FOOD STANDARDS
Australia New Zealand
Te Mana Kounga Kai – Ahitereiria me Aotearoa

**Supporting Document 1**

# RISK AND TECHNICAL ASSESSMENT REPORT

# BETA-GALACTOSIDASE DERIVED FROM *BACILLUS CIRCULANS* AS A PROCESSING AID

## RISK ASSESSMENT SUMMARY

Application A1032 seeks approval for the use of β-galactosidase derived from *Bacillus circulans* as a processing aid.

The *Australia New Zealand Food Standards Code* (the Code) currently permits the use of a number of microbial enzymes as processing aids in the manufacture of food. Four micro-organisms are currently listed the Code as permitted sources of the enzyme, β-galactosidase (EC. 3.2.1.23).

The risk assessment has considered the micro-organism identity, the safety of *B. circulans* and the enzyme preparation of β-galactosidase, and its technological suitability. The enzyme has been demonstrated to perform the specified reactions with lactose under the described process and manufacturing conditions. Based on the available data, it was concluded that the submitted studies did not reveal any hazard-related concerns with the enzyme or source micro-organism that would preclude the listing of β-galactosidase derived from *B. circulans* as a food processing aid.

## UNCERTAINTIES IN THE RISK ASSESSMENT

Uncertainties surrounding the definitive taxonomic identification of the source micro-organism (see Section 3.2) and potential allergenicity of the enzyme (see Sections 4.2 & 5.4.4) were identified. However, as noted in this report additional information and scientific discourse was able to ameliorate these safety concerns.

## CONCLUSIONS OF THE RISK ASSESSMENT

- There were no safety concerns identified for the enzyme preparation, the enzyme itself or the source micro-organism.
- As no hazards were identified for β-galactosidase derived from *B. circulans* ATCC 31382, or the micro-organism itself, no health standard was considered necessary.
- The Acceptable Daily Intake (ADI) for β-galactosidase from *B. circulans* ATCC 31382 is 'not specified'.
- The precise taxonomic identity of the source micro-organism is uncertain. Consequently it is considered that the most informative name would be *B. circulans* ATCC 31382.
- The properties of the enzyme β-galactosidase from the micro-organism *B. circulans* ATCC 31382 comply with the general enzyme specifications published by JECFA.

- The enzyme β-galactosidase performs its specified technological functions under the process and manufacturing conditions.
- There is no detectable soybean protein in the enzyme preparation and no enzyme activity or soybean protein in the GOS preparation

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# 1 INTRODUCTION

Application A1032 submitted by Friesland Foods BV (future legal entity 'FrieslandCampina Domo') seeks approval for the use of the enzyme β-galactosidase (lactase) derived from *Bacillus circulans* as a processing aid.

The systematic name for this enzyme is β-D-galactoside galactohydrolase (EC 3.2.1.23, CAS No. 9031-11-2). This enzyme hydrolyses the break down as well as formation of, β-galactosidic bonds in sugars such as lactose. Under conditions of high lactose concentrations the enzyme will use lactose as an alternative acceptor to water resulting in the formation of galacto-oligosaccharides (GOS). The enzyme may perform both hydrolysis and polymerisation with the equilibrium dependent on both the type of enzyme and the reaction conditions. For the preparationion of β-galactosidase, *B. circulans* is cultivated in a fermentation process and the microbial extracellular enzyme is isolated and purified. The commercial enzyme preparation consists of two β-galactosidase isoforms that have high hydrolysis specificity to β-1,4 linkages. The enzyme preparation has been observed to have no other enzymatic activities.

No residual enzyme activity remains in the finished GOS preparation as the enzyme is inactivated by acid and heat reactions and then removed by a filtration step. The enzyme is intended to be used in the manufacture of galacto-oligosaccharides (GOS) from lactose. GOS contain two to five molecules of galactose and one glucose molecule connected through glycosidic bonds. The tested β-galactosidase preparation had a minimum enzyme activity of 5000 lactose LU/g.

The enzyme is currently listed in the *Australia New Zealand Food Standards Code* (the Code) in the Table to Clause 17 (Permitted enzymes of microbial origin) of Standard 1.3.3 (Processing Aids) as derived from the moulds *Aspergillus niger* and *Aspergillus oryzae*, and the yeast *Kluyveromyces lactis* and *Kluyveromyces marxianus*. *B. circulans* is not currently listed in the Code.

The Applicant has noted that the *B. circulans* strain (ATCC 31382) is a naturally occurring, non-GMO and non-pathogenic strain, and has a history of use in the preparation of commercial enzymes for use in the food industry. While there was no information from *The International Programme on Chemical Safety* (IPCS) for CAS No. 9031-11-2, the Scientific Committee for Food (SCF, 2007) has reported on the safety of *B. circulans* as an enzyme source.

