ORIGINAL SUBMISSION

Clasado Inc
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Pittsburgh, PA 15241
United States

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July 29, 2013

RECEIVED
SEP 4 2013
OFFICE OF
FOOD ADDITIVE SAFETY

Dr. Moraima Ramos-Valle
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Dear Dr. Ramos-Valle:

Re: GRAS Exemption Claim for BbgIV *Beta*-galactosidase Enzyme Preparation

In accordance with proposed 21 CFR §170.36 [Notice of a claim for exemption based on a Generally Recognized as Safe (GRAS) determination] published in the *Federal Register* [62 FR 18938 (17 April 1997)], I am submitting one hard copy and one electronic copy (on CD), as the notifier [Clasado Inc., 1620 Pinetree Drive Pittsburgh, PA, 15241, USA], a Notice of the determination, on the basis of scientific procedures, that BbgIV *Beta*-galactosidase Enzyme Preparation, produced by Biocatalysts Limited for Clasado Inc., as defined in the enclosed documents, is GRAS under specific conditions of use as a processing-aid in the manufacture of a food ingredient, and therefore, is exempt from the premarket approval requirements of the *Federal, Food, Drug and Cosmetic Act*. Information setting forth the basis for the GRAS determination, which includes detailed information on the notified substance and a summary of the basis for the GRAS determination, as well as a consensus opinion of an independent panel of experts in support of the safety of BbgIV *Beta*-galactosidase Enzyme Preparation under the intended conditions of use, also are enclosed for review by the agency.

Should you have any questions or concerns regarding this GRAS Notice, please do not hesitate to contact me at any point during the review process so that we may provide a response in a timely manner.

Sincerely,

(b) (6)

Michael Moore
Business Manager, North America
Clasado Inc.
Tel: 412-831-7220
Mike.Moore@clasado.us

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Pittsburgh, PA 15241
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July 29, 2013

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Should you have any questions or concerns regarding this GRAS Notice, please do not hesitate to contact me at any point during the review process so that we may provide a response in a timely manner.

Sincerely,

(b) (6)

Michael Moore
Business Manager, North America
Clasado Inc.
Tel: 412-831-7220
Mike.Moore@clasado.us

August 29, 2013

RECEIVED
SEP 4 2013
OFFICE OF
FOOD ADDITIVE SAFETY

Dr. Moraima Ramos-Valle
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Dear Dr. Ramos-Valle,

RE: Certification of Virus-Free Document – (GRAS Exemption Claim for BbglV *Beta*-galactosidase Enzyme Preparation)

I hereby certify that the enclosed electronic files for the petition entitled, "BbglV *Beta*-galactosidase Enzyme Preparation" were scanned for viruses prior to submission and are thus certified as being virus-free using McAfee VirusScan 8.8.

Sincerely,

(b) (6)

Judy Vowles
Manager, Word Processing

August 29, 2013

Dr. Moraima Ramos-Valle
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Dear Dr. Ramos-Valle,

RE: Certification of Virus-Free Document – (GRAS Exemption Claim for BbgIV *Beta*-galactosidase Enzyme Preparation)

I hereby certify that the enclosed electronic files for the petition entitled, "BbgIV *Beta*-galactosidase Enzyme Preparation" were scanned for viruses prior to submission and are thus certified as being virus-free using McAfee VirusScan 8.8.

Sincerely,

Judy Vowles
Manager, Word Processing

GRAS Exemption Claim for BbgIV *Beta*-galactosidase Enzyme Preparation

Submitted to:

Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition (CFSAN)
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD
U.S.A. 20740-3835

Submitted by:

Clasado Inc.
1620 Pinetree Drive
Pittsburgh, PA
15241
USA

July 3, 2013

RECEIVED
SEP 4 2013
OFFICE OF
FOOD ADDITIVE SAFETY

GRAS Exemption Claim for BbgIV *Beta*-galactosidase Enzyme Preparation

Submitted to: Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition (CFSAN)
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD
U.S.A. 20740-3835

Submitted by: Clasado Inc.
1620 Pinetree Drive
Pittsburgh, PA
15241
USA

July 3, 2013

GRAS Exemption Claim for BbgIV *Beta*-galactosidase Enzyme Preparation

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I GRAS Exemption Claim

A Claim of Exemption from the Requirement for Premarket Approval Pursuant to Proposed 21 CFR §170.36(c)(1) [62 FR 18938 (17 April 1997)]

Clasado Inc. hereby claims that the use of BbgIV *beta*-galactosidase enzyme preparation in foods, as described in Section I.D below, is exempt from the requirement of premarket approval of the *Federal Food, Drug, and Cosmetic Act* because we have determined that such uses are Generally Recognized as Safe (GRAS).

Signed,

(b) (6)

3RD JULY 2013

Graham C. Waters
CEO
Clasado Inc.
graham.waters@clasado.com.mt

Date

B Name and Address of Notifier

Clasado Inc.
1620 Pinetree Drive
Pittsburgh, PA
15241
USA
Tel: 1-412-831-7220
Fax: 1-412-831-2274

C Common Name of the Notified Substance

BbgIV *beta*-galactosidase enzyme preparation

D. Conditions of Intended Use in Food

D.1 Technological Function and Food Applications

The BbgIV *beta*-galactosidase enzyme preparation is used as a processing-aid in the manufacture of Bimuno galacto-oligosaccharides manufactured by Clasado Inc. The transgalactosidic activity of BbgIV *beta*-galactosidase is used for the synthesis of galacto-oligosaccharides by transfer of D-galactosyl units on the D-galactose substituent of lactose.

D.2 Maximum Level of Use

The BbgIV *beta*-galactosidase enzyme preparation is used at the minimum level required to achieve the desired effect and used in accordance with the principles of current Good Manufacturing Practice (cGMP). Prior to large-scale production, the enzyme preparation is assayed for hydrolytic activity, and then assessed on a laboratory scale to determine the rate of glucose production under large scale synthesis conditions. The optimum enzyme dosage rate and duration of the full scale factory synthesis is determined from the laboratory scale synthesis. The addition rate of the enzyme is typically between 0.1 and 0.22 g per kg of lactose in the production of Bimuno galacto-oligosaccharides.

E Basis for the GRAS Determination

Pursuant to 21 CFR § 170.30 of the *Code of Federal Regulations* (CFR) (U.S. FDA, 2012), BbgIV *beta*-galactosidase enzyme preparation has been determined by Clasado Inc. to be GRAS on the basis of scientific procedures.

F Availability of Information

The data and information that serve as the basis for this GRAS Notification will be sent to the U.S. Food and Drug Administration (FDA) upon request, or will be available for review and copying at reasonable times at the offices of:

Clasado Inc.
1620 Pinetree Drive
Pittsburgh, PA
15241
USA

Should the FDA have any questions or additional information requests regarding this notification, Clasado Inc. will supply these data and information.

II Detailed Information about the Identity of the Substance

II.A Identity

BbgIV *beta*-galactosidase is a homo-dimer with a predicted molecular weight of 235.4 kDa for the dimer (117.7 kDa per subunit), and a molecular weight of 248 kDa when determined by size exclusion chromatography (Goulas *et al.*, 2009). *Beta*-galactosidases catalyze the hydrolysis of *beta*-galactosides by breaking *beta*-D-galactosidic linkages within simple disaccharides (lactose) and complex galacto-oligosaccharides, liberating galactose and glucose moieties. At high concentrations of the substrate (*e.g.*, lactose), *beta*-galactosidases also are able to catalyze the synthesis of galacto-oligosaccharides by transfer of one or more D-galactosyl units

onto the D-galactose substituent of lactose (Goulas *et al.*, 2007). Detailed biochemical characterization of BbgIV *beta*-galactosidase is presented by Goulas *et al*. (2009).

Common or Usual Name:	BbgIV *beta*-galactosidase
Synonyms:	lactase (ambiguous); *beta*-lactosidase
Chemical Abstracts Service (CAS) Number:	9031-11-2
Molecular weight:	235.4 kDa

Specificity: *Beta*-galactosidases catalyze the hydrolysis of terminal non-reducing *beta*-D-galactose residues in *beta*-D-galactosides (*e.g.*, lactose). At high concentrations of the substrate (lactose), the transferase activity of the *beta*-galactosidase predominates resulting in the production of galacto-oligosaccharides from lactose by the transfer of one or more D-galactosyl units onto the D-galactose substituent of lactose (Tzortzis *et al.*, 2005a,b). Biochemical characterization of BbgIV *beta*-galactosidase has been published by Goulas *et al.* (2009). The relative and specific activity of *Bifidobacterium bifidum* recombinant BbgIV *beta*-galactosidase is presented in Table II.A-1 below.

Table II.A-1 Percent Relative and Specific Activity in U/mg of *Bifidobacterium bifidum* Recombinant BbgIV *Beta*-galactosidase Towards Different Galactosides

Substrate	% Relative Activity	U/mg
Lactose	100	190
β-D-(1→6) galactobiose	4.7±0.9	9.0
β-D-(1→4) galactobiose	-	-
β-D-(1→4) galactosyllactose	-	-
N-Acetyllactosamine	61.1±1.2	116.0
Lacto-N-Biose I	-	-
Lactulose	3.5±0.5	6.6
3-Fucosyllactose	-	-

Table adapted from Goulas *et al*., 2009
± Standard deviation, n=3; symbol (-) Not detected.

Amino Acid Sequences: Using CLUSTAL 2.1 software, multiple sequence alignment comparisons of the wild-type *bbgIV beta*-galactosidase gene from *Bifidobacterium bifidum* NCIMB41171 (Genbank accession number: DQ443548.2) with the synthetic gene expressed in *Escherichia coli* BL21(DE3) were conducted (Larkin *et al*., 2007). Although the codon usage was optimized for synthetic gene expression in *E. coli*, the amino acid sequences of both encoded proteins were identical.

II.B Production Organism

BbgIV *beta*-galactosidase is synthesized by a recombinant strain of *E. coli* BL21(DE3) expressing a plasmid encoded synthetic *bbgIV* gene from *B. bifidum* NCIMB 41171. The strain has been assigned the laboratory designation *E. coli* BglA MCB3 (Biocatalysts Limited. Wales,

United Kingdom). The recombinant organism was produced using standard biotechnology practices applied to safe laboratory strains of *E. coli* currently used for the production of biotechnology products used by the industrial, pharmaceutical, and food industries. The strain is considered genetically stable when cultured under standard laboratory practices.

II.B.1 Host Organism

The host organism used for production of the recombinant strain is *E. coli* BL21(DE3). This strain is a common laboratory strain used for expression of biotechnology products. The strain was obtained from a commercial culture collection (Life Technologies Cat #: C6000-03) and therefore has an established and verifiable identity. The complete gene sequence of *E. coli* BL21(DE3) was published in 2009 by Jeong *et al*. (2009), and comprehensive bioinformatic analyses of the organism are described by Studier *et al*. (2009).

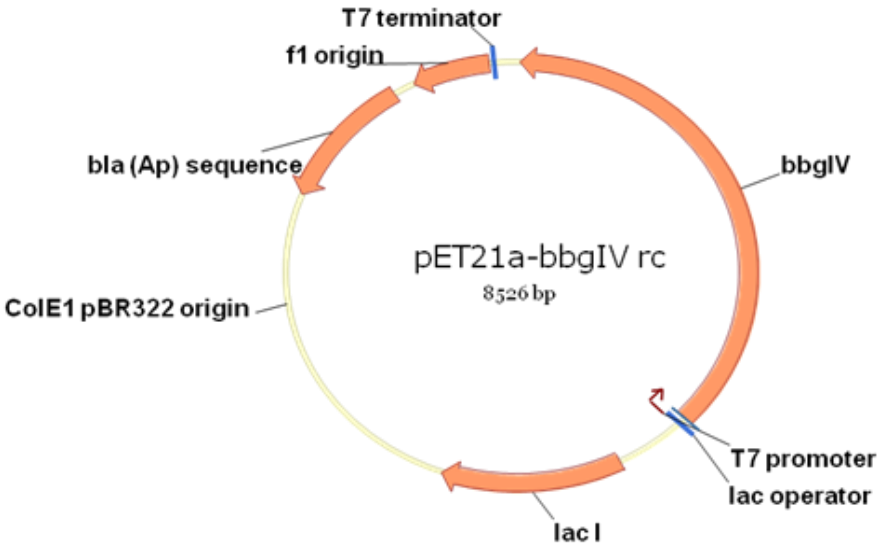
II.B.2 Donor Organism

Recombinant *E. coli* BL21(DE3) contains the pET-21a-d(+) vector (Novagen) encoding a synthetic *bbgIV* gene from *B. bifidum* NCIMB 41171. The sequence was optimized for expression by *E. coli* BL2(DE3). The plasmid expression product encodes for a protein that is 100% homologous to the wild-type *bbgIV* protein synthesized by the donor strain. Information characterizing the molecular cloning and biochemical characterization of BbgIV *beta*-galactosidase from *B. bifidum* NCIMB 41171 is presented by Goulas *et al.* (2007). *B. bifidum* NCIMB 41171 was originally isolated from a fecal sample obtained from a healthy volunteer, and the strain is maintained within a commercial culture collection (NCIMB) and has an established and verifiable identity.

