group mean values for absolute kidney weight or kidney:brain weight ratio, and no corresponding microscopic findings in the kidneys, and the change was therefore considered to be incidental and unrelated to treatment. Nasal lesions consistent with gavage associated oesophageal reflux were observed in a number of rats, and were considered to be due to the gavage procedure rather than the test article. No other treatment-related lesions were discovered on histopathological examination.

It was concluded that the No Observed Adverse Effect Level for the lactase used in this study was 1000 mg/kg bw/day total protein, which is equivalent to 1416.4 mg/kg bw/day TOS.

3.3.3 Genotoxicity assays

As for toxicology studies in animals, safety of CB108 Lactase may be inferred from rat studies performed using a lactase with an identical amino acid sequence, identified as lactase BIF917.

Bacterial reverse mutation assay (Bioreliance 2014) Regulatory status: GLP; compliant with OECD Section 4 (Part 471) and with Redbook 2000.

The test systems for this assay were *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* WP2 uvrA. The test article, an aqueous solution of lactase enzyme with a protein concentration of 133.13 mg/mL, was diluted as required with the vehicle/negative control article, distilled water. Positive controls for assays without metabolic activation by addition of S9 mix were 2-nitrofluorene for TA98, Acridine Mutagen ICR-191 (6-Chloro-9-[3-(2-chloroethylamino)propylamino]-2-methoxyacridine dihydrochloride) for TA1537, and N-methyl-N-nitro-N-nitrosoguanidine for TA100, TA1535 and WP2 uvrA. For

assays with metabolic activation, the positive control article was 2-aminoanthracene for all bacterial strains.

The first experiment conducted was a dose rangefinder. The dose levels tested were 1.5, 5.0, 15, 50, 150, 500, 1500 and 5000 µg/plate. Each test was conducted in duplicate. Most tests were conducted by the treat and plate method because it has a rinsing step to remove the test article, which potentially contains histidine which could cause false positive results, from the bacteria prior to plating. The plate incorporation method was used only for the positive control with *E. coli* in the presence of S9 activation. For the dose rangefinder by the treat and plate method, S9 mix or sham mix, tester strain, vehicle, and test article or positive control article were added to a tube pre-heated to 37±2°C. The contents of the tube were mixed by vortex and incubated for 1 h at 37°C with shaking. The contents of the tube were then centrifuged and the supernatant was removed. The tester strain was resuspended in 0.9% saline and an aliquot was added to molten selective top agar at 45°C. This mixture was overlaid onto the surface of minimal bottom agar. After the overlay had solidified, the plates were inverted and incubated for 48 to 72 hours at 37°C. Revertant bacterial colonies were counted. No positive mutagenic responses were observed with any of the tester strains with or without S9 activation, and no precipitate was observed.

Based on the findings of the initial toxicity-mutation assay, the doses of lactase used in the confirmatory mutagenicity assay were 15, 50, 150, 500, 1500 and 5000 µg/plate. The confirmatory mutagenicity assay was conducted in triplicate according to the same method as that used for the dose rangefinder. No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation. The dose levels tested were 15, 50, 150, 500, 1500 and 5000 µg per plate. Neither precipitate nor toxicity was observed.

It was concluded that under the conditions of this study, the test article did not cause a positive mutagenic response with any of the tester strains in either the presence or absence of metabolic activation.

In Vitro *Mammalian Chromosome Aberration Test in Human Peripheral Blood Lymphocytes. GLP; OECD Section 4 (Part 473)*

The test system for both the preliminary study and the definitive study comprised peripheral blood lymphocytes from a healthy non-smoking woman in her twenties. The donor had no recent history of radiotherapy, viral infection or the administration of drugs. The vehicle/negative control article was sterile water. Positive control articles were mitomycin C in the absence of metabolic activation and cyclophosphamide in the presence of metabolic activation by S9 mix. Prior to the start of each study, peripheral blood lymphocytes were prepared by being cultured in complete medium with 1% phytohemagglutinin at 37°C in a humidified atmosphere of 5 % CO2 in air for 44-48 hours.

Dose levels of lactase from the preliminary study were 1.5, 5.0, 15, 50, 150, 500, 1500 and 5000 µg/mL. Cells were exposed to the test article for 4 and 20 hours in the non-activated test system and for 4 hours in the S9-activated test system. All cells were harvested 20 hours after treatment initiation. Visible precipitate was observed in treatment medium at 5000 µg/mL at the start and the conclusion of the preliminary study, and haemolysis was observed at the same dose level at the conclusion of the study in the S9-activated 4-hour and the non-activated 20-hour treatment groups.