## Objectives of the assessment

In proposing to amend the Code to include β-galactosidase derived from *B. circulans* as a processing aid, the objectives of this risk assessment were to:

- Assess the risk to public health and safety;
- Assess the technical function of the enzyme.

## 1.2 Terminology

In this assessment report, the enzyme has been referred to as 'β-galactosidase' although various other names for the enzyme have been used in the common and scientific literature. The Code currently lists the enzyme (EC 3.2.1.23) as β-galactosidase. The activity of this enzyme has been measured in lactase units (LU)[1] as lactase is another commonly used name for this enzyme.

## 1.3 Chemistry

| | |
|---|---|
| Systematic name: | β-D-galactoside galactohydrolase |
| IUBMB Enzyme Nomenclature: | EC 3.2.1.23 |
| C.A.S number: | 9031-11-2 |
| Common name: | β-galactosidase |
| Other names: | lactase, β-gal, β-lactosidase, maxilact, hydrolact, β-D-lactosidase, S 2107, lactozyme, trilactase, β-L-galactanase, oryzatym and sumiklat. |

**β-galactosidase** is a hydrolase enzyme that catalyses the hydrolysis of β-galactosides (e.g. lactose) into monosaccharides (e.g. galactose and glucose), where the glucosidic group on the non-reducing β-D-galactose residue is replaced by a hydroxyl group (an acceptor group).

β-galactosidase is also known to catalyse the transglycosylation of sugars, that is, when a sugar moiety is an acceptor instead of the water molecule. This then leads to the synthesis of new oligosaccharides (Yamamoto *et al*., 2004). Some of the enzymes have high specificity to synthesize oligosaccharides of specific chain length and orientation of the linkage. For example, β-galactosidase sourced from *B. circulans* shows high hydrolysis specificity to β-1,4 linkages and in turn yields β-1,4 linked galactosyl oligosaccharides (GOS) by transglycosylation, while β-galactosidase sourced from *Aspergillus oryzae gives* β-1,6 GOS. Figure 1 illustrates the preparationion of β-1,4 linked GOS specifically produced by *B. circulans a*nd β-1,6 GOS by other microbial enzymes.

*B. circulans* ATCC 31382 was found to produce three β-galactosidase isoforms, galactosidase-I, β-galactosidase-II and galactosidase-III. However, only two β-galactosidases, galactosidase-I and β-galactosidase-II were purified from the commercial enzyme preparation, Biolacta®N5 (Daiwa Kasei, Japan). They showed a similar isoelectric point (pI) of 4.5 and the same optimum pH of 6.0, but they differ in substrate specificity and galactosyl transferring activity, with β-galactosidase-II having higher transferring activity rate than galactosidase-I (Ito & Sasaki, 1997). These two enzymes specifically hydrolyse β-1,4 galactoside bonds but not β-1,3 bonds.

[1] One lactose unit (LU) was defined as the quantity of enzyme that liberates 1 µmole of glucose per minute at the early stage of the reaction at 40°C, pH 6.0. When lactose is hydrolysed by lactase, it is converted into glucose and galactose. The lactase activity is determined by measuring the amount of liberated glucose.

β-galactosidase-III from *B. circulans* was not found in the Biolacta® enzyme preparation. It is a much smaller enzyme and it catalyses β1-3 galactoside bonds. It was assumed that either the culture condition was not suitable for the preparationion of β-galactosidase-III under the fermentation conditions of Biolacta® or the enzyme might be removed during the ultrafiltration step of Biolacta® preparationion.