II.B.3 Construction of Recombinant Strain

The BbgIV *beta*-galactosidase gene from *Bifidobacterium bifidum* NCIMB41171 (Genbank accession number: DQ443548.2) was synthesized and codon optimized by GeneArt (Germany). The gene was cloned into the T7 promoter expression vector, pET21a (Invitrogen), and the pET21a-BbgIV plasmid was then transformed into *Escherichia coli* BL21(DE3) (Biocatalysts Limited. Wales, United Kingdom) (see Figure II.B.3-1). *E. coli* BL21(DE3) was grown on nutrient medium containing the antibiotic ampicillin for selection of the plasmid construct. Expression of the 235.4 kDa *beta*-galactosidase enzyme as encoded by the pET21a-BbgIV plasmid is under control of the Lac repressor gene (*lacI*), and therefore synthesis of the enzyme occurs following induction with isopropylthio-β-galactoside (IPTG), a lactose analog.

Figure II.B.3-1 Expression Cassette



II.C Manufacturing

II.C.1 Raw Materials/Processing-aids

BbgIV *beta*-galactosidase is produced using a batch fed fermentation system, and the fermentation medium contains de-mineralized water, yeast extract, peptones, minerals, pH regulators, ampicillin, IPTG, sugar-based carbon sources, and anti-foaming and flocculation aids; these materials are standard materials used in the enzyme industry. Ampicillin, IPTG, and magnesium sulphate are added to the media post-sterilization. Ampicillin is used during fermentation to maintain stable growth of the recombinant strain during fermentation, and IPTG is used to induce expression of the BbgIV *beta*-galactosidase gene. The suitable use of IPTG and antibiotics during fermentation of enzyme preparations has been reviewed previously (GRN 126) (Innovase LLC, 2003). The removal of residual fermentation chemicals is achieved by washing and dia-filtration procedures and the absence of ampicillin in the finished enzyme preparation is verified for each lot of material (See Table II.D-1). All raw materials and processing-aids are food grade ingredients and where applicable comply with limits established in the Food Chemicals Codex (FCC). Anti-foaming and flocculation additives are used in accordance with cGMP, and have been previously determined to be safe and suitable for use in the production of enzyme preparations (GRN 428) (DSM Food Specialties, 2012).

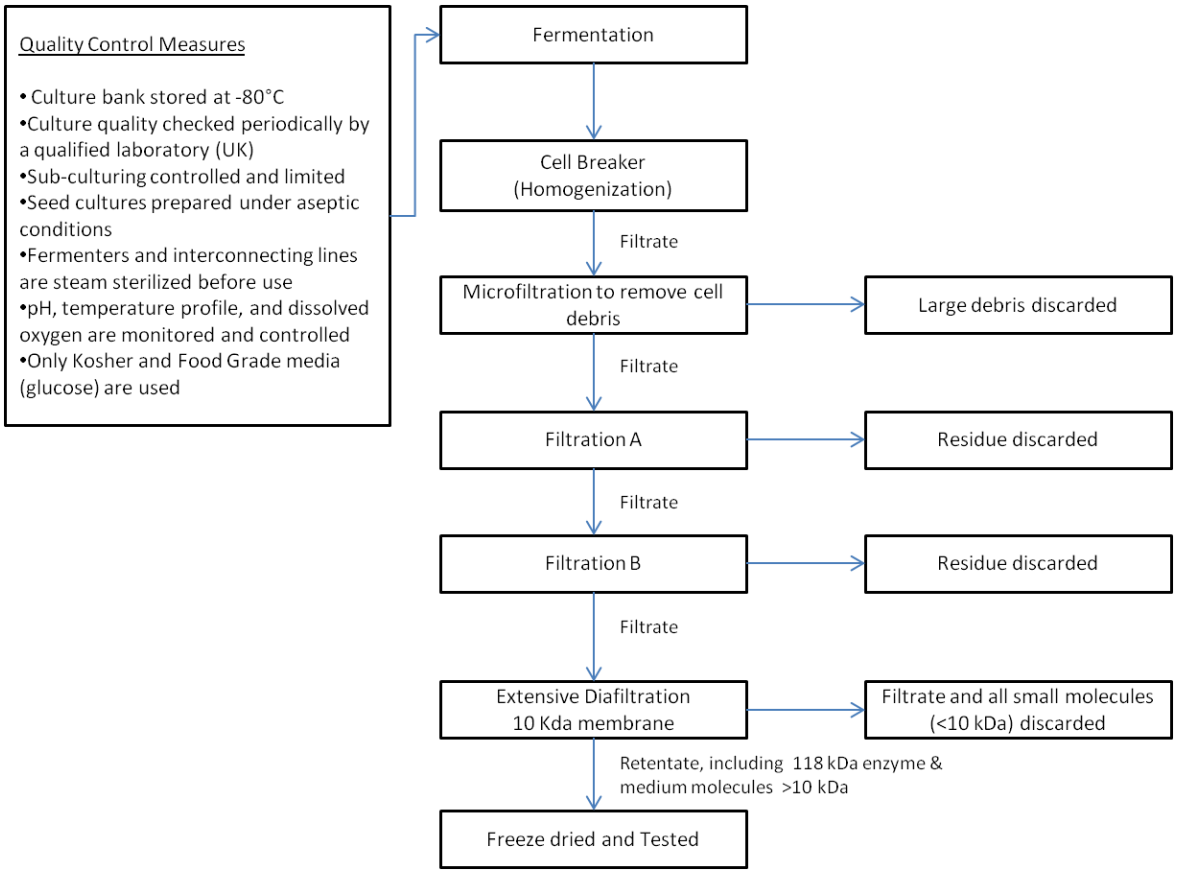
II.C.2 Fermentation and Recovery

As mentioned above, the production strain is grown in an aqueous solution containing appropriate sources of carbon, nitrogen, mineral salts and other miscellaneous organic and inorganic compounds together with 100 µg/mL of the antibiotic, ampicillin. A series of control

measures are in place to prevent contamination by foreign microorganisms and to ensure that the fermentation conditions remain optimal. These measures include storage of the culture bank at -80°C, quality control checks on the culture by an accredited laboratory, the control and limiting of sub-culturing, the preparation of seed cultures under aseptic conditions, the sterilization of fermenters and interconnecting lines prior to use, the monitoring and control of pH, temperature, and dissolved oxygen, and the use of Kosher and food grade media.

The BbgIV *beta*-galactosidase enzyme is liberated from the production strain following fermentation by cell disruption. Homogenized biomass is then treated with buffered solutions, and residual solids are removed by a series of sedimentation and filtration processes designed to remove debris greater than the molecular weight of the BbgIV *beta*-galactosidase enzyme. The resulting filtrate is then concentrated using ultra-filtration and dia-filtered to remove components smaller than the 10 KDa molecular weight cut-off of the membrane. Finally, the retentate is filtered through a 0.22 µm bacto-filtration unit and freeze-dried with sucrose to produce a dry, light beige powder product. Prior to release, the enzyme product is tested to ensure compliance with the final product specifications. An overview of the manufacturing process is presented in Figure II.C.2-1.

Figure II.C.2-1 Overview of Manufacturing Process and Quality Control



II.D Specifications for Food Grade Material

Appropriate food grade specifications have been established for the BbgIV *beta*-galactosidase enzyme preparation (see Table II.D-1 below). The specifications are consistent with the purity and microbial limits established for enzyme preparations in the FCC, 8th Edition and by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (JECFA, 2006; FCC, 2012). All methods of analysis utilized are internationally recognized or have been validated by Biocatalysts (see Table II.D-1 below).

Each batch of BbgIV *beta*-galactosidase enzyme preparation is certified to contain a minimum hydrolytic activity of 19,000 U/g on a dry weight basis. Limits for heavy metal and microbial contamination are provided and microbial analyses demonstrating the absence of the production organism in the finished product is included. Other analyses to confirm the absence of undesirable carry-over products of fermentation include measures for ampicillin and periodic screening for aflatoxin contamination also is conducted.

Table II.D-1 Specifications for BbgIV *Beta*-galactosidase Enzyme Preparation

Specification Parameter	Specification	Method
Appearance	Off White to Brown	Visual
Appearance In Solution (600 nm)	Clear	Visual
pH (1% aqueous solution)	6.5 to 7.5	
Galactosidase Activity (U/g dw)	19,000	Assay Procedure G904P, BbgIV Galactosidase Assay
Total Organic Solids (%)*	NS	By difference calculation
Moisture (%)	<5	MA100 Sartorius moisture analysis
Ash (%)*	<5	C001 BS4401 Part 1, ISO 936:1998
Protein (mg/mL)*	NS	Bradford
Antibiotic activity*	Absent	Delvotest® SP-NT
Heavy Metal Specifications*		
Cadmium (ppm)	<1	I/046 ICPMS/010
Lead (ppm)	<3	I/046 ICPMS/010
Mercury (ppm)	<0.1	I/046 ICPMS/010
Arsenic (ppm)	<1	I/046 ICPMS/010
Microbiological Specifications		
Total Viable Count (cfu/g)	<50,000	M001; ISO 4833 2003; APHA, 2001 - Technical Committee on Microbiological Methods for Foods; Roberts *et al.*, 2003; Manual (Bridson, 1998)
Coliforms (cfu/g)	<1	M005; ISO 4832:2006; BS 4285-3.7 1987; Roberts *et al.*, 2003; Manual (Bridson, 1998)
Coagulase Positive *Staphylococcus* (cfu/g)	<10	M013; ISO 6888-1 1999; APHA, 2001 - Technical Committee on Microbiological Methods for Foods; Roberts *et al.*, 2003; Manual (Bridson, 1998)
Salmonella (in 40g)	Absent	M041; ISO 6579 2002; E.C. No 2073/2005 (EC, 2005); Oxoid Manual (Bridson, 1998)

Table II.D-1 Specifications for BbgIV *Beta*-galactosidase Enzyme Preparation

Specification Parameter	Specification	Method
Escherichia coli	Absent	M012
Yeasts (cfu/g)	<100	M022; BS/ISO 21527-1 2008; Marks & Spencer, 2008; Roberts *et al.*, 2003; Oxoid Manual (Bridson, 1998)
Moulds (cfu/g)	<100	M022; BS/ISO 21527-1 2008; Marks & Spencer, 2008; Roberts *et al.*, 2003; Oxoid Manual (Bridson, 1998)
Thermophilic Aerobic Spore formers (in 1g)	Absent	M022; BS/ISO 21527-1 2008; Marks & Spencer, 2008; Roberts *et al.*, 2003; Oxoid Manual (Bridson, 1998)
Thermophilic Anaerobic spore formers (in 1g)	Absent	M022; BS/ISO 21527-1 2008; Marks & Spencer, 2008; Roberts *et al.*, 2003; Oxoid Manual (Bridson, 1998)
Process organism (in 30g)	Absent	M022; BS/ISO 21527-1 2008; Marks & Spencer, 2008; Roberts *et al.*, 2003; Oxoid Manual (Bridson, 1998)

cfu = colony forming unit; dw = dry weight; U = enzymatic units; NS = not specified – for information only; ppm = parts per million
*Testing conducted on a periodic basis or following a significant change to supplier or process conditions.

Analysis of 3 non-consecutive lots of the BbgIV *beta*-galactosidase enzyme preparation demonstrates that the manufacturing process as described in Section II.C produces a consistent product that meets established specifications. A summary of the analytical results is presented below in Table II.D-2.