As a result of the findings in the preliminary study, dose levels in the definitive study were 100, 200, 550, 700, 850, 1000, 1250, 1500 µg/mL for the non-activated 4-hour treatment group; 25, 50, 100, 200, 450, 500, 550 µg/mL for the S9-activated 4-hour treatment group;

and 10, 25, 50, 75, 100, 125, 150, 200 µg/mL for the non-activated 20-hour treatment group. All assays were conducted in duplicate.

In the non-activated 4-hour exposure group, at 850 µg/mL, which was the highest dose level evaluated microscopically, mitotic inhibition was 58%, relative to the vehicle control. The dose levels selected for analysis of chromosome aberrations were 200, 550, and 850 µg/mL. The percentage of cells with structural or numerical aberrations in the test article-treated group was not significantly increased relative to vehicle control at any dose level.

For the S9-activated 4-hour exposure group, at 500 µg/mL, the highest test dose level evaluated microscopically, mitotic inhibition was 51%, relative to the vehicle control. The dose levels selected for analysis of chromosome aberrations were 100, 200, and 500 µg/mL. The percentage of cells with structural or numerical aberrations in the test article-treated group was not significantly increased relative to vehicle control at any dose level.

For the non-activated 20-hour exposure group, at 100 µg/mL, the highest test dose level evaluated microscopically, mitotic inhibition was 53%, relative to the vehicle control. The dose levels selected for analysis of chromosome aberrations were 25, 50, and 100 µg/mL. The percentage of cells with structural or numerical aberrations in the test article-treated group was not significantly increased relative to vehicle control at any dose level.

For all three exposure groups, the positive control article induced a significant increase in chromosomal aberrations, confirming the validity of the assays. It was concluded that under the conditions of the assay, lactase did not induce structural or numerical chromosome aberrations.

3.3.4 Bioinformatics concerning potential for toxicity

A BLAST search for homology of the lactase sequence was performed against the complete Uniprot database, with a threshold E-value of 0.1. The majority of matches were β-galactosidases. None of the closest 1000 database matches was annotated as either a toxin or a venom.

A further BLAST search for homology of the mature lactase sequence was performed against the Uniprot animal toxin databases. There were no matches, indicating that the lactase sequence does not share homology with any known protein toxin or venom.

3.3.5 Potential for allergenicity

A full-length sequence alignment search was conducted against known allergens in the Food Allergy Research and Resource Program Allergen Online database (Version 18A), with the E-value set to <0.1. No matches were found.

An 80 amino acid sliding window search was also conducted against the same database, with the homology set to >35%. There was one match, a pollen sequence from the sycamore, *Platanus orientalis*. However the E-value of 1.2×10^3 indicated that this match was not significant.

A case report of allergy to oral lactase was located, that of Viosin and Borici-Mazi (2016). A woman with self-diagnosed adult-onset lactose intolerance developed bilateral orbital swelling, throat constriction and shortness of breath after consuming a tablet of lactase originating from *Aspergillus niger*. A skin test confirmed the allergy. Viosin and Borici-Mazi cited an earlier case of a patient developing an allergic reaction after consuming a tablet of lactase originating from *Aspergillus oryzae* (Binkley, 1996; as cited by Viosin and Borici-Mazi

2016) as well as cases of pharmaceutical workers developing lactase sensitisation after exposure to lactase powder by inhalation or dermal exposure. It is apparent that allergy to lactase following oral exposure is very rare, and case reports are limited to consumption of lactase supplements, which may be expected to represent much higher exposure than would occur due to the use of this enzyme. The extent of homology between the lactases that caused allergic reactions and this lactase is unknown.

Nitrogen sources used in the fermentation medium during manufacture of the enzyme may include soy protein, and glucose and sorbitol products derived from wheat. Both soy and wheat are considered to be major food allergens.

The applicant has commissioned analysis of batches of the enzyme for the presence of soy proteins. All samples were below the limit of detection, <2.5 ppm, of a soy protein ELISA test. Assuming a worst case of 2.5% soy protein in the enzyme, at the recommended use rate of 0.1% enzyme product in food manufacture the greatest possible amount of soy protein in the final food would be 2 -3 ppb. EFSA (2014) noted that Minimal Eliciting Doses (MEDs) and Minimal Observed Eliciting Doses (MOEDs) for soy were in the range 4 to 88 mg, and one study recorded a NOAEL of 2 mg. The risk of allergic reaction to soy protein through use of this enzyme is therefore considered to be low.