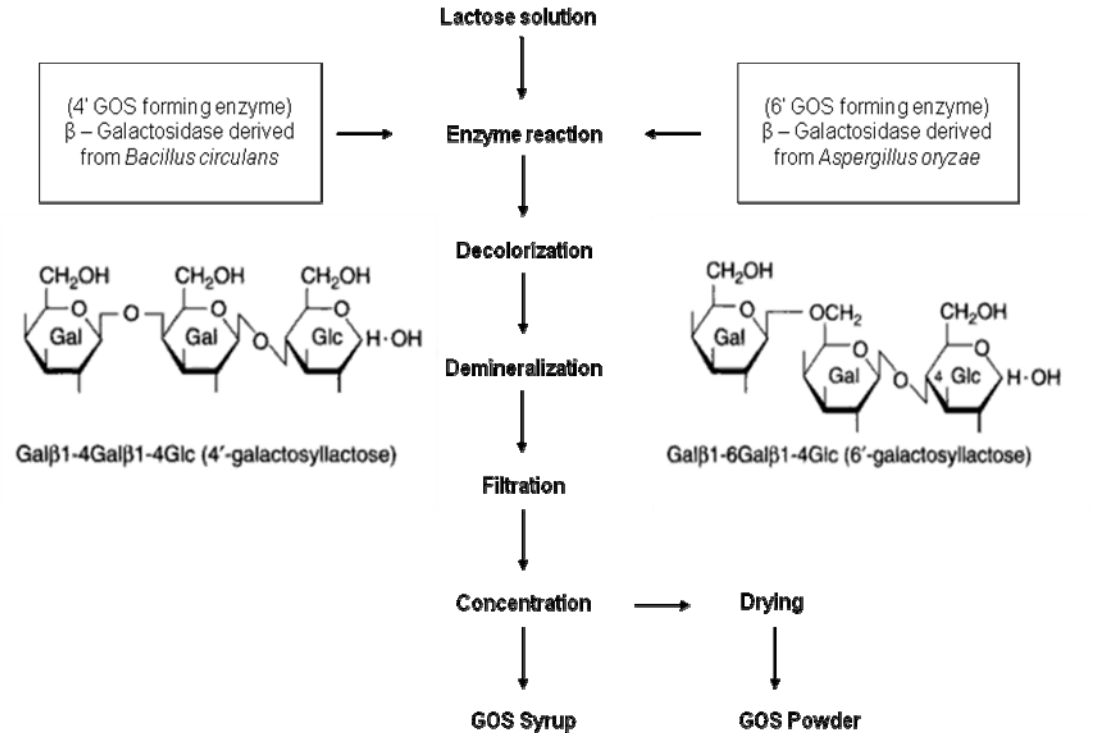


Figure 1: Industrial preparationion process for GOS (modified from Taniguchi, 2005).

## 2 KEY RISK ASSESSMENT QUESTIONS

To meet the objectives of this risk assessment, the following key questions have been posed.

1. Is the new microbial source, *B. circulans* safe for producing β-galactosidase?
2. What is the risk to the public from the use of β-galactosidase derived from *B. circulans* as a processing aid?
3. Does the enzyme perform its technical function as specified?
4. Does the final enzyme preparation contain any allergenic materials?

## 2.1 Response to risk assessment questions

*Question 1: Is the new microbial source,* B. circulans *safe for producing β-galactosidase?*

Risk assessment based on microbiological analysis concluded that this micro-organism is not a toxigenic species (Section 3.1). The single, oral administration of live *B. circulans* to mice was not associated with any toxicity when assessed up to 14 days after inoculation (Section 4.3). The Applicant has noted that this micro-organism has a history of safe use in the preparation of commercial enzymes for the food industry. The latter is supported by a number of international organisations, foreign governments (Main report, S2.2) and a report by the European Commission's Scientific Committee for Food (SCF, 2007). Overall, there were no concerns with the safety of *B. circulans,* when used as a source of β-galactosidase.

*Question 2: What is the risk to the public from the use of β-galactosidase derived from* B. circulans *as a processing aid?*

No safety concerns with the use of this enzyme as a processing aid were raised. The same enzyme, from other microbial sources, is already listed in the Code as a processing aid (Table to Clause 17 of Standard 1.3.3). The Applicant has indicated that the enzyme is removed via acid/heat treatment and filtration from the final GOS preparation (Section 5.4.4). Any residual enzymic protein would be in the form of inactivated enzyme, which would be metabolised like any other protein in the gastrointestinal tract. The enzyme preparation did not contain any detectable mycotoxins (aflatoxin B1, ochratoxin A, sterigmatocystin, zearalenone and T-2 toxin) or antibiotic activity to *S. aureus, E. coli*, *B. cereus, B. circulans* (ATCC 4516), *Streptococcus pyogenes,* and *Serratia marcescens* (Section 4.6). Overall, there were no concerns with the safety of the enzyme preparation or the enzyme itself when used as a processing aid.

*Question 3: Does the enzyme perform its technical function as specified?*

The enzyme, β-galactosidase, is used as a food processing aid in the preparationion of galacto-oligosaccharides (GOS) (Section 5.4.3). β-galactosidase was reported as the most employed enzyme for the industrial preparationion of GOS (Neri *et al.*, 2009). β-galactosidase from the commercial enzyme preparation (Biolacta®N5), has transgalactosylation activity specifically selected for the preparationion of GOS of a quality desired by the Applicant. GOS produced by Biolacta® consists mainly of 2-4 monomer units.