Table II.D-2 Summary of the Product Analysis for 3 Lots of BbgIV *Beta*-galactosidase Enzyme Preparation

Specification Parameter	Specification	Manufacturing Lot		
		2B60001	31230597	31331938
Appearance	Light golden brown powder	Brown	Brown	Brown
Appearance In Solution (600nm)	Clear	Clear	Clear	Clear
pH (1% aqueous solution)	6.5 to 7.5	7.46	7.5	7.25
Galactosidase Activity (U/g) dry weight	19,000	25,184	50,353	19,172
Total Organic Solids (%)*	NS	95.86	96.91	96.42
Moisture (%)	<5	3.94	2.99	3.38
Ash (%)*	<5	0.2	0.1	0.2
Protein (mg/mL)*	-	0.845	0.857	0.871
Antibiotic activity*	Absent	Absent	Absent	Absent
Heavy Metal Specifications*				
Cadmium (ppm)	<1	0.02	<0.01	<0.01
Lead (ppm)	<3	1.54	0.779	1.12
Mercury (ppm)	<0.1	0.05	<0.05	<0.05
Arsenic (ppm)	<1	<0.06	<0.06	<0.06
Microbiological Specifications				
Total Viable Count (cfu/g)	<50,000	<10	<10	<10
Coliforms (cfu/g)	<1	<1	<1	<1

Table II.D-2 Summary of the Product Analysis for 3 Lots of BbgIV *Beta*-galactosidase Enzyme Preparation

Specification Parameter	Specification	Manufacturing Lot		
		2B60001	31230597	31331938
Coagulase Positive *Staphylococcus* (cfu/g)	<10	<10	<10	<10
Salmonella (in 40g)	Absent	Absent	Absent	Absent
Escherichia coli	Absent	Absent	Absent	Absent
Yeasts (cfu/g)	<100	<10	<10	<10
Moulds (cfu/g)	<100	<10	<10	<10
Thermophilic Aerobic Spore formers (in 1g)	Absent	Absent	Absent	Absent
Thermophilic Anaerobic spore formers (in 1g)	Absent	Absent	Absent	Absent
Process organism (in 30g)	Absent	Absent	Absent	Absent

cfu = colony forming unit; U = enzymatic units; NS = not specified – for information only; ppm = parts per million; U = units
Enzyme preparation also contains residual citrate or phosphate buffers and sucrose as a stabilizing agent.
*Testing conducted on a periodic basis or following a significant change to supplier or process conditions.

II.E Additional Characterization

II.E.1 *Beta*-lactamase Genetic Material

The BbgIV *beta*-galactosidase gene from *Bifidobacterium bifidum* NCIMB41171 was cloned into a T7 promoter expression vector, pET21a, which also contains the gene sequence for *beta*-lactamase. The *beta*-lamase gene confers resistance to lactam antibiotics, such as ampicillin. Transfer of the intact ampicillin resistance gene to the enzyme preparation will be prevented by the activity endogenous endo/exonucleases that are released during cell disruption, which result in the degradation of cellular DNA sequences. The use of flocculation aids also are anticipated to remove DNA during purification of the enzyme preparation, especially long-chain intact genes. The *beta*-lactamase gene is encoded with a plasmid which possesses and *E. coli* origin of replication and is therefore unable to replicate in other hosts. Finally, the theoretical size of the *beta*-lactamase is 31.4 kDa, and as the filters used for purification of Bimuno syrup exclude molecules >10 kDA, transference of intact gene sequences of the *beta*-lactamase gene into the finished food ingredient will be prevented. Testing for residual traces of the full length beta-lactamase gene was conducted in accordance with Good Manufacturing Practices on three lots of galacto-oligosaccharides produced using the BbgIV enzyme preparation (ILS Genomics, Morrisville, NC). Galacto-oligosaccharide samples were solubilized in phosphate buffered saline (PBS) at a concentration of 1 mg/ml prior to residual DNA testing. Detection and quantification of the *E. coli* beta-lactamase gene was conducted using quantitative PCR assay. Optimization of the qPCR conditions and preparation of the standard curve was conducted using pET21 plasmid DNA. Extraction efficiency was determined by a “spike in” of target DNA to 2 of the aliquots for each sample prior to extraction Qualification. Primers and probes used for the target specific assay is shown in Table II.E-1.

Table II.E-1 qPCR Assay Primers and Probes

Primer Name	Primer Sequence	Type
B-lactamase_F	5'-CGCCCCGAAGAACGTTT-3'	Forward
B-lactamase_R	5'-CGCGCCACATAGCAGAACTT-3'	Reverse
B-lactamase_probe	5'-6FAM-CAATGATGAGCACTTTT-MGBNFQ-3'	Probe

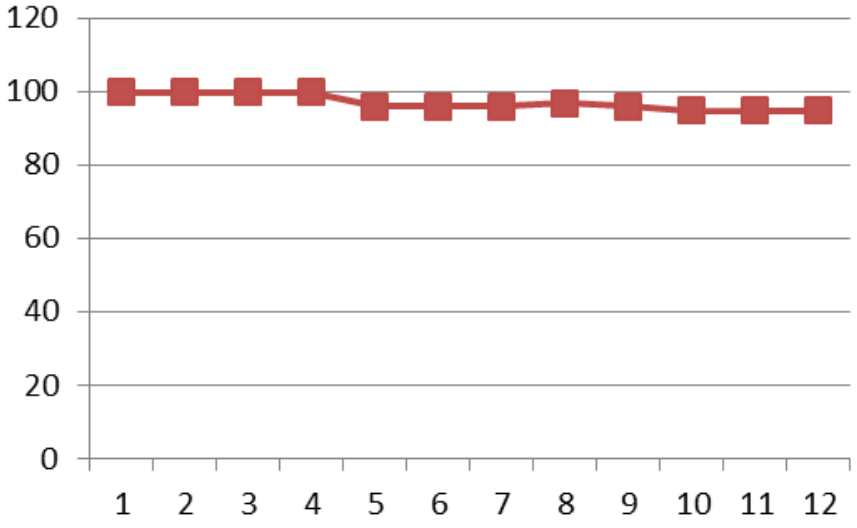
For the 3 syrup galacto-oligosaccharide samples the detected DNA mass was less than the standard curves limit of quantification (LOQ) value of 0.00625 pg. It was therefore concluded that under the conditions of intended use of the enzyme preparation for the manufacture of galacto-oligosaccharides, there is no risk for introduction of the intact beta-lactamase gene into the food supply.

II.F Stability

One batch of the BbgIV *beta*-galactosidase enzyme preparation (Galactosidase 904P batch 31230597) was stored at -18°C in a temperature monitored freezer and assessed at monthly intervals using a galactosidase test method.

As demonstrated in Figure II.F-1 below, the batch of BbgIV *beta*-galactosidase enzyme displayed consistent activity under the recommended storage conditions (*i.e.*, -18°C) and remained within the specification of +/- 10% of the initial activity. The shelf-life has been set at 12 months based on the results of this study and previous work carried out during the development of the enzyme preparation.

Figure II.F-1 Stability of BbgIV *Beta*-galactosidase Enzyme Preparation (G904P) at -18°C over 12 Months (y-axis = enzyme hydrolysis activity; x-axis = time in months)



III Self-Limiting Levels of Use

The use of BbgIV *beta*-galactosidase enzyme preparation is self-limiting due to the cost of the enzyme preparation and the concentration required for optimal synthesis of galacto-oligosaccharides.

IV Detailed Summary of the Basis for GRAS Determination

The determination that BbgIV *beta*-galactosidase enzyme preparation is GRAS is on the basis of scientific procedures. The intended use of BbgIV *beta*-galactosidase enzyme preparation in the manufacture of Bimuno galacto-oligosaccharides, as described in Section D, is based on generally available information demonstrating that host and donor organisms are derived from non-toxicogenic non-pathogenic strains, and use of appropriate production controls employed during the manufacture of the enzyme preparation and Bimuno galacto-oligosaccharides. Specifically, the use of a 10 kDA filter at 2 stages of the manufacturing processes, which ensures that transfer of the recombinant organism and other metabolites/processing-aids originating from the fermentation into Bimuno galacto-oligosaccharides does not occur. Safety is further corroborated by generally available information supporting the safety of *beta*-galactosidase enzyme preparations currently used in food production. A summary of the data and information supporting the safety of the BbgIV *beta*-galactosidase enzyme preparation is provided in Sections IV.A and IV.B below.

IV.A Probable Exposure

IV.A.1 Enzyme Residues in Bimuno Galacto-oligosaccharides

The BbgIV *beta*-galactosidase enzyme preparation is used as a processing aid in the manufacture of Bimuno galacto-oligosaccharides and is removed from the finished product using ultra filtration through a 10 kDa membrane. Bimuno galacto-oligosaccharides manufactured using the BbgIV *beta*-galactosidase enzyme preparation are absent protein at detectable concentrations (see Table II.D-2). Accordingly, dietary exposure to the enzyme preparation from intended food uses will not occur.

IV.B Information to Support the Safety of the Enzyme

IV.B.1 Safety of the Production Strain

As described in Section II.C, the enzyme is obtained by fermentation using *E. coli* BL21 (DE3) expressing the BbgIV *beta*-galactosidase gene from *Bifidobacterium bifidum* NCIMB 41171. The GRAS use of this enzyme is intended for exclusive application to the manufacture of Bimuno galacto-oligosaccharides as manufactured by Clasado Ltd. As discussed in Section II.C.2, the genomic stability of the organism is maintained by the use of master and working

cultures, and the fermentation procedure is subject to strict quality control measures. Isolation of the enzyme from the organism requires disruption of the cell using homogenization procedures, and the residual cell matter is removed using a series of filtration steps, followed by a final dia-filtration (<10 kDa) for removal of small molecular weight metabolites and all residual fermentation media. During the production of Bimuno galacto-oligosaccharides, constituents within the BbgIV *beta*-galactosidase enzyme preparation are removed from the galacto-oligosaccharides using ultra-filtration through a 10 kDa membrane. Through the use of appropriate filtration procedures employed during the manufacture of the enzyme and the finished galacto-oligosaccharide ingredient, all constituents produced during fermentation, including the enzyme preparation, are excluded from the finished product (Bimuno galacto-oligosaccharides). The host and donor strains used for production of the recombinant organism were determined to be safe and suitable (non-toxicogenic/non-pathogenic) for the production of galacto-oligosaccharides. A discussion of generally available information supporting the safety of the host and donor organisms is presented in Sections IV.B.2 and IV.B.3 respectively.

IV.B.2 Safety of the Host Organism – *Escherichia coli* BL21(DE3)

IV.B.2.1 History of Safe Use

Escherichia coli are commensal residents of the gut microflora of humans and numerous animal species and are members of *Enterobacteriaceae* taxonomic family of gram-negative non-spore-forming facultative anaerobes. *Escherichia coli* strains are taxonomically grouped into 5 different ECOR phylogroups (A, B1, B2, D, and E) based on sequence similarity of housekeeping genes (Archer *et al*., 2011). Human commensal strains are typically found in Group A or B1, with non-related pathogenic strains classified under Group B2, D, and E. Among thousands of *E. coli* isolates, three group A strains laboratory strains, strains K-12, B, C and their derivatives, are designated as Risk Group 1 organisms in biological safety guidelines (Daegelen *et al.*, 2009; Archer *et al.*, 2011). Under the National Institute for Health (NIH) guidelines for research involving recombinant DNA molecules, RG1 organisms are classified as "*Agents not associated with disease in healthy adult humans*" (CDC/NIH, 2009). Of these strains, *E. coli* K-12, and the B derivatives (*e.g*., REL606, BL21) are among the most widely used strains for production of biotechnology products for production of industrial, pharmaceutical and food biotechnology preparations. For example, chymosin obtained by *Escherichia coli* K-12 expressing the bovine chymosin gene has been affirmed GRAS by the U.S. Food and Drug Administration (FDA) for use in food applications (U.S. FDA, 2012), and recombinant human insulin derived using *E. coli* K-12 fermentation was the first approved biotechnology drug for human use. Of the 151 recombinant proteins approved for use as human pharmaceuticals by the FDA and European Medicines Evaluation Agency up to 2009, 45 products were synthesized using safe *E. coli* laboratory strains, including K-12 and BL21(DE3) (Ferrer-Miralles *et al*., 2009). *Escherichia coli* strains K-12 and BL21 are derived from human commensal isolates (Daegelen *et al*., 2009).

Despite the long-history of apparent safe use of *E. coli* BL21(DE3) for use in production of recombinant proteins of biotechnology, including protein derived pharmaceutical preparations, publically available authorizations and/or safety opinions pertaining to the use of this strain for production of additives/ingredients intended for addition to food are not available. An assessment of the pathogenicity and toxicogenic potential of *E. coli* BL21(DE3) is therefore presented below.

IV.B.2.2 Pathogenicity

As discussed by Pariza and Johnson (2001) "…*food enzyme preparations rarely contain viable production organisms. Hence the issue of pathogenicity is largely moot as regards food enzyme production strains*." Nevertheless, it is common practice to characterize the pathogenic potential of the host strain, and a brief overview of information relevant to the pathogenicity of *E. coli BL21*(DE3) is presented below.