The applicant has also measured the wheat protein persisting in sorbitol or glucose used in fermentation. Using the Thermo Scientific Coomassie Plus assay, a colorimetric method, all samples contained less than the LOD of 3 ppm, with the exception of one glucose sample that contained 10 ppm. This value was therefore used to construct a worst-case exposure. Glucose syrup is diluted approximately 50% in the fermentation mix, and therefore the fermentation mix would contain at most 5 ppm wheat protein, which would result in 5 ppb protein in the final food processed with an enzyme product at 0.1%. EFSA (2014) noted that the lowest MED/MOED recorded for wheat allergy is 2.6 mg wheat protein. The risk of allergic reaction from residual wheat protein in the enzyme is therefore considered to be low.

3.3.6 Approvals by other regulatory agencies

CB108 Lactase is included on a positive list in France, and the applicant received a letter of approval from Denmark in 2015.

In November 2015, the US FDA responded with no questions to a GRAS notification (GRN 579) for Lactase BIF917, which as previously stated has an identical amino acid sequence to CB108 lactase and which has the same the same IUBMB number 3.2.1.23, for use in the production of galacto-oligosaccharide for infant formula and in the production of fresh dairy products.

4 Conclusion

There are no public health or safety concerns for the general population associated with the use of β-galactosidase from *Bacillus subtilis* as a food processing aid.

The modification involving the insertion of the lactase gene has been shown to be stably inherited.

A lactase with an identical amino acid sequence showed no evidence of genotoxicity in a bacterial reverse mutation assay or a chromosomal aberration assay. In a 90-day oral gavage study in rats, the NOAEL was the highest dose tested, 1000 mg/kg bw/day total protein, which is equivalent to 1416.4 mg/kg bw/day TOS. The TMDI is calculated to be 2.25

mg/kg bw/day TOS. From these values, the Margin of Exposure (MoE) is approximately 630.

Bioinformatic data indicated a lack of homology with known toxins or allergens. Batch analyses showed that levels wheat and soy residues from the fermentation medium were below the limit of detection in the enzyme preparation. The risk of allergic reaction to wheat or soy in the enzyme preparation is considered low.

Based on the reviewed data it is concluded that in the absence of any identifiable hazard an Acceptable Daily Intake (ADI) 'not specified' is appropriate. A dietary exposure assessment was therefore not required.

5 References

Biavati, B, Mattarelli, P (2012): Genus I. Bifidobacterium. In Goodfellow et al. (Eds.): Bergey's Manual® of Systematic Bacteriology. Volume 5 The Actinobacteria, Part A. 2nd edition. New York, NY: Springer New York, pp. 171–206

de Boer A, Diderichsen B (1991) On the safety of *Bacillus subtilis* and *B. amyloliquefaciens*: a review. Applied Microbiology and Biotechnology 36(1):1–4

EFSA (2007) Introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA. Opinion of the Scientific Committee (Question No EFSA-Q-2005-293. The EFSA Journal 587:1–16

EFSA (2014) Scientific opinion on the evaluation of allergenic foods and food ingredients for labelling purposes. EFSA Journal 2014 doi:10.2903/j.efsa.2014.NNNN

Food Chemicals Codex 6th Edition (2018), The United States Pharmacopeia, United States Pharmacopeial Convention, Rockville, MD.

Food Chemicals Codex 9th Edition (2014), The United States Pharmacopeia, United States Pharmacopeial Convention, Rockville, MD.

JECFA (2006) General specifications and considerations for enzyme preparations used in food processing.

Taylor et al. The Allergen Bureau of Australia and New Zealand, and Houben G (2014). Establishment of Reference Doses for residues of allergenic foods: Report of the VITAL Expert Panel. Food Chem Toxicol 63: 9-17

US EPA (1997) Final risk assessment of *Bacillus subtilis*. Accessed 29 October 2018.

Voisin MR and Borici-Mazi R (2016). Anaphylaxis to supplemental oral lactase enzyme. Allergy Asthma Clin Immunol (2016) 12:66 DOI 10.1186/s13223-016-0171-8