ORIGINAL SUBMISSION

KELLER AND HECKMAN LLP
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RECEIVED
APR 26 2016
OFFICE OF
FOOD ADDITIVE SAFETY

Writer's Direct Access
Richard F. Mann
(202) 434-4229
mann@khlaw.com

#649
GRN 000649

April 25, 2016

Via FedEx

Antonia Mattia, Ph.D., Director
Division of Biotechnology and GRAS Notice Review (HFS-225)
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Pkwy
College Park, MD 20740

Re: GRAS Notification for β-galactosidase Enzyme Preparation

Dear Dr. Mattia:

We respectfully submit the attached GRAS Notification on behalf of our client, GenoFocus, Inc. (GenoFocus), for a β-galactosidase enzyme preparation derived from *Bacillus circulans* (*B. circulans*) expressed in *Bacillus subtilis* (*B. subtilis*). The β-galactosidase enzyme will be used as a processing aid in the production of galacto-oligosaccharides (GOS), which, in turn, may be used in a variety of food applications (including products consumed by adults and infants (children < 1 year of age)).[1] The attached GRAS Notification provides a review of the information related to the intended uses and manufacturing and safety of the ingredient.

This GRAS Notification includes confidential and proprietary commercial information. We respectfully request that FDA treat designated portions of this GRAS Notification as confidential business information (CBI) exempt from disclosure under 5 U.S.C. § 552(b)(4). We have included three (3) hard copies of the complete GRAS Notification for your review, along with three (3) hard copies of the GRAS Notification with CBI redacted. We also have enclosed a separate document specifically designating the information that GenoFocus considers to be CBI within the GRAS Notification. Should the Agency have any questions or concerns about the

[1] GOS produced with the use of the GenoFocus β-galactosidase enzyme preparation will meet the specifications provided in GRN No. 236 and is intended to be used in the same food applications covered in that GRAS Notice. See GRN No. 236, at Tables II-4 and II-5 on pages 000025-000026; and Table 1 on page 000004.

information that GenoFocus has designated as CBI, we respectfully request that you contact us to discuss these issues prior to filing the Notification.

GenoFocus has determined that its β-galactosidase enzyme preparation is generally recognized as safe (GRAS) based on scientific procedures in accordance with 21 C.F.R. § 170.30(b) and in conformance with the guidance issued by the Food and Drug Administration (FDA) under *proposed* 21 C.F.R. § 170.36, 62 Fed. Reg. 18938 (Apr. 17, 1997). Therefore, the use of the β-galactosidase enzyme preparation in the production of GOS as described in this GRAS Notification is exempt from the requirement of premarket approval as set forth in the Federal Food, Drug, and Cosmetic Act.

The analytical data, published studies, and information that are the basis for this GRAS determination are available for FDA review and copying at reasonable times at Keller and Heckman LLP, 1001 G Street, NW, Suite 500W, Washington, DC 20001, or will be sent to FDA upon request.

We look forward to the Agency's review of this submission and would be happy to provide Agency officials with any information they may need to complete their assessment. Thank you for your attention to this matter.

Sincerely,

(b) (6)

Richard F. Mann

Enclosures

GRN 000649

COMPLETE VERSION – CONFIDENTIAL BUSINESS INFORMATION NOT REDACTED

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FOOD ADDITIVE SAFETY

Form Approved: OMB No. 0910-0342; Expiration Date: 02/29/2016
(See last page for OMB Statement)

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

GENERALLY RECOGNIZED AS SAFE *(GRAS)* NOTICE

FDA USE ONLY

GRN NUMBER	DATE OF RECEIPT
ESTIMATED DAILY INTAKE	INTENDED USE FOR INTERNET
NAME FOR INTERNET	
KEYWORDS	

RECEIVED
APR 28 2016
OFFICE OF FOOD ADDITIVE SAFETY

Transmit completed form and attachments electronically via the Electronic Submission Gateway *(see Instructions)*, OR Transmit completed form and attachments in paper format or on physical media to: Office of Food Additive Safety *(HFS-200)*, Center for Food Safety and Applied Nutrition, Food and Drug Administration, 5100 Paint Branch Pkwy., College Park, MD 20740-3835.

PART I – INTRODUCTORY INFORMATION ABOUT THE SUBMISSION

1. Type of Submission *(Check one)*
☒ New ☐ Amendment to GRN No. _____ ☐ Supplement to GRN No. _____

2. ☐ All electronic files included in this submission have been checked and found to be virus free. *(Check box to verify)*

3a. For New Submissions Only: Most recent presubmission meeting *(if any)* with FDA on the subject substance *(yyyy/mm/dd)*: _____

3b. For Amendments or Supplements: Is your amendment or supplement submitted in response to a communication from FDA? *(Check one)* ☐ Yes ☐ No If yes, enter the date of communication *(yyyy/mm/dd)*: _____

PART II – INFORMATION ABOUT THE NOTIFIER

1a. Notifier

Name of Contact Person	Position
Jeong Hyun Kim	Principal Scientist

Company *(if applicable)*: GenoFocus, Inc.