The pathogenicity of *E. coli* strain BL21 was evaluated by Chart *et al*. (2000). Groups of 5 BALB/c mice were administered $1x10^6$ cfu of viable BL21 bacteria in saline *via* the oral or peritoneal routes and animals were then euthanized following a 7-day observation period. All animals were evaluated for viable bacteria in spleen, liver and kidney tissue. A positive control group was administered an equivalent dose of *Salmonella enteritidis* (P132344), a *Salmonella* strain known to be highly virulent to BALB-c mice. Mice dosed with *Salmonella* developed poor health and were euthanized. Animals administered BL21 displayed normal health throughout the observation period, and viable colonies of BL21 could not be recovered from the tissues sampled. The authors also evaluated the pathogenicity of BL21 in 1-day-old chicks, an animal model susceptible to invasion of the gut mucosa by non-pathogenic organisms. Birds were administered $1x10^2$ cfu of viable BL21 or the *E. coli* pathogen (EQ1). The birds were euthanized at 24, 48, and 72 hours post-inoculation. Gross pathology, histology and bacteriology were conducted on the liver, spleen, and caeca. Transient colonization of the liver with BL21 was observed, and colonization of the liver and spleen with EQ1 was observed. The authors reported that failure of BL21 to colonize the spleen was indicative that the strain is sensitive to macrophage mediated clearance. Colonization of the caeca was observed for BL21 and EQ1 at 24 hours; however, colonization of the ceaca was more persistent with EQ1. Similar observations were reported in colonization studies conducted by the authors in 4-month-old Merino crossbred sheep administered $2.5x10^9$ viable BL21 or EQ1. The authors concluded that *E. coli* BL21 was a poor colonizer of the gastrointestinal tract of animals highly susceptible to establishment of persistent infection. The observations by Chart and colleagues demonstrating that *E. coli* BL21 exhibits limited capacity to colonize the gastrointestinal tract of animals is expected to be relevant to transgenic strains produced from the organism. Transgenic modifications of commercial interest are intended to optimize production of commercial protein of interest (*e.g*., enzyme) and would not be expected to improve viability of the organism in the gastrointestinal tract. In fact such genetic modifications, which typically

optimize production of the intended recombinant protein over all other metabolic functions of the cell, typically place the strain at a metabolic disadvantage to natural organisms in the environment. Such organisms are usually fastidious in their nutritional requirements that they could not survived outside the laboratory (Junod, 2007).

A comparison of the survival of *E. coli* BL21 with the human pathogen *E. coli* E20513 (O111:H2) in rabbit serum also was evaluated by the authors (Chart *et al.*, 2000). *Escherichia coli* BL21 was susceptible to serum complement mediated cell lysis, an observation that was consistent with SDS-PAGE profiles demonstrating that BL21 does not express long-chain lipopolysaccharides (LPS) involved in evading complement-host interactions. Bioinformatic observations also have demonstrated that BL21 does not contain functional/intact gene sequences encoding an O antigen polysaccharide (Jeong *et al.*, 2009). In contrast to the susceptibility of BL21 to complement mediated cell lysis, E20513 (O111:H2), an O-antigen positive organism that synthesizes long-chain LPS, was able to evade complement mediated killing and survival was observed in normal and heat inactivated serum. Based on these observations the authors concluded that *E. coli* "*BL21, did not have the well-recognized pathogenic mechanisms required by strains of E. coli causing the majority of enteric infections.*"

IV.B.2.3 Toxicogenicity

The species *E. coli* is well established to contain members with pathogenic and toxicogenic determinants. *Escherichia coli* is one of the most extensively investigated microorganisms, and the majority of toxins produced by this species and related members (*e.g.*, Shigella) are well characterized (Sears and Kaper, 1996; Chart, 1998, 2000; Nataro and Kaper, 1998; Qadri *et al.*, 2005). *Escherichia coli* toxins characterized to date for the species include the heat stable enterotoxins (*e.g.*, EAST-1, STa, STI, ST_p, ST_h, STb, STII), heat labile enterotoxins (*e.g.*, LT-1, LT-II), cytolethal distending toxin, cytotoxic necrotizing factors (*e.g.*, CNF1, CNF2) and the Shiga-toxins (Stx, SLT-1, SLT-2) (Sears and Kaper, 1996). Toxins produced by pathogenic members of *E. coli* have acute effects, typically resulting in diarrheagenic responses in infected individuals/animals. The absence of toxin production by *E. coli* BL21 is demonstrated in the feeding studies reviewed above in Section IV.B.2.2, where oral and/or intraperitoneal administration of *E. coli* BL21 to mice, 1-day-old chicks, and sheep was without evidence of intolerance or toxicity (Chart *et al*., 2000).

As discussed, toxins synthesized by pathogenic strains of *E. coli* have been extensively characterized, and established/putative genetic determinants associated with toxicogenicity have in many cases been elucidated (Sears and Kaper, 1996). The entire genome of *E. coli* BL21(DE3) was characterized by Jeong *et al*. (2009), and the whole genomic sequence of the strain was compared in detail to the genomes of strains *E. coli* K-12 (MG1655) and *E. coli* REL606 using bioinformatic analyses (Studier *et al*., 2009). Consistent with the long-history of safe use of these strains in the laboratory, and the history of safe use of *E. coli* K-12 in the

production of food additives and pharmaceutical preparations, known *E. coli* enterotoxins or related pathogenic determinants were not identified by the authors.

Based on the unique ability of BL21 to efficiently integrate and express foreign DNA, and the recognized absence of known toxins produced by the strain, BL21 has been the strain of choice for use toxicology studies of biotechnology derived proteins intended for use in agricultural practice. A significant number of short-term and subchronic toxicity evaluations have been conducted with recombinant proteins produced by *E. coli* BL21(DE3) without evidence of confounding toxicity from toxins originating from the organism during fermentation (Mathesius *et al*., 2009; Guimarães *et al*., 2010; Quemada *et al*., 2010). This extensive history of use for production of recombinant proteins further support the conclusion that *E. coli* BL21(DE3) can be concluded to be a non-toxicogenic strain.

IV.B.3 Safety of the Donor Organism – *Bifidobacterium bifidum*

The BbgIV *beta*-galactosidase gene as expressed by recombinant *E. coli* BL21(DE3) was obtained from *Bifidobacterium bifidum* NCIMB 41171. The cloning of the *bbgIV* gene from *Bifidobacterium bifidum* NCIMB 41171 was reported by Goulas *et al*. (2007), and comprehensive comparative analyses on the purification and biochemical characterization of BbgIV *beta*-galactosidase also have been reported by the authors (Goulas *et al*., 2009). Clasado has demonstrated that the BbgIV *beta*-galactosidase protein expression product as synthesized by the company's recombinant strain of *E. coli* BL21 is 100% homologous at the protein sequence level (see Section II.B.2).

Bifodobacterium are gram positive, heterofermentive, non-motile, non-spore forming organisms, and most members of this genus are strict anaerobes. *Bifidobacterium* are a major genera and normal residents of the gut microflora of humans and many animals, and colonization of the infant gut by various species of *Bifidobacterium*, including *Bifidobacterium bifidum*, occurs early in life. *Bifidobacterium bifidum* NCIMB 41171 was isolated from a fecal sample obtained from a healthy human volunteer (Tzortzis *et al.*, 2005a,b).

The safety of select members of the genus *Bifidobacterium* for use in food was evaluated by the European Food Safety Authority (EFSA) using the Qualified Presumption of Safety (QPS) process. This assessment process recognizes that many microorganisms have long-histories of safe use by the food industry, and is based on 4 essential pillars of information: established identity, body of knowledge, possible pathogenicity, and end use. Following a review of the available information relevant to the safety of *B. bifidum*, EFSA concluded that the current food uses of this species do not present cause for safety concern. *Bifodobacterium bifidum* was granted QPS status in 2007 without qualification on use, and to date the QPS status of this species has been maintained following yearly assessment as required under the QPS system (EFSA, 2007; EFSA, 2012). The conclusion that usual consumption patterns of *Bifodobacterium bifidum* from traditional food uses of the species is safe, is consistent with the

opinions expressed in multiple safety reviews of *Bifidobacteria* published by qualified Experts in food safety and microbiology (Holzapfel *et al.*, 1998; Vanderhoof and Young, 1998; Saarela *et al.*, 2002; Borriello *et al.*, 2003; Picard *et al.*, 2005; Boyle *et al.*, 2006).

IV.B.4 Safety of the Enzyme Product

The BbgIV *beta*-galactosidase gene (*bbgIV*) used for manufacture of recombinant *E. coli* BL21(DE3) was cloned from *B. bifidum* NCIMB 41171 as described by Goulas *et al.* (2007). *Bifidobacterium bifidum* NCIMB 41171 is a human commensal derived from a fecal isolate obtained from a healthy volunteer. Active *beta*-galactosidases of microbial (*e.g.*, *Bifodobacterium* and *Lactobacillus* species) and human origin are naturally present within the gastrointestinal tract of all individuals. Enzymes present within Lactic acid bacteria (*e.g.*, *Lactobacillus* and *Bifidobacterium*) also have long-histories of safe use for production of fermented foods. As discussed by Pariza and colleagues, toxicity is an unusual property among proteins and "… *whereas there are thousands of proteins in food, only a tiny fraction exhibit toxigenic potential by the oral route. These known toxigenic proteins differ greatly in structure from commercially available food enzymes.*" (Pariza and Foster, 1983; Pariza and Johnson, 2001). Pariza and Foster also note that the results of exhaustive literature reviews conducted by the FDA, pertaining to the safety of microbial and non-microbial enzymes used in food production, support the position that enzymes from non-toxicogenic, non-pathogenic organisms are safe to consume (Pariza and Foster, 1983). Whole cell isolates and enzyme preparations containing *beta*-galactosidases (EC 3.2.1.23) have been evaluated in 90-day repeat dose gavage toxicity studies in groups of male and female Sprague-Dawley rats (GRNs 285, 334) (GTC Nutrition, 2009; Yakult Pharmaceutical Industry Co., Ltd., 2010). These studies were conducted in accordance with accepted international guidelines for toxicity testing of chemicals, and results from these experiments demonstrated that the *beta*-galactosidase preparations were non-toxic in this rat species. No observed adverse effect levels greater than 10 mL yeast extract (providing >25,000 mU of *beta*-galactosidase activity)/kg body weight/day and 13,150 mg *beta*-galactosidase enzyme preparation/kg body weight/day (potency of the test article was 5,200 SU/g) in male and female rats, the highest doses tested have been determined. In addition, 2 *beta*-galactosidase preparations have been affirmed GRAS by the FDA for use as direct food ingredients for the hydrolysis of lactose in accordance with 21 CFR 184.1387 and 21 CFR 184.1388 (U.S. FDA, 2012).

BbgIV *beta*-galactosidase from *Bifidobacterium bifidum* NCIMB 41171 was non-mutagenic in the bacterial reverse mutation assay (Ames test) using various *S.* Typhimurium strains TA98, TA100, TA102, TA1535, TA1537 at doses up to 5,000 µg/plate in the presence and absence of metabolic activation (Clasado, data on file).

BbgIV *beta*-galactosidase enzyme preparation was evaluated using the Pariza-Johnson decision tree (Pariza and Johnson, 2001) and was concluded to be safe and acceptable for use in food.

IV.B.5 Allergenicity

As reported by Pariza and Foster (1983), "*Allergies and primary irritations from enzymes used in food processing should be considered a low priority item of concern except in very unusual circumstances.*" BbgIV *beta*-galactosidase is obtained from *Bifidobacterium bifidum*, a species naturally present in the human gastrointestinal tract. The widespread exposure of all humans, at all ages of life, to the enzyme from commensal *Bifidobacterium* within the gastrointestinal tract is therefore expected, and allergenicity concerns are not expected.

The Allergen Online database version 13 (Updated February 12, 2013) was used to conduct a preliminary screen of BbgIV *beta*-galactosidase from *Bifidobacterium bifidum* NCIMB 41171 (GI:90822504) for relevant matches against to known putative allergens. This database is maintained by the Food Allergy Research and Resource Program of the University of Nebraska. A FASTA3 overall search of Allergen Online was conducted using default settings (E cutoff = 1 and maximum alignments of 20). As shown in Table IV.B.5-1 poor matches (E values 0.42 to 9.6) to a number of putative allergens were identified, with matches to grass pollen being the most common. No matches to any of the 8 major allergens were identified. No matches to BbgIV *beta*-galactosidase sharing greater than 50% identity over its length relative to putative allergens within the database were identified, which indicates that the potential of cross-reactivity to allergens listed in Table IV.B.5-1 is low (Aalberse, 2000).