*Question 4: Does the final enzyme preparation contain any allergenic materials?*

This enzyme was considered unlikely to pose an allergenic risk due to its homology to other β-galactosidases already in the Code (Section 4.2). Therefore it is not necessary to perform an analysis of homology of this enzyme. Although soybean meal is used as a fermentation medium in the preparationion of the enzyme preparation, there was not detectable soybean protein present in the final preparation (LOD = 1 mg/kg) (Section 5.4.4). Overall, the preparation containing β-galactosidase from *B. circulans* was not considered to pose an allergenic risk to consumers.

# 3 MICROBIOLOGICAL ASSESSMENT

## 3.1 Introduction

Members of the *Bacillus* sp. are widely distributed in the environment and are widespread in soil and decomposing vegetation (Sharpe *et al*., 1989). The two major human pathogens within the *Bacillus* genus are *B*. *anthracis* and *B*. *cereus*. Sharpe *et al*. (1989) noted that other *Bacillus* species are saprophytic, do not produce toxins and are not regarded as pathogenic to humans.

However, recent epidemiological evidence from foodborne outbreaks of illness has suggested that some *Bacillus* spp. may be of more human health importance than previously recognised (Jenson and Moir, 2003). Screening for the presence of genes for toxin preparationion or cytotoxicity suggests that only a small percentage of *Bacillus* spp. apart from
*B*. *anthracis* and *B*. *cereus* may be able to produce toxins (Beattie and Williams, 1999, From *et al*., 2005).

The Applicant has proposed to use a wild-type strain of bacteria (LOB 377) identified to belong to the genus *Bacillus*. The strain is one that was originally isolated from soil (Iida *et al*., 1980) and was found to produce lactase (β-galactosidase).

Traditionally the first step in characterising a species of bacteria, including *Bacillus,* is a combination of morphological and biochemical methods. Iida *et al*. (1980) used two taxonomic keys developed by the United States Department of Agriculture to determine the species of *Bacillus* of the wild-type strain (Gordon, 1989). The first key focused on biochemical factors while the second key included morphological information, such as the shape of the spore. Three potential species were identified using the keys: *B*. *megaterium*, *B*. *stearothermophilus* and *B*. *circulans*. *B*. *circulans* was selected as the most likely species due to consideration of the cell size (excludes *B*. *megaterium*) and growth temperature (excludes *B*. *stearothermophilus*).

## 3.2 Identification of the host organism

The wild strain described by Iida *et al*. (1980) was submitted to the American Type Culture Collection (ATCC) as *Bacillus circulans* and assigned the number ATCC 31382.

*B*. *circulans* has been described as a 'complex' rather than a species due to the phenotypic heterogeneity between strains (Sneath, 1986). Nakamura and Swezey (1983) identified at least 10 DNA homology groups within the taxon. A summary of the biochemical tests for *B*. *circulans* ATCC 31382 taken from Iida *et al*. (1980) and the 10 DNA homology groups is presented in Table 1 (Sneath, 1986) based on the information from Nakamura and Swezey (1983). A comparison of the biochemical results for ATCC 31382 against each of the homology groups revealed that there was no clear homology group that had the same biochemical profile.

The ATCC performs a number of biochemical and molecular tests to authenticate the strains in the collection. In the case of Gram-positive aerobic spore formers, such as *Bacillus* sp., this involves a total of 66 biochemical characterisation tests including substrate utilisation, NaCl tolerance, pH and temperature growth ranges (ATCC Authentication of Prokaryotes at ATCC). On the basis of these tests the ATCC 31382 strain has been designated as "*Bacillus* sp. deposited as *B*. *circulans*" and not *B*. *circulans*, as suggested by Iida *et al*. (1980). This designation would indicate that the ATCC was not able to determine a specific species for the strain.

**Table 1: Differential characteristics of DNA homology groups of *Bacillus circulans* complex (Sneath, 1986)**

| Characteristic | *B. circulans* ATCC31382 | DNA Homology groups | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | A | B | C | D | E | F | G | H | I | K |
| Anaerobic growth | + | + | + | – | – | + | + | – | + | + | + |
| Growth in 5% NaCl | – | + | – | – | – | d | – | – | + | – | d |
| Growth at pH 5.6 | – | + | + | + | + | + | d | d | – | + | + |
| Growth with lysozyme | ND | – | – | – | – | – | – | + | + | – | + |
| Hydrolysis of | | | | | | | | | | | |
| Casein | Slight | – | + | – | d | d | – | – | – | – | – |
| Starch | + | + | – | + | + | + | + | – | + | + | + |
| Nitrate reduced to nitrite | + | – | – | – | – | – | – | – | + | – | + |
| pH in V-P broth <5.5 | <6.0 | + | – | – | – | d | + | – | + | – | + |
| Utilization of | | | | | | | | | | | |
| Citrate | + | – | – | – | – | – | – | – | – | + | – |
| Fumarate | ND | + | + | + | – | – | – | + | – | + | – |
| Acid from | | | | | | | | | | | |
| L-Arabinose | + | + | + | + | + | + | + | – | + | + | + |
| D-Mannose | + | + | + | + | + | + | + | – | + | + | + |
| Sorbitol | ND | + | + | – | – | d | d | – | – | + | – |
| Mol% G+C of DNA | ND | 37-39 | 45-46 | 47-49 | 47-48 | 48-50 | 47-50 | 50-52 | 50-52 | 53-54 | 53 |