Table IV.B.5-1 Overall FASTA3 Search of Allergen Online Database Version 13 with bbgIV *Beta*-galactosidase [*Bifidobacterium bifidum* NCIMB 41171]

Sequence G.I. #	Organism	Description	Length	E Score	% Identity	Amino acid Alignment
168419914	*Paspalum notatum*	Pas n 1 allergen precursor	265	0.42	24.2	149
126385	*Lolium perenne*	Pollen allergen Lol p 1	263	1.2	24.8	137
190613941	*Prunus dulcis*	putative allergen Pru du 2.02	241	1.3	32.1	84
167472847	*Quercus alba*	pollen allergen Que a 1 isoform	159	1.5	27.6	76
25091406	*Prunus persica*	Thaumatin-like protein 2	242	1.8	31.0	84
190613903	*Prunus dulcis* x *Prunus persica*	putative allergen Pru p 2.02	242	1.8	31.0	84
168316	*Lolium perenne*	pollen allergen	263	2	24.8	137
7271239	*Malassezia sympodialis*	allergen	179	2.2	34.8	46
1582250	*Phleum pratense*	allergen Phl p I	262	3.6	21.5	214
25361513	*Fusarium culmorum*	helix-loop-helix protein	450	3.7	24.4	119
45823012	*Phleum pratense*	unnamed protein product, partial	240	4.5	21.6	231
168314	*Lolium perenne*	pollen allergen, partial	252	5.5	25.8	132
75274600	*Lolium perenne*	Pollen allergen	263	5.7	27.1	133

Table IV.B.5-1 Overall FASTA3 Search of Allergen Online Database Version 13 with bbgIV *Beta*-galactosidase [*Bifidobacterium bifidum* NCIMB 41171]

Sequence G.I. #	Organism	Description	Length	E Score	% Identity	Amino acid Alignment
3901094	*Phleum pratense*	pollen allergen Phl pI	263	6.7	21.7	217
57283137	*Nicotiana tabacum*	villin 1, partial	559	7	32.7	52
33149333	*Dactylis glomerata*	group 1 allergen Dac g 1.01 precursor, partial	240	8.4	21.4	201
338930674	*Paspalum notatum*	group 13 grass pollen allergen	391	9.5	30.1	73
338930676	*Paspalum notatum*	group 13 grass pollen allergen, partial	393	9.5	30.1	73
338930672	*Paspalum notatum*	group 13 grass pollen allergen, partial	395	9.6	30.1	73

An 80 amino acid sliding window (segments 1-80, 2-81, 3-82, *etc*.) also was used to scan the amino acid sequence of BbgIV *beta*-galactosidase against the allergen database using FASTA to search for matches of 35% identity or more. This 35% identity for 80 amino acid segments is a suggested guideline proposed by Codex for evaluating proteins in genetically modified crops (Codex, 2003; Goodman *et al*., 2008). As shown in Table IV.B.5-2 below the results of the FASTA3 alignments of all possible 80 amino acid segments of BbgIV *beta*-galactosidase against all putative allergen sequences in the database were all less than the 35% threshold over 80 amino acids. Based on the information presented herein it was therefore concluded that the intended food uses of BbgIV *beta*-galactosidase do not raise allergenic risks.

Table IV.B.5-2 80 mer Sliding Window Search Results for with bbgIV *Beta*-galactosidase [*Bifidobacterium bifidum* NCIMB 41171]

Database	AllergenOnline Database v13 (February 12, 2013)
Input Query	>gi|90822504|gb|ABE00939.1| bbgIV *beta*-galactosidase [Bifidobacterium bifidum NCIMB 41171] mnttddqrkngdpivspsipttawladprvyavhrldahsdhacwsrspvdgestnlr qsldgewrvrvetaptgrfpdgtsdgpdwisdvsplfaapgfddssfsrvqvpshlet agllapqyvnvqypwdghedpkapaipehghvavyrrefdadgevaqavregrpvtlt fqgaataiyvwlngsfvgyaedsftpsefdvtdaikvdgnvlavvcyeyssaswledq dfwrlhglfrsvelnarpaahiadlhadadwdlatsrgslsldvlidgaanaatvdfa lwdkngtivwhtatkadgtlhaeaeiddaapwsaerpdlyelsvtlldadgkvletar trigfrhvaiedgilklngkrlvfrgvnrhefdcrrgraiteedmlwdirfmkrhnin avrtshypnqsrwyelcdeygiylidetnlethgswnspgdipvgtsvpgddeawlga cidrldsmilrdrnhpsvlvwslgnesyagevlkamsahahrldpgrpvhyegvnwnh aydgisdfesrmyakpaeiqdwlehgdergeaskpfvsceymhamgnscgglsefidl eryerysggfiwdyidqglvqrlpdgserlsvggewgdrptdyefvgngivfadrtps pkaqevkqlyspvklapdghgvtienrnlfagtdgyvfaarlledgheiwhadyrfdv aagdtqhhdiafpdidadgdtrevtyevdlllaeatawapagyelafgqltgtlnpeq ditetshdddgratrtlsrwnagirrddeeillsrtqggivswkrddremvirrpelv tfrpltdndrgnhsgfdraawfaagryaivtetkihesddglvaeyqyeladpnhtpv svtyhvnsdmrmqltveypgnatdmaslpafgiewelpgeydrlryygpgpeetyrdr kqggklgiwdatakasmapylmvqetgshedvrwleatdiqghglrvtqrgdrhftas llpwntytieaarrhedlpkprhnylrllaaqmgvggddswgapvhtayqlpagrplt ldvnleli

Table IV.B.5-2 80 mer Sliding Window Search Results for with bbgIV *Beta*-galactosidase [*Bifidobacterium bifidum* NCIMB 41171]	
Length	1052
Number of 80 mers	973
Number of Sequences with hits	0

IV.C Expert Panel Evaluation

Clasado Inc. has determined that BbgIV *beta*-galactosidase enzyme preparation is GRAS for use as a processing-aid at levels consistent with cGMP at the minimum levels necessary for synthesis of Bimuno galacto-oligosaccharides meeting previously established food grade specifications, on the basis of scientific procedures. This GRAS determination is based on data generally available in the public domain pertaining to the safety of BbgIV *beta*-galactosidase enzyme preparation, as discussed herein, and on consensus among a panel of experts (the Expert Panel) who are qualified by scientific training and experience to evaluate the safety of food ingredients. The Expert Panel consisted of the following qualified scientific experts: Prof. Joseph F Borzelleca Ph.D. (Virginia Commonwealth University School of Medicine), Prof. Michael W. Pariza Ph.D. (University of Wisconsin), and. Prof. Steven L. Taylor Ph.D. (University of Nebraska).

The Expert Panel, convened by Clasado Inc., independently and critically evaluated all data and information presented herein, and also concluded that BbgIV *beta*-galactosidase enzyme preparation was GRAS for use as a processing-aid at levels consistent with cGMP at the minimum levels necessary for synthesis of Bimuno galacto-oligosaccharides meeting previously established food grade specifications, based on scientific procedures. A summary of data and information reviewed by the Expert Panel, and evaluation of such data as it pertains to the proposed GRAS use of BbgIV *beta*-galactosidase enzyme preparation is presented in Appendix A.

IV.D Conclusion

Based on the above data and information presented herein, Clasado Inc. has concluded that the intended food use of BbgIV *beta*-galactosidase enzyme preparation, as described in Section D, is GRAS based on scientific procedures. General recognition of Clasado Inc.'s GRAS determination is supported by the unanimous consensus rendered by an independent Panel of Experts, qualified by experience and scientific training, to evaluate the use of BbgIV *beta*-galactosidase enzyme preparation in food, who similarly concluded that the intended use of BbgIV *beta*-galactosidase enzyme preparation described herein is GRAS.

BbgIV *beta*-galactosidase enzyme preparation therefore may be marketed and sold for its intended purpose in the U.S. without the promulgation of a food additive regulation under Title 21 of the CFR.

V References

Aalberse RC (2000). Structural biology of allergens. J Allergy Clin Immunol 106(2):228-238. Cited In: Goodman and Wise, 2006.

APHA (2001). *Compendium of Methods for the Microbiological Examination of Foods, 4th edition*. Washington (DC): APHA Technical Committee on Microbiological Methods for Foods. Cited In: Food Analytical Laboratories Ltd., 2011 [Microbiology Method M001].

Archer CT, Kim JF, Jeong H, Park JH, Vickers CE, Lee SY et al. (2011). The genome sequence of *E. coli* W (ATCC 9637): comparative genome analysis and an improved genome-scale reconstruction of *E. coli*. BMC Genomics 12:9. doi:10.1186/1471-2164-12-9.

Borriello SP, Hammes WP, Holzapfel W, Marteau P, Schrezenmeir J, Vaara M et al. (2003). Safety of probiotics that contain lactobacilli or bifidobacteria. Clin Infect Dis 36(6):775-780.

Boyle RJ, Robins-Browne RM, Tang ML (2006). Probiotic use in clinical practice: What are the risks? Am J Clin Nutr 83(6):1256-1264.

Bridson EY, editor (1998). *Oxoid Manual 8th edition*. Hampshire, Engl.: Oxoid Ltd. Available at: http://sst-web.tees.ac.uk/external/u0003076/Food_micro/oxoidmanual.pdf.

BS 4285-3.7 (1987). *Microbiological Examination for Dairy Purposes. Enumeration of Coliform Bacteria*. London, Engl.: British Standards Institution (BSi). Cited In: Food Analytical Laboratories Ltd., 2011 [Microbiology Method M005].

BS/ISO 21527-1 (2008). *Microbiology of Food and Animal Feeding Stuffs – Horizontal Method for the Enumeration of Yeasts and Moulds. Part 1: Colony Count Technique in Products With a Water Activity Greater Than 0.95*. London, Engl.: British Standards Institution (BSi) / Geneva, Switz.: International Organization for Standardization (ISO). Cited In: Food Analytical Laboratories Ltd., 2011 [Microbiology Method M022].

CDC/NIH (2009). *Biosafety in Microbiological and Biomedical Laboratories, 5th edition*. Atlanta (GA): Centers for Disease Control (CDC) / Bethesda (MD): National Institutes of Health (NIH). Available at: http://www.cdc.gov/biosafety/publications/bmbl5/index.htm.

Chart H (1998). Toxigenic *Escherichia coli*. Symp Ser Soc Appl Microbiol 84(27):77S-86S.

Chart H (2000). VTEC enteropathogenicity. Symp Ser Soc Appl Microbiol (29):12S-23S.

Chart H, Smith HR, La Ragione RM, Woodward MJ (2000). An investigation into the pathogenic properties of *Escherichia coli* strains BLR, BL21, DH5alpha and EQ1. J Appl Microbiol 89(6):1048-1058.

Codex (2003). Codex Alimentarius Commission. Alinorm 03/04: Joint FAO/WHO Food Standard Programme, Codex Alimentarius Commission, Twenty-Fifth Session, Rome, 30 June-5 July, 2003. Appendix III, Guideline for the conduct of food safety assessment of foods derived from recombinant-DNA plants and Appendix IV, Annex on the assessment of possible allergenicity, pp. 47-60.

Daegelen P, Studier FW, Lenski RE, Cure S, Kim JF (2009). Tracing ancestors and relatives of *Escherichia coli* B, and the derivation of B strains REL606 and BL21(DE3). J Mol Biol 394(4):634-643.

DSM Food Specialties (2012). *GRAS Notification for an Asparaginase from a Genetically Modified Strain of Aspergillus Niger*. (Submitted as U.S. FDA, 2012–GRN 428) (Internal DSM Reference: #00058237; Identifier no: DSM1126-001). Prepared by The Netherlands: DSM Food Specialties for submission to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety (HFS-200). Available at: http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=428.

EC (2005). Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. Off J Eur Union 48(L338):1-26. Available at: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2005:338:0001:0026:EN:PDF.

EFSA (2007). Opinion of the Scientific Committee on a request from EFSA on the introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA (question no EFSA-Q-2005-293, adopted on 19 November 2007 by European Food Safety Authority). EFSA J 587:1-16. Available at: http://www.efsa.europa.eu/en/efsajournal/pub/587.htm.

EFSA (2012). EFSA Panel on Biological Hazards (BIOHAZ); Scientific Opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed (2012 update) (question no EFSA-Q-2012-00218, adopted on 6 December 2012 by European Food Safety Authority). EFSA J 10(12):3020. [84 pp.] doi:10.2903/j.efsa.2012.3020. Available at: http://www.efsa.europa.eu/en/efsajournal/pub/3020.htm.

FCC (2012). Enzyme preparations. In: *Food Chemicals Codex, 8th edition*. Rockville (MD): United States Pharmacopeial Convention (USP), pp. 375-380.

Ferrer-Miralles N, Domingo-Espín J, Corchero JL, Vázquez E, Villaverde A (2009). Microbial factories for recombinant pharmaceuticals. Microb Cell Fact 8:17. doi: 10.1186/1475-2859-8-17.

Food Analytical Laboratories Ltd. (2011). *[Various Methods]*. Food Analytical Laboratories Ltd.

Goodman RE, Vieths S, Sampson HA, Hill D, Ebisawa M, Taylor SL, van Ree R (2008). Allergenicity assessment of genetically modified crops--what makes sense? Nat Biotechnol 26(1):73-81. doi: 10.1038/nbt1343.

Goodman RE, Wise J (2006). *Bioinformatic Analysis of Proteins in Golden Rice 2 to Assess Potential Allergenic Cross-Reactivity* (Study No. BIO-02-2006). Lincoln (NE): Food Allergy Research and Resource Program, Food Science and Technology, University of Nebraska. Available at: http://www.allergenonline.org/Golden%20Rice%202%20Bioinformatics%20FARRP%202006.pdf.

Goulas T, Goulas A, Tzortzis G, Gibson GR (2009). Comparative analysis of four β-galactosidases *from Bifidobacterium bifidum* NCIMB41171: purification and biochemical characterisation. Appl Microbiol Biotechnol 82(6):1079-1088.

Goulas TK, Goulas AK, Tzortzis G, Gibson GR (2007). Molecular cloning and comparative analysis of four β-galactosidase genes from *Bifidobacterium bifidum* NCIMB41171. Appl Microbiol Biotechnol 76(6):1365-1372.

GTC Nutrition (2009). *Galactooligosaccharide GRAS Notice*. (Submitted as U.S. FDA, 2009–GRN 285). Prepared by Golden (CO): GTC Nutrition for submission to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety (HFS-200). Available at: http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=graslisting&id=285.

Guimarães LM, Farias DF, Muchagata RC, de Magalhães MQ, Campello CC, Rocha TL et al. (2010). Short-term evaluation in growing rats of diet containing *Bacillus thuringiensis* Cry1Ia12 entomotoxin: nutritional responses and some safety aspects. J Biomed Biotechnol 2010:doi:pii: 630267. 10.1155/2010/630267.

Holzapfel WH, Haberer P, Snel J, Schillinger U, Huis in't Veld JH (1998). Overview of gut flora and probiotics. Int J Food Microbiol 41(2):85-101.

Innovase LLC (2003). *GRAS Notification for BD5088 alpha-bylase Enzyme Preparation, Derived From Pseudomonasflourescens Biovar I, Expressing a Gene Encoding an Optimized Thermococcales alpha-amylase*. (Submitted as U.S. FDA, 2003–GRN 126). Prepared by San Diego (CA): Innovase LLC for submission to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=126.

ISO 4832:2006 (2006). *Microbiological Examination of Food and Animal Feeding Stuffs. Enumeration of Coliforms-Colony Count Technique*. Geneva, Switz.: International Organization for Standardization (ISO). Cited In: Food Analytical Laboratories Ltd., 2011 [Microbiology Method M005].

ISO 4833 (2003). *Microbiological Examination of Food and Animal Feeding Stuffs. Enumeration of Micro-Organisms-Colony Count Technique at 30oc. Part 1*. Geneva, Switz.: International Organization for Standardization (ISO). Cited In: Food Analytical Laboratories Ltd., 2011 [Microbiology Method M001].

ISO 6579: 2002+A1 (2007). *Microbiological Examination of Food and Animal Feeding Stuffs. Horizontal Method Detection of Salmonella spp*. Geneva, Switz.: International Organization for Standardization (ISO). Cited In: Food Analytical Laboratories Ltd., 2011 [Microbiology Method M041].

ISO 6888-1 (1999). *Microbiological Examination of Food And Animal Feeding Stuffs. Enumeration of Staphylococcus Aureus by Colony Count Technique*. Geneva, Switz.: International Organization for Standardization (ISO). Cited In: Food Analytical Laboratories Ltd., 2011 [Microbiology Method M013].

JECFA (2006). Enzyme preparations (general specifications & considerations). In: *Combined Compendium of Food Additive Specifications [Online Edition]*. (Formerly: FAO Food and Nutrition Paper). Rome, Italy: Food and Agriculture Organization of the United Nations (FAO), Joint FAO/WHO Expert Committee on Food Additives (JECFA), pp. xxi-xxv. Available at: http://www.fao.org/ag/agn/jecfa-additives/docs/enzymes_en.htm.

Jeong H, Barbe V, Lee CH, Vallenet D, Yu DS, Choi SH et al. (2009). Genome sequences of *Escherichia coli* B strains REL606 and BL21(DE3). J Mol Biol 394(4):644-652.

Junod SW (2007). *Celebrating a Milestone: FDA's Approval of First Genetically-Engineered Product*. (Originally appeared in FDLI's Update Magazine, Sep./Oct.). Washington (DC): Food and Drug Law Institute (FDLI). College Park (MD): U.S. Food and Drug Administration (U.S. FDA). Available at: http://www.fda.gov/AboutFDA/WhatWeDo/History/ProductRegulation/SelectionsFromFDLIUpdateSeriesonFDAHistory/ucm081964.htm

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H et al. (2007). Clustal W and Clustal X version 2.0. Bioinformatics 23(21):2947-2948.

Marks & Spencer (2008). Method 3.8 Enumeration of general yeasts and moulds. In: *Manual of Microbiological Methods (September 2008) Web Issue*. Marks & Spencer. Cited In: Food Analytical Laboratories Ltd., 2011 [Microbiology Method M022].

Mathesius CA, Barnett JF, Cressman RF, Ding J, Carpenter C, Ladics GS et al. (2009). Safety assessment of a modified acetolactate synthase protein (GM-HRA) used as a selectable marker in genetically modified soybeans. Regul Toxicol Pharmacol 55(3):309-320.

Nataro JP, Kaper JB (1998). Diarrheagenic *Escherichia coli*. Clin Microbiol Rev 11(1):142-201. & [Erratum, 11(2):403].

Pariza MW, Foster EM (1983). Determining the safety of enzymes used in food processing. J Food Prot 46(5):453-468.

Pariza MW, Johnson EA (2001). Evaluating the safety of microbial enzyme preparations used in food processing: update for a new country. Regul Toxicol Pharmacol 33(2):173-186.

Picard C, Fioramonti J, Francois A, Robinson T, Neant F, Matuchansky C (2005). Review article: bifidobacteria as probiotic agents — physiological effects and clinical benefits. Aliment Pharmacol Ther 22(6):495-512.

Qadri F, Svennerholm AM, Faruque AS, Sack RB (2005). Enterotoxigenic *Escherichia coli* in developing countries: epidemiology, microbiology, clinical features, treatment, and prevention. Clin Microbiol Rev 18(3):465-483.

Quemada H, Zarka K, Pett W, Bothyma G, Felcher K, Mirendil H et al. (2010). Safety evaluation of the Cry11a1 protein found in the transgenic potato 'SpuntaG2'. J Am Soc Hortic Sci 135(4):325-332.

Roberts D, Hooper W, Greenwood M (2003). *Practical Food Microbiology. Methods for the Examination of Food for Micro-Organisms of Public Health Significance, 3rd edition*. Toronto (ON) / New York (NY): John Wiley. Cited In: Food Analytical Laboratories Ltd., 2011 [Microbiology Method M001].

Saarela M, Mättö J, Mattila-Sandholm T (2002). Safety aspects of *Lactobacillus* and *Bifidobacterium* species originating from human oro-gastrointestinal tract or from probiotic products. Microb Ecol Health Dis 14(4):233-240.

Sears CL, Kaper JB (1996). Enteric bacterial toxins: mechanisms of action and linkage to intestinal secretion. Microbiol Rev 60(1):167-215.

Studier FW, Daegelen P, Lenski RE, Maslov S, Kim JF (2009). Understanding the differences between genome sequences of *Escherichia coli* B strains REL606 and BL21(DE3) and comparison of the E. coli B and K-12 genomes. J Mol Biol 394(4):653-680.

Tzortzis G, Goulas AK, Gee JM, Gibson GR (2005b). A novel galactooligosaccharide mixture increases the bifidobacterial population numbers in a continuous in vitro fermentation system and in the proximal colonic contents of pigs in vivo. J Nutr 135(7):1726-1731

Tzortzis G, Goulas AK, Gibson GR (2005a). Synthesis of prebiotic galactooligosaccharides using whole cells of a novel strain, Bifidobacterium bifidum NCIMB 41171. Appl Microbiol Biotechnol 68(3):412-416.

U.S. FDA (2012). *U.S. Code of Federal Regulations (CFR). Title 21—Food and Drugs (Food and Drug Administration)*. Washington (DC): U.S. Government Printing Office (GPO). Available at: http://www.gpo.gov/fdsys/browse/collectionCfr.action?collectionCode=CFR [See Table for CFR sections].

CFR Sections Referenced (Title 21—Food and Drugs)		
Part	**Section §**	**Section Title**
170—Food additives	170.30	Eligibility for classification as generally recognized as safe (GRAS)
184—Direct food substances affirmed as generally recognized as safe	184.1387	Lactase enzyme preparation from Candida pseudotropicalis
	184.1388	Lactase enzyme preparation from Kluyveromyces lactis

Vanderhoof JA, Young RJ (1998). Use of probiotics in childhood gastrointestinal disorders. J Pediatr Gastroenterol Nutr 27(3):323-332.

Yakult Pharmaceutical Industry Co., Ltd. (2010). *Generally Recognized as Safe (GRAS) Determination for the Use of Galacto-Oligosaccharides (GOS) in Foods and Infant Formulas.* (Submitted as U.S. FDA, 2010–GRN 334). Prepared by Bethesda (MD): Spherix Consulting, Inc. for Tokyo, Japan: Yakult Pharmaceutical Industry Co., Ltd. for submission to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety (HFS-200). Available at: http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=334.

Expert Panel Report on the Generally Recognized as Safe Determination of BbgIV Beta-galactosidase Enzyme Preparation for Use in the Manufacture of Bimuno® Galacto-oligosaccharides

June 17, 2013

Clasado Incorporated (Clasado) intends to use a BbgIV *beta*-galactosidase enzyme preparation produced from recombinant *Escherichia coli* BL21(DE3) expressing the *bbgIV* gene from *Bifidobacterium bifidum* NCIMB 41171, for the manufacture of the Bimuno® galacto-oligosaccharides. Under the conditions of intended use in food, BbgIV *beta*-galactosidase herein referred to as "the enzyme preparation," will be used as a processing-aid at levels consistent with current Good Manufacturing Practice (cGMP) at the minimum levels necessary for synthesis of Bimuno galacto-oligosaccharides meeting previously established food grade specifications. Clasado convened a panel ("Expert Panel") of independent scientists, qualified by their scientific training and relevant national and international experience, to conduct a critical and comprehensive evaluation of the available pertinent data and information on BbgIV *beta*-galactosidase, and determine whether the aforementioned use of the enzyme preparation is Generally Recognized as Safe (GRAS) based on scientific procedures. The Expert Panel consisted of the following individuals: Prof. Joseph F Borzelleca Ph.D. (Virginia Commonwealth University School of Medicine), Prof. Michael W. Pariza Ph.D. (University of Wisconsin), and. Prof. Stephen L. Taylor Ph.D. (University of Nebraska).

The Expert Panel, independently and collectively, critically evaluated a dossier provided by Clasado **("Documentation Supporting the Determination that BbgIV Beta-galactosidase Enzyme Preparation is Generally Recognized as Safe (GRAS) for use in the Production of Bimuno® Galacto-oligosaccharides")**, which included a summary of all available scientific data and information, both favorable and unfavorable, relevant to the safety of the intended food use of Clasado's enzyme preparation. This information was prepared in part from a comprehensive search of the scientific literature through April 2013 and also included information characterizing the manufacture of the enzyme preparation, supporting analytical data, intended conditions of use, and estimated exposure under the intended uses. In addition, the Expert Panel evaluated other information deemed appropriate or necessary.

Following its independent critical evaluation, the Expert Panel unanimously concluded that the use of Clasado's BbgIV *beta*-galactosidase enzyme preparation meeting appropriate food-grade specifications and manufactured consistent with cGMP, is GRAS for the manufacture of Bimuno galacto-oligosaccharides based on scientific procedures. A summary of the basis for the Expert Panel's conclusion is provided below.