Symbols: -, 90% or more of strains are negative; +, 90% or more of strains are positive; d, 11-89% of strains are positive; ND, no data; V-P, Voges-Proskauer

## 3.3 Conclusion

The Applicant has indicated that the source micro-organism for the preparationion of β-galactosidase is *Bacillus circulans*. However, the exact taxonomic identity of the micro-organism as specified by the Applicant is unclear. Nonetheless, as the micro-organism has been deposited with the ATCC as ATCC 31382 by the enzyme producer and this specified source has been used in the various safety studies submitted in this Application (A1032), there is sufficient information to identify the micro-organism with a reasonable level of confidence. Thus, to provide an unambiguous assignment of the micro-organism's nomenclature with the possibility of enhanced taxonomic identification in the future, *Bacillus circulans* ATCC 31382 is proposed for drafting into the Code.

# 4 CHEMICAL SAFETY ASSESSMENT

## 4.1 Evaluation of the safety studies

In support of this application, the following toxicological studies were submitted:

- Acute dose toxicity study in mice with an enzyme preparation;
- Acute dose toxicity study in mice with the bacteria;
- Repeat-dose oral toxicity, 90 day study in rats with an enzyme preparation;
- A *S. typhimurium*/*E. coli* reverse mutation assay (Ames test) with an enzyme preparation;
- A chromosomal aberration assay using Chinese Hamster Ovary cells with an enzyme preparation;
- Micronucleus assay in mice with an enzyme preparation.

The toxicological studies used a β-galactosidase preparation derived from a technical grade stock powder labelled as BIOLACTA® or BIOLACTA® N5. Reference to this stock powder was made throughout the submitted toxicological studies. This stock powder contained approximately 30% lactase (β-galactosidase) and 70% lactose. Total protein was determined to be ≤21% from batch analysis of Biolacta® N5. There were no specific data about the quantity of β-galactosidase protein in the finished GOS preparation (see below for discussion of the allergenicity of the enzyme).

## 4.2 Potential allergenicity of β-galactosidase

There were no empirical data on the potential allergenicity of β-galactosidase derived specifically from the host bacterium, *B. circulans* ATCC 31382. The Code currently lists four other sources of β-galactosidase. These sources of β-galactosidase were negative for immunological significant sequence homology, after an assessment using the FAO-WHO decision tree for the Structural Database of Allergenic Proteins (Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology, 2001).Thus, given the amino acid homology of these enzymes with the enzyme from *B. circulans*, it was considered unlikely that it would pose an allergenic risk.

## 4.3 Acute toxicity studies

**Title:** Acute toxicity test in mice of BIOLACTA®, stock powder.
**Test facility:** Japan Food Research Laboratories, Tokyo, Japan.
**Testing period:** 28 Jan – 8 Mar, 1991.
**Report Date:** 8 Mar, 1991.
**GLP status:** Nil

Fasted mice (ICR, male and female, n = 10/sex) were given a single oral (gavage) dose of β-galactosidase (2000 mg/kg bw) using purified water as the vehicle. No control group was employed. Bodyweight was measured prior to and weekly after dosing. Animals were sacrificed 14 days after dosing. The study report noted that there were no deaths, clinical signs or reductions in body weight in either sex. No abnormalities were noted at necropsy, however, no details were given about the level of examination undertaken at necropsy.

While there were no raw data available for independent evaluation, this was not considered a serious limitation that would negate the conclusion. Thus, based on the absence of significant findings, the β-galactosidase preparation (BIOLACTA®) did not induce acute toxicity in mice at a limit dose of 2000 mg/kg bw PO.

**Title:** Pathogenicity study of lactase (LT) producing bacteria (*Bacillus circulans*) in mice.
**Test facility:** Gifu Research Laboratories, JBS Inc. Gifu, Japan.
**Testing period:** 15 Feb – 1 Mar, 2005
**Study No.:** JBS-05-MPOG-014
**Report Date:** 31 Mar, 2005.
**GLP status:** Nil

Mice (ICR, male and female, n = 5/sex) were given a single oral inoculation of either the SKML medium (control group) or live *B. circulans*[2] ($7.0 \times 10^8$ CFU/animal). Body weight was measured prior to dosing and at Days 1, 3, 7, 10 and 14 after dosing. Animals were sacrificed 14 days after dosing. The following organs were examined for macroscopic and histological changes: brain, lung, liver, spleen and kidney.