BbgIV *beta*-galactosidase [*B. bifidum* NCIMB 41171] (EC 3.2.1.23), is a homo-dimer with a molecular weight of 248 kDa determined by size exclusion chromatography (Goulas *et al.*, 2009). The enzyme catalyzes the hydrolysis of *beta*-D-galactosidic linkages within simple disaccharides (*e.g.*, lactose) and longer-chain galacto-oligosaccharides. At high concentrations of the substrate (*i.e.*, lactose), the enzyme also can transfer of one or more D-galactosyl units onto the D-galactose substituent of lactose, and is therefore used by Clasado for the synthesis of the Bimuno galacto-oligosaccharide ingredients. Detailed biochemical studies characterizing the enzyme activity and specificity of the enzyme have been described previously (Goulas *et al*., 2009). The enzyme preparation that is the subject of Clasado's GRAS determination is produced using a recombinant strain of *E. coli* BL21(DE3) expressing the BbgIV *beta*-galactosidase gene (*bbgIV*) from *B. bifidum* NCIMB 41171. The BbgIV *beta*-galactosidase gene from *B. bifidum* NCIMB 41171 (Genbank accession number DQ443548.2) was synthesized and codon optimized for expression in *E. coli* by third-party experts (GeneArt, Germany) and cloned into a commercial expression vector (pET21a). The pET21a-BbgIV plasmid was then transformed into *E. coli* BL21(DE3). Selection of the transformed clones was achieved by screening for strains demonstrating resistance to ampicillin. The identities of all genes present within the expression cassette hosted by the recombinant organism were available to the Expert Panel. The Expert Panel reviewed information demonstrating that the codon optimized sequence present within the expression cassette is expected to encode for a recombinant BbgIV *beta*-galactosidase that is 100% homologous to that produced by wild-type *B. bifidum* NCIMB 41171. Studies demonstrated that the functional equivalence of recombinant BbgIV *beta*-galactosidase from *E. coli* expression systems is equivalent to those found within wild type *B. bifidum* NCIMB 41171 (Goulas *et al.*, 2007, 2009). The Expert Panel concluded that the production organism was sufficiently characterized for use in the safety assessment of the enzyme.

Data and information detailing the fermentation procedure used for production of the enzyme preparation were presented to the Expert Panel. A batch fed fermentation system is employed and the fermentation broth includes de-mineralized water, yeast extract, soy peptone, glucose, mineral salts and antifoaming aids. Ampicillin is used during fermentation to stabilize growth of the organism, and isopropylthio-β-galactoside (IPTG) is used for induction of recombinant enzyme expression. Quality control measures include the use of master and working cultures with limited sub-culturing for maintenance of genomic and phenotypic stability of the organism. All fermenters and interconnecting lines, seed cultures, and fermentation broths are sterilized before use. Liberation of BbgIV *beta*-galactosidase is achieved by homogenization of the cells, and the cellular debris is removed using (b) (4) and (b) filtration methods. Removal of all small molecules originating from the fermentation process, including fermentation aids and metabolites of fermentation, is achieved *via* the use of extensive washing and filtration through a 10 kDa filter. The final solution is then filter-sterilized through a 0.22 µm bacto-filtration unit.

Appropriate food grade specifications were established by Clasado for the enzyme preparation. The specifications include limits for enzyme activity, pH, total organic solids, protein, ash, heavy metals, and microbial contamination. Each lot of the enzyme preparation is certified to be free of the process organism. Additional limits confirming the absence of antibiotics and aflatoxin contamination also are included with the product specifications. Clasado states that all methods of analysis utilized are conducted using validated methods. The Expert Panel reviewed the results from 3 non-consecutive commercial lots of the enzyme preparation confirming that the manufacturing process produces a consistent material in conformance with the product specifications. The recombinant strain contains a *beta*-lactamase encoded with the plasmid vector. This vector contains an *E. coli* origin of replication and is therefore unable to replicate in other hosts. During the cell disruption stage of the manufacturing process, release of endogenous endonucleases and exonucleases are expected to degrade cellular DNA, and residual DNA/RNA are removed from solution during processing through the use of flocculation aids and filtration procedures. Ultra-filtration using a 10 kDa filter during production of Bimuno galacto-oligosaccharides also will prevent transfer of any residual DNA into the finished product. Using quantitative PCR (qPCR) analysis methods, three commercial lots of galacto-oligosaccharides, as manufactured by Clasado using the BbgIV enzyme preparation, were analyzed for the presence of intact full-length copies of the antibiotic resistance gene. All samples were negative for the gene at a detection limit of 68 pg per sample.

It is the Expert Panel's understanding that the BbgIV *beta*-galactosidase enzyme preparation is intended for exclusive use in the production of Bimuno galacto-oligosaccharides as manufactured by Clasado. Exclusion of the enzyme preparation from Clasado's galacto-oligosaccharides is achieved by the use of ultra-filtration. Protein analyses conducted on 4 lots of galacto-oligosaccharide syrup demonstrate the absence of protein above a detection limit of 0.1%. Accordingly, the consuming population will not be exposed to the enzyme preparation from food uses of Bimuno galacto-oligosaccharides.

Data and information characterizing the safety of the enzyme preparation, host, and donor organism, were reviewed by the Expert Panel. Since the enzyme preparation is intended for exclusive use for the production of Bimuno galacto-oligosaccharides, the Expert Panel considered production controls employed during the manufacture of the enzyme preparation and Bimuno galacto-oligosaccharides, specifically, the use of a 10 kDA filter at two stages of the manufacturing process, would be sufficient to establish that transfer of the recombinant organism and other metabolites/processing-aids originating from the fermentation into Bimuno galacto-oligosaccharides would not occur. This conclusion is supported by analytical data demonstrating the absence of the recombinant organism in the enzyme preparation and data confirming the absence of ampicillin residues and *E. coli* cell-wall derived lipopolysaccharides in Bimuno galacto-oligosaccharides. Analytical data demonstrating the absence of protein at a

detection limit of 0.1% in Bimuno galacto-oligosaccharides further demonstrate that dietary exposure to the enzyme preparation from intended food uses will not occur.

The Expert Panel critically evaluated the safety of the host strain, *E. coli* BL21(DE3), used for production of the recombinant organism. *Escherichia coli* BL21(DE3) is a common laboratory strain with an established history of safe use in industrial biotechnology and is classified as a Risk Group 1 organism, *agents not associated with disease in healthy adult humans*, in biological safety guidelines (Archer *et al*., 2011). The strain is taxonomically categorized as a group A strain, and is genetically distinct from non-related pathogenic strains that are typically categorized within groups B2, D, and E (Archer *et al*., 2011). The Expert Panel noted that the *E. coli* strain, *E. coli* K-12 also is categorized as a group A strain, and this strain has an established history of safe use by the food industry for production of food additives. Chymosin produced by recombinant *E. coli* K-12 is GRAS affirmed by the U.S. Food and Drug Administration for use in specific food applications (U.S. FDA, 2012). The Expert Panel also understands that *E. coli* K-12 is widely used by food manufacturers globally for the production of recombinant strains used in the production of food ingredients. Although *E. coli* K-12 and *E. coli* BL21(DE3) are not closely related strains, both organisms are members of the group A classification and completed gene sequences of both strains have been published (Blattner *et al.*, 1997; Jeong *et al*., 2009; Archer *et al*., 2011). In addition, comprehensive comparative bioinformatic evaluations of the gene sequences of *E. coli* BL21(DE3) and *E. coli* K-12 have been conducted (Studier *et al.*, 2009). Genetic differences between *E. coli* BL21(DE3) and *E. coli* K-12 of pathogenic or toxicological significance have not been reported in these evaluations, and no known *E. coli* toxin has been reported (Studier *et al*., 2009).

The pathogenicity of *E. coli* strain BL21 was evaluated by Chart *et al*. (2000). Groups of 5 BALB/c mice were administered a single gavage dose of $1x10^6$ cfu of viable BL21 bacteria and monitored over a 7-day observation period. All animals were evaluated for viable bacteria in spleen, liver and kidney tissue. Animals administered BL21 displayed normal health throughout the observation period, and viable colonies of BL21 could not be recovered from the tissues sampled. The authors also evaluated the pathogenicity of BL21 in 1-day-old chicks, an animal model susceptible to invasion of the gut mucosa by non-pathogenic organisms. Birds were administered $1x10^2$ cfu of viable BL21 or the *E. coli* pathogen (EQ1). The birds were euthanized at 24, 48, and 72 hours post-inoculation. Gross pathology, histology and bacteriology were conducted on the liver, spleen, and caeca. As expected, transient colonization of the liver with BL21 was observed, and colonization of the liver and spleen with EQ1 was observed. The authors reported that failure of BL21 to colonize the spleen was indicative that the strain is sensitive to macrophage mediated clearance. Semi-quantitative measures of gut colonization using cloacal swabbing resulted in low-level colonization for BL21 strains in 50% of the chicks at week 6. This observation was in contrast to observations in chicks administered an avian adapted O78 pathogen where high-level colonization was

observed under identical conditions at week 6 in 50% of the birds. In persistence studies in 4-month-old Merino crossbred sheep administered $2.5x10^9$ viable BL21 or EQ1, viable isolates of BL21 could not be detected at anytime. *In vitro* observations by the authors demonstrating the absence of long-chain lipopolysaccharides, iron regulated proteins, fimbriae, siderophores and hemaglutination activity further support that strain BL21 lacks pathogenic genotypic and phenotypic traits that are common in virulent strains of *E. coli*. The authors concluded that *E. coli* "*BL21, did not have the well-recognized pathogenic mechanisms required by strains of E. coli causing the majority of enteric infections.*"

The Expert Panel noted that studies by Chart and colleagues demonstrating that *E. coli* BL21 exhibits limited capacity to colonize the gastrointestinal tract of animals is an expected outcome in microorganisms that have been subjected to extensive genetic manipulation. Such genetic modifications are intended to optimize production of an intended recombinant protein of interest (*e.g.*, enzyme) over all other metabolic and genetic capacities of the organism; these changes typically place the strain at a significant metabolic disadvantage to natural organisms in the environment. Fermentation organisms produced using both traditional and recombinant gene manipulation techniques are typically so fastidious in their nutritional requirements that they could not survived outside the laboratory (Junod, 2007).

The Expert Panel concluded that there was sufficient generally available published information to conclude that the host organism, *E. coli* BL21(DE3) was non-pathogenic and non-toxicogenic and therefore suitable and safe for use in the production of enzyme preparations intended for use in food applications.

The Expert Panel also critically evaluated information supporting the safety of the donor strain, *B. bifidum* NCIMB 41171, used for cloning the BbgIV *beta*-galactosidase gene. The Expert Panel noted that the cloned gene used for production of the expression cassette is well characterized, and exogenous protein expression products synthesized by recombinant *E. coli* BL21(DE3) would be limited to the synthesis of BbgIV *beta*-galactosidase. *Bifidobacterium bifidum* NCIMB 41171 is a human commensal strain, and strains of *Bifidobacterium bifidum* are commonly used as probiotic ingredients (Mogensen *et al.*, 2002). The safety of *Bifidobacterium* species used in food production has been reviewed extensively by various qualified experts including the FEEDAP Panel of the European Food Safety Authority (Holzapfel *et al.*, 1998; Saarela *et al.*, 2002; Borrielo *et al.*, 2003; Picard *et al.*, 2005; Boyle *et al.*, 2006; EFSA, 2007, 2012; Vankerckhoven *et al*., 2008). *Bifidobacterium bifidum* was granted QPS status by the European Food Safety Authority in 2007 without qualification on use, a determination that has been maintained following yearly review (EFSA, 2007, 2012). Based on its critical evaluation of the extensive information characterizing the safety of the *Bifodobacterium* genus, the Expert Panel concluded that cloning of the *bbgIV* gene from *Bifidobacterium bifidum* NCIMB 41171 would not be a safety concern as there are no known toxic, allergenic, or other undesirable

effects of toxicological concern that have been identified in the literature for members of this species or genus.

The Expert Panel critically evaluated information submitted by Clasado characterizing the toxicity of *beta*-galactosidase enzyme preparations. Whole cell isolates and enzyme preparations containing *beta*-galactosidases (*i.e*., EC 3.2.1.23) were evaluated in 90-day subchronic feeding toxicity studies using male and female Sprague-Dawley rats. These studies, conducted in accordance with GLP and internationally accepted guidelines for the toxicity testing of chemicals (*i.e*., OECD guideline 408), demonstrated that the *beta*-galactosidase preparations were non-toxic in this rat species, with no observed adverse effect levels of greater than 10 mL yeast extract (providing >25,000 mU of *beta*-galactosidase activity)/kg body weight/day and 13,150 mg *beta*-galactosidase enzyme preparation/kg body weight/day (potency of the test article was 5,200 SU/g) in male and female rats, the highest doses tested (U.S. FDA, 2009, 2010). The Expert Panel also noted that *beta*-galactosidase preparations have a long-history of safe use in the United States as a direct food ingredient for use in the hydrolysis of lactose in milk preparations (U.S. FDA, 2012). The Expert Panel considered enzyme preparations used for food applications to be of low toxic potential (Pariza and Foster 1983; Pariza and Johnson, 2001). The Expert Panel evaluated the BbgIV *beta*-galactosidase enzyme preparation using the Pariza-Johnson decision tree and concluded that it was safe and acceptable for use in food (see Attachment A).

Since a search of the published amino acid sequence of BbgIV *beta*-galactosidase, using the web-based database Allergen Online (version 13), for relevant matches to known putative allergens did not identify relevant matches[1] suggesting that BbgIV *beta*-galactosidase from *B. bifidum* NCIMB 41171 would be potentially allergenic, the Expert Panel concluded that the allergenicity of the enzyme preparation should not be a health concern. The current history of safe use of *beta*-galactosidases as direct food ingredients for hydrolysis of lactose in milk products corroborates the conclusion that *beta*-galactosidase preparations are of low allergenic potential.