There were no deaths, clinical signs or reductions in body weight during the observation period. There were no treatment-related, untoward macroscopic or histological findings at necropsy.

Overall, oral inoculation of $7.0 \times 10^8$ CFU of live *B. circulans* did not induce acute toxicity in mice when assessed up to 14 days after inoculation.

## 4.4 Repeat dose toxicity study

**Title:** A 90-day oral toxicity study of lactase (LT) concentrate in rats.
**Test facility:** Bozo Research Center, Inc., Tokyo, Japan.
**Testing period:** May – Aug 2004
**Study No.:** B-5324
**Report Date:** 27 Jan, 2005.
**GLP status:** Nil[3]

Rats (Sprague Dawley, 12/sex/group) were given 0 (water for injection), 3288, 6575 or 13150 mg/kg bw/day lactase[4] concentrate orally for 91 days (13 weeks).

Dose justification was based on the results of a 2-week oral toxicity study with lactase (LT) concentrate in rats (Study No.: B186; Not provided to FSANZ). Doses of 2775, 5550 and 11100 mg/kg bw/day (activity 6160 LSU/g)[5] were reported not to be associated with toxicity. Thus, the high dose selected for the present study was selected at 13150 mg/kg (activity 3820 LU/g), and the lower doses at 2-fold reductions.

There were no deaths, clinical signs or reductions in body weight and food consumption. There were no adverse findings from urinalysis, ophthalmological, haematological or clinical

[2] Strain not identified but culture was supplied by Daiwa Kasei K.K., Lot No.: LT41I12F.
[3] The study was conducted in compliance with: "Partial Revision of Testing Facilities Regulated in Ordinance No. 4 for Studies on New Chemical Substances and for Provision on Items, etc. of Investigation of Harmful Effects of Designated Chemical Substances", Joint-Signature Notification, KanHoAn No. 41 of the Planning and Coordinating Bureau, Environmental Agency, Japan, SeEiHatsu No. 268 of the Environmental Health Bureau, Ministry of Health and Welfare, Japan and Heisei 12-02-14 KiKyoku No. 1 of the Basic Industries Bureau, Ministry of International Trade and Industry, Japan, March 1, 2000, Partial Revision on January 24, 2001. No OECD compliance noted in study.
[4] Lactase concentrate supplied by Daiwa Kasei K.K. Contains 8.8% w/w of lactase, with a potency of 5200 LSU/g or 3820 LU/g. Lactase is an equivalent name for β-galactosidase.
[5] An alternative measure of enzyme activity utilises o-Nitrophenyl-β-galactopyranoside (ONPG) as a substrate. When ONPG is hydrolysed by lactase, it is converted to o-Nitrophenol and galactose. The lactase activity for this assay is determined by measuring the amount of liberated o-Nitrophenol. One lactase unit (LSU) is defined as the amount of enzyme that liberates 1 µmol of o-Nitrophenol per min at the early stage of the reaction at 40°C, pH 6.0.

chemistry examinations. Organ weight measurements at necropsy showed an increase in the absolute** (728 mg) and relative weights* (140 mg/100 g bw) of salivary glands in HD males *cf* control males (631 mg and 124 mg/100 g bw, respectively[6]). However, the effect was not dose related and there was no histopathological correlate (assessed) and was thus considered not to be toxicologically significant. There were no significant gross or histological findings at necropsy.

Overall, a NOAEL was established at 13150 mg/kg bw/day PO for the lactase concentrate in rats.

## 4.5 Genotoxicity studies

The results of three studies on genotoxicity with β-galactosidase (BIOLACTA® N5) have been summarized in Table 2. Overall, no genotoxic potential was identified with β-galactosidase derived from *B. circulans*.

**Table 2: Genotoxicity of β-galactosidase (BIOLACTA®).**

| Assay | Test system | Concentration/dose | Result |
|---|---|---|---|
| Ames | *S. typhimurium*, TA98, TA100, TA1523 & TA 1537; *E. coli* WP2 *uvr*A | Test 1: 10 to 5000 µg/plate, ± S9, deionised water (vehicle).<br>Test 2: 50 to 200 µL/plate of 100 mg/mL extract, +S9, dimethylsulphoxide (vehicle). | Negative<br>GLP status: Nil<br>Standard: The Ministry of Labour, Japan, 1991.<br>Valid assay: appropriate negative & positive controls were used (Satoa, 1993). |
| Chromosomal aberration | CHO cells | Test 1: 50 to 5000 µg/mL, ± S9.<br>Test 2: 10-1000 µg/mL, ± S9. | Negative<br>GLP compliant<br>OECD guideline 473.<br>Positive controls: mitomycin (-S9) & cyclophosphamide (+S9)<br>(de Vogel & Verhagen, 1995) |
| Micronucleus | Mouse (Swiss, 15/sex/group)<br>Assessed up to 72h post dose. | 0, 2000 mg/kg bw IP | Negative<br>GLP compliant.<br>OECD guideline 474.<br>Positive control: mitomycin (1.5 mg/kg bw)<br>(Verhagen, 1995) |

Purity of chemical substance (β-galactosidase): 27.7%. Impurities: Proteinase < 0.1%.