[1] Relevant matches were defined as hits ≥35% identity within 80 amino acid sliding window segments. This 35% identity for 80 amino acid segments is a suggested guideline proposed by Codex for evaluating proteins in genetically modified crops (Codex, 2003, Gooodman *et al*., 2008).

Conclusion

We, the members of the Expert Panel, have independently and collectively critically evaluated the information summarized above and conclude that Clasado's BbgIV *beta*-galactosidase enzyme preparation produced by fermentation from recombinant *E. coli* BL21(DE3) expressing the *bbgIV* gene from *B. bifidum* NCIMB 41171, meeting appropriate food-grade specifications and manufactured in accordance with current Good Manufacturing Practice, is safe and suitable for its intended use in the production of Bimuno galacto-oligosaccharides

We, the members of the Expert Panel, have independently and collectively critically evaluated the information summarized above and conclude that Clasado's BbgIV *beta*-galactosidase enzyme preparation produced by fermentation from recombinant *E. coli* BL21(DE3) expressing the *bbgIV* gene from *B. bifidum* NCIMB 41171, meeting appropriate food-grade specifications and manufactured in accordance with current Good Manufacturing Practice, is Generally Recognized as Safe (GRAS) based on scientific procedures for its intended use in the production of Bimuno galacto-oligosaccharides.

It is our opinion that other qualified experts would concur with these conclusions.

(b) (6)

29 June 2013

Prof. Joseph F. Borzelleca Ph.D.
Virginia Commonwealth University School of Medicine

Date

Prof. Michael W. Pariza Ph.D.
University of Wisconsin

Date

Prof. Stephen L. Taylor Ph.D.
University of Nebraska

Date

Conclusion

We, the members of the Expert Panel, have independently and collectively critically evaluated the information summarized above and conclude that Clasado's BbglV *beta*-galactosidase enzyme preparation produced by fermentation from recombinant *E. coli* BL21(DE3) expressing the *bbglV* gene from *B. bifidum* NCIMB 41171, meeting appropriate food-grade specifications and manufactured in accordance with current Good Manufacturing Practice, is safe and suitable for its intended use in the production of Bimuno galacto-oligosaccharides

We, the members of the Expert Panel, have independently and collectively critically evaluated the information summarized above and conclude that Clasado's BbglV *beta*-galactosidase enzyme preparation produced by fermentation from recombinant *E. coli* BL21(DE3) expressing the *bbglV* gene from *B. bifidum* NCIMB 41171, meeting appropriate food-grade specifications and manufactured in accordance with current Good Manufacturing Practice, is Generally Recognized as Safe (GRAS) based on scientific procedures for its intended use in the production of Bimuno galacto-oligosaccharides.

It is our opinion that other qualified experts would concur with these conclusions.

Prof. Joseph F. Borzelleca Ph.D.
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Date

(b) (6)

Prof. Michael W. Pariza Ph.D.
University of Wisconsin

5 July 2013
Date

Prof. Stephen L. Taylor Ph.D.
University of Nebraska

Date

Conclusion

We, the members of the Expert Panel, have independently and collectively critically evaluated the information summarized above and conclude that Clasado's BbglV *beta*-galactosidase enzyme preparation produced by fermentation from recombinant *E. coli* BL21(DE3) expressing the *bbglV* gene from *B. bifidum* NCIMB 41171, meeting appropriate food-grade specifications and manufactured in accordance with current Good Manufacturing Practice, is safe and suitable for its intended use in the production of Bimuno galacto-oligosaccharides

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Prof. Joseph F. Borzelleca Ph.D. Virginia Commonwealth University School of Medicine	Date
Prof. Michael W. Pariza Ph.D. University of Wisconsin	Date
(b) (6) [redacted] Prof. Stephen L. Taylor Ph.D. University of Nebraska	28 June 2013 Date

REFERENCES

Archer CT, Kim JF, Jeong H, Park JH, Vickers CE, Lee SY et al. (2011). The genome sequence of *E. coli* W (ATCC 9637): comparative genome analysis and an improved genome-scale reconstruction of *E. coli*. BMC Genomics 12:9. doi:10.1186/1471-2164-12-9.

Blattner FR, Plunkett G, Bloch CA, Perna NT, Burland V, Riley M et al. (1997). The complete genome sequence of *Escherichia coli* K-12. Science 277(5331):1453-1462.

Borriello SP, Hammes WP, Holzapfel W, Marteau P, Schrezenmeir J, Vaara M et al. (2003). Safety of probiotics that contain lactobacilli or bifidobacteria. Clin Infect Dis 36(6):775-780.

Boyle RJ, Robins-Browne RM, Tang ML (2006). Probiotic use in clinical practice: What are the risks? Am J Clin Nutr 83(6):1256-1264.

Chart H, Smith HR, La Ragione RM, Woodward MJ (2000). An investigation into the pathogenic properties of *Escherichia coli* strains BLR, BL21, DH5alpha and EQ1. J Appl Microbiol 89(6):1048-1058.

EFSA (2007). Opinion of the Scientific Committee on a request from EFSA on the introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA (question no EFSA-Q-2005-293, adopted on 19 November 2007 by European Food Safety Authority). EFSA J 587:1-16. Available at: http://www.efsa.europa.eu/en/efsajournal/pub/587.htm.

EFSA (2012). EFSA Panel on Biological Hazards (BIOHAZ); Scientific Opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed (2012 update) (question no EFSA-Q-2012-00218, adopted on 6 December 2012 by European Food Safety Authority). EFSA J 10(12):3020. [84 pp.] doi:10.2903/j.efsa.2012.3020. Available at: http://www.efsa.europa.eu/en/efsajournal/pub/3020.htm.

Goulas TK, Goulas AK, Tzortzis G, Gibson GR (2007). Molecular cloning and comparative analysis of four β-galactosidase genes from *Bifidobacterium bifidum* NCIMB41171. Appl Microbiol Biotechnol 76(6):1365-1372.

Goulas T, Goulas A, Tzortzis G, Gibson GR (2009). Comparative analysis of four β-galactosidases *from Bifidobacterium bifidum* NCIMB41171: purification and biochemical characterisation. Appl Microbiol Biotechnol 82(6):1079-1088.

Holzapfel WH, Haberer P, Snel J, Schillinger U, Huis in't Veld JH (1998). Overview of gut flora and probiotics. Int J Food Microbiol 41(2):85-101.

Jeong H, Barbe V, Lee CH, Vallenet D, Yu DS, Choi SH et al. (2009). Genome sequences of *Escherichia coli* B strains REL606 and BL21(DE3). J Mol Biol 394(4):644-652.

Junod SW (2007). *Celebrating a Milestone: FDA's Approval of First Genetically-Engineered Product*. (Originally appeared in FDLI's Update Magazine, Sep./Oct.). Washington (DC): Food and Drug Law Institute (FDLI). College Park (MD): U.S. Food and Drug Administration (U.S. FDA). Available at: http://www.fda.gov/AboutFDA/WhatWeDo/History/ProductRegulation/SelectionsFromFDLIUpdateSeriesonFDAHistory/ucm081964.htm

Mogensen G, Salminen S, O'Brien J, Ouwehand A, Holzapfel W, Shortt C et al. (2002). Inventory of microorganisms with a documented history of use in food. In: *Health Benefits and Safety Evaluation of Certain Food Components*. (Bulletin of the International Dairy Federation, No. 377). Brussels, Belgium: International Dairy Federation (IDF), pp. 10-19.

Pariza MW, Foster EM (1983). Determining the safety of enzymes used in food processing. J Food Prot 46(5):453-468.

Pariza MW, Johnson EA (2001). Evaluating the safety of microbial enzyme preparations used in food processing: update for a new country. Regul Toxicol Pharmacol 33(2):173-186.

Picard C, Fioramonti J, Francois A, Robinson T, Neant F, Matuchansky C (2005). Review article: bifidobacteria as probiotic agents — physiological effects and clinical benefits. Aliment Pharmacol Ther 22(6):495-512.

Saarela M, Mättö J, Mattila-Sandholm T (2002). Safety aspects of *Lactobacillus* and *Bifidobacterium* species originating from human oro-gastrointestinal tract or from probiotic products. Microb Ecol Health Dis 14(4):233-240.

Studier FW, Daegelen P, Lenski RE, Maslov S, Kim JF (2009). Understanding the differences between genome sequences of *Escherichia coli* B strains REL606 and BL21(DE3) and comparison of the E. coli B and K-12 genomes. J Mol Biol 394(4):653-680.

U.S. FDA (2009). *GRN No. 285 - Galacto-oligosaccharides.* (GRAS Notice Inventory). Submitted by Golden (CO)*:* GTC Nutrition to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=285.

U.S. FDA (2010). *GRN No. 334 - Galacto-oligosaccharides*. (GRAS Notice Inventory). Submitted by Tokyo, Japan: Yakult Pharmaceutical Industry Co., Ltd. to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=334.

U.S. FDA (2012). *U.S. Code of Federal Regulations (CFR). Title 21—Food and Drugs (Food and Drug Administration)*. Washington (DC): U.S. Government Printing Office (GPO). Available at: http://www.gpo.gov/fdsys/browse/collectionCfr.action?collectionCode=CFR [See Table for CFR sections].

CFR Sections Referenced (Title 21—Food and Drugs)		
Part	**Section §**	**Section Title**
184—Direct food substances affirmed as generally recognized as safe	184.1387	Lactase enzyme preparation from Candida pseudotropicalis
	184.1388	Lactase enzyme preparation from Kluyveromyces lactis

Vankerckhoven V, Huys G, Vancanneyt M, Vael C, Klare I, Romond M-B et al. (2008). Biosafety assessment of probiotics used for human consumption: recommendations from the EU-PROSAFE project. Trends Food Sci Technol 19(2):102-114.

ATTACHMENT A

Pariza-Foster Decision Tree Analysis

This analysis is based on the Decision Tree of MW Pariza and EA Johnson (2001): *Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing: Update for a New Century*, Regulatory Toxicology and Pharmacology, 33:173-186. Decision points that do not pertain are included for completeness but crossed out.

1. Is the production strain genetically modified?
If yes, go to 2. If no, go to 6. **YES**

2. Is the production strain modified using rDNA techniques?
If yes, go to 3. If no, go to 5. **YES**

3. Issues relating to the introduced DNA are addressed in 3a–3e.

3a. Do the expressed enzyme product(s) which are encoded by the introduced DNA have a history of safe use in food?
If yes, go to 3c. If no, go to 3b **YES**

~~3b. Is the NOAEL for the test article in appropriate short-term oral studies sufficiently high to ensure safety?~~
~~If yes, go to 3c. If no, go to 12.~~

3c. Is the test article free of transferable antibiotic resistance gene DNA?
If yes, go to 3e. If no, go to 3d. **YES**

~~3d. Does the resistance gene(s) code for resistance to a drug substance used in treatment of disease agents in man or animal? If yes, go to 12. If no, go to 3e.~~

3e. Is all other introduced DNA well characterized and free of attributes that would render it unsafe for constructing microorganisms to be used to produce food-grade products?
If yes, go to 4. If no, go to 12. **YES**

4. Is the introduced DNA randomly integrated into the chromosome?
If yes, go to 5. If no, go to 6. **NO**

~~5. Is the production strain sufficiently well characterized so that one may reasonably conclude that unintended pleiotropic effects which may result in the synthesis of toxins or other unsafe metabolites will not arise due to the genetic modification method that was employed?~~
~~If yes, go to 6. If no, go to 7.~~

6. Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure? **YES, *Escherichia coli* BL21(DE3) has been repeatedly tested *via* evaluation procedures that meet and exceed the criteria of this evaluation procedure.**
If yes, the test article is ACCEPTED. If no, go to 7. **TEST ARTICLE IS ACCEPTED**
~~7. Is the organism nonpathogenic?~~
~~If yes, go to 8. If no, go to 12.~~

~~8. Is the test article free of antibiotics?~~

~~If yes, go to 9. If no, go to 12.~~

~~9. Is the test article free of oral toxins known to be produced by other members of the same species?~~
~~If yes, go to 11. If no, go to 10.~~

~~10. Are the amounts of such toxins in the test article below levels of concern?~~
~~If yes, go to 11. If no, go to 12.~~

~~11. Is the NOAEL for the test article in appropriate oral studies sufficiently high to ensure safety?~~
~~If yes, the test article is ACCEPTED.~~

~~12. An undesirable trait or substance may be present and the test article is not acceptable for food use. If the genetic potential for producing the undesirable trait or substance can be permanently inactivated or deleted, the test article may be passed through the decision tree again.~~

SUBMISSION END