## 4.6 Toxin and antibacterial activity analyses

The β-galactosidase preparation was shown not to contain the following mycotoxins above the respective detection limits: aflatoxin B1 (0.5 ppb), ochratoxin A (0.5 ppb), sterigmatocystin (20 ppb), zearalenone (50 ppb) or T-2 toxin (200 ppb) (Mycotoxin Research Association, Yokohama, Japan, 1991).

[6] * $p < 0.05$; ** $p < 0.01$.

Furthermore, the β-galactosidase preparation was not found to possess any antibacterial activity to: *S. aureus* (ATCC 5538); *E. coli* (ATCC 11229); *B. cereus* (ATCC 2); *B. circulans* (ATCC 4516); *Streptococcus pyogenes* (ATCC 12344); and *Serratia marcescens* (ATCC 14041) (Japan Food Research Laboratories, Tokyo, Japan, 1991).

## 4.7 Conclusion

Application A1032 seeks approval for the use of β-galactosidase (lactase) from *Bacillus circulans* as a processing aid. The enzyme will be used as a processing aid only, and any residue would be in the form of inactivated enzyme, which would be metabolised like any other protein in the gastrointestinal tract.

The chemical hazard assessment concluded that:

- There was no evidence of toxicity by the enzyme extract in an acute dose toxicity study in mice at doses of up to and including 2000 mg/kg bw, PO of β-galactosidase;
- There was no evidence of toxicity by the bacterium in an acute dose toxicity study in mice orally inoculated with live *B. circulans(*tested at 7.0 x $10^8$ CFU);
- A NOAEL was established at 13150 mg/kg bw/day PO in rats for the enzyme extract with an activity of 3820 LU/g, when administered for 13 weeks;
- There was no evidence that the enzyme preparation had any genotoxic potential when assessed in two *in vitro* assays and a single *in vivo* assay.

Based on the available data, it was concluded that no hazard could be identified for β-galactosidase derived from *B. circulans,* or the micro-organism itself. In view of the acid lability and sensitivity to proteolytic digestion following oral ingestion of β-galactosidase, the absence of a hazard may have been reasonably anticipated. As no health standards were considered necessary for this enzyme, the ADI entry should be considered as 'not specified'.

# 5 FOOD TECHNOLOGY ASSESSMENT

## 5.1 Introduction

The Applicant has sought the listing of a new microbial source, *B. circulans* ATCC 31382, for the preparationion of β-galactosidase, in Table to Clause 17 of Standard 1.3.3 (Food Processing Aids) of the *Australia New Zealand Food Standards Code* (the Code). Four other microbial sources (*Aspergillus niger, A. oryzae, Kluyveromyces marxianus, K. lactis*) have been approved as a source of β-galactosidase.

## 5.2 Technological function of the enzyme

The enzyme, β-galactosidase, is used as a food processing aid in the preparationion of galacto-oligosaccharides (GOS). It was reported to be the most employed enzyme for the industrial preparationion of GOS (Neri *et al*., 2009). Under specific process conditions and a high lactose concentration, the lactose becomes the acceptor group during the transglycosylation process. This results in polymerisation in addition to hydrolysis, in which the lactose molecule is attached to the galactose residue and forms a GOS.

The two β-galactosidases, galactosidase-I and β-galactosidase-II, from the commercial enzyme preparation (Biolacta® N5) have transgalactosylation activity specifically selected for the preparationion of GOS of a quality desired by the Applicant. GOS produced by Biolacta® consist mainly of 2-4 monomer units.

## 5.3 Method of analysis

The β-galactosidase activity is measured using Daiwa's method (Exhibit 10 in A1032). When lactose is hydrolysed by β-galactosidase, it is converted into glucose and galactose. The β-galactosidase activity was determined by measuring the amount of liberated glucose. The β-galactosidase (also known as lactase) activity is defined by the amount of enzyme that liberates 1 µmol of glucose per min at the early stage of the reaction at 40$^{o}$C, pH 6.0, and expressed as lactase units (LU).

## 5.4 Manufacturing

### 5.4.1 The preparationion process of β-galactosidase

The Applicant has an exclusive agreement with Daiwa Kasei K. K., Japan for the supply of Biolacta® N5, the commercial form of β-galactosidase from *B. circulans* ATCC 31382.

β-galactosidase is an extracellular enzyme. β-galactosidase derived from *B. circulans* ATCC 31382 is produced by means of fermentation. Soybean meal is used as a fermentation medium. The enzyme preparationion process was comprised of a cultivation step followed by several filtration and purification steps. The Applicant has stated that the enzyme was separated from other proteins and enzymes present in the medium or cell content by filter-presses and micro-filters. The isolated enzyme is sprayed dried and standardised with lactose to obtain a preparation with consistent enzymatic activity units prior to packaging. The process and manufacturing conditions were described in detail in US Patent 4237230 (Iida, 1980).

The commercial preparation, Biolacta® N5, has been observed to have no other enzymatic activities. The final composition of the β-galactosidase concentrate is given in Table 3.

**Table 3: Composition of averages of five batches of β-galactosidase concentrate**

| Constituent | Concentration (g/ 100 g) |
|---|---|
| Moisture | 4.5 |
| Protein | 69.3 |
| Fat/oil | < 0.1 |
| Carbohydrate | 9.4 |
| Ash | 16.9 |

### 5.4.2 Specifications

The Applicant has stated that the specification for β-galactosidase derived from *B. circulans* ATCC 31382 complies with the international specifications relevant for enzymes, which were prepared by the FAO/WHO Expert Committee on Food Additives at its 67$^{th}$ Meeting (2006) for publication in FAO JECFA Monographs 3 (2006). This specification reference ia a primary specification source listed in clause 2 of Standard 1.3.4 – Identity and Purity of the Code. An enzyme preparation specification for an enzyme batch was provided by the Applicant (Table 4).

**Table 4: Specification for β-galactosidase provided by the Applicant**

| Parameter | Specification |
|---|---|
| **Lactase activity** | not less than 5,000 LU/g |
| ***Heavy metals (as Pb)** | Not more than 40 µg/g |
| ***Arsenic (as As)** | Not more than 3 µg/kg |
| **Viable bacteria count** | Not more than $10^4$ /g |
| **Coliforms** | Not more than 30 /g |
| ***Salmonella*** | Negative in 25 g |
| ***E. coli*** | Negative in 25 g |
| **Commercial preparation (**Biolacta® N5) | |
| **Description** | A light brown powder |
| **Composition** | 30 % β-galactosidase concentrate**: 70% lactose |
| **Shelf life** | 6 months under room temperatures and sealed conditions after shipment |

*these are tested using the concentrated material before standardisation.
** composition of β-galactosidase concentrate is given in Table 3.

### 5.4.3 Preparationion of Galacto-Oligosaccharides

Biolacta® N5 is added to the lactose solution adjusted to pH 7. The reaction is carried out at 60°C for 12 hours. After heating, the reaction mixture is treated with active carbon, and applied to an ion-exchange column. The eluate is concentrated to obtain a syrup preparation. It was noted by Taniguchi (2005) that more than 55% of the added lactose is converted to GOS and the sugar composition of the preparationion is tetrasaccharides (15%), trisaccharides (23.5%), transferred disaccharides (20%) and galactose (1.3%).

### 5.4.4 Potential allergenicity of the enzyme preparation

Soybean meal is used as a fermentation medium in the preparationion of the enzyme preparation. Daiwa Kasei K. K. has advised (letter dated 26 August 2009) that the quantity of protein derived from soybean remaining in the enzyme concentrate was not more than the detection limit of 1.0 mg/kg.

During the preparationion of Vivinal®GOS, the enzyme β-galactosidase is inactivated by acid and heat reactions and then removed by filtration. The Applicant has shown results that demonstrated that β-galactosidase activity is not present in the final GOS preparation. The Applicant has advised that trace amounts of the enzyme protein could remain in GOS but they cannot differentiate between milk proteins and the enzyme protein. The presence of milk proteins in the GOS preparation results from the use of a milk fraction rich in lactose as a starting material for the GOS preparationion and therefore there may be some residual milk proteins at end of the process.

## 5.5 Conclusion

The enzyme, β-galactosidase produced by *B. circulans* ATCC 31382, meets the stated purpose of the Application, i.e. it has been demonstrated to perform the specified reactions with lactose under the described process and manufacturing conditions.
According to the Applicant it is the preferred enzyme for the preparationion of their GOS preparations and it meets the international specification for enzymes (FAO/WHO, 2006).
The Applicant claims that there is no detectable soybean protein in the enzyme preparation or enzyme activity in the GOS preparation.

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