Mailing Address *(number and street)*: 65, Techno 1-ro, Yuseong-gu

City	State or Province	Zip Code/Postal Code	Country
Daejeon	N/A	34014	Korea

Telephone Number	Fax Number	E-Mail Address
+82-42-4483(302)		jhkim@genofocus.com

1b. Agent or Attorney *(if applicable)*

Name of Contact Person	Position
Richard F. Mann	Partner

Company *(if applicable)*: Keller and Heckman LLP

Mailing Address *(number and street)*: 1001 G St. NW, Suite 500W

City	State or Province	Zip Code/Postal Code	Country
Washington	DC	20001	

Telephone Number	Fax Number	E-Mail Address
202-434-4229	202-434-4646	mann@khlaw.com

PART III – GENERAL ADMINISTRATIVE INFORMATION

1. Name of Substance
ß-galactosidase enzyme preparation

2. Submission Format: *(Check appropriate box(es))*
☐ Electronic Submission Gateway
☐ Electronic files on physical media with paper signature page
☒ Paper
If applicable give number and type of physical media

3. For paper submissions only:
Number of volumes 1
Total number of pages 25

4. Does this submission incorporate any information in FDA's files by reference? *(Check one)*
☒ Yes *(Proceed to Item 5)* ☐ No *(Proceed to Item 6)*

5. The submission incorporates by reference information from a previous submission to FDA as indicated below *(Check all that apply)*
☒ a) GRAS Notice No. GRN 236
☐ b) GRAS Affirmation Petition No. GRP
☐ c) Food Additive Petition No. FAP
☐ d) Food Master File No. FMF
☐ e) Other or Additional *(describe or enter information as above)*

6. Statutory basis for determination of GRAS status *(Check one)*
☒ Scientific Procedures *(21 CFR 170.30(b))* ☐ Experience based on common use in food *(21 CFR 170.30(c))*

7. Does the submission (including information that you are incorporating by reference) contain information that you view as trade secret or as confidential commercial or financial information?
☒ Yes *(Proceed to Item 8)*
☐ No *(Proceed to Part IV)*

8. Have you designated information in your submission that you view as trade secret or as confidential commercial or financial information *(Check all that apply)*
☒ Yes, see attached Designation of Confidential Information
☒ Yes, information is designated at the place where it occurs in the submission
☐ No

9. Have you attached a redacted copy of some or all of the submission? *(Check one)*
☒ Yes, a redacted copy of the complete submission
☐ Yes, a redacted copy of part(s) of the submission
☐ No

PART IV – INTENDED USE

1. Describe the intended use of the notified substance including the foods in which the substance will be used, the levels of use in such foods, the purpose for which the substance will be used, and any special population that will consume the substance *(e.g., when a substance would be an ingredient in infant formula, identify infants as a special population).*

The ß-galactosidase enzyme preparation will be used as a processing aid in the production of galacto-oligosaccharides (GOS), which, in turn, may be used in a variety of food applications (including products consumed by adults and infants (children <1 year of age)). The inclusion rate for the β-galactosidase enzyme preparation is up to 0.3% (by percentage of the lactose starting material that is used to produce GOS).The enzyme will be removed by ultrafiltration during GOS production and thus the finished GOS will not contain detectable concentrations of the enzyme protein.

2. Does the intended use of the notified substance include any use in meat, meat food product, poultry product, or egg product? *(Check one)*
☐ Yes ☒ No

PART V – IDENTITY

1. Information about the Identity of the Substance

	Name of Substance[1]	Registry Used *(CAS, EC)*	Registry No.[2]	Biological Source *(if applicable)*	Substance Category *(FOR FDA USE ONLY)*
1	ß-galactosidase	CAS IUBMB	9031-11-2 3.2.1.23	*B. subtilis* expressing gene from *B. circulans*	
2					
3					

[1] Include chemical name or common name. Put synonyms *(whether chemical name, other scientific name, or common name)* for each respective item (1 - 3) in Item 3 of Part V *(synonyms)*

[2] Registry used e.g., CAS *(Chemical Abstracts Service)* and EC *(Refers to Enzyme Commission of the International Union of Biochemistry (IUB), now carried out by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB))*

2. Description

Provide additional information to identify the notified substance*(s)*, which may include chemical formula*(s)*, empirical formula*(s)*, structural formula*(s)*, quantitative composition, characteristic properties *(such as molecular weight(s))*, and general composition of the substance. For substances from biological sources, you should include scientific information sufficient to identify the source *(e.g., genus, species, variety, strain, part of a plant source (such as roots or leaves), and organ or tissue of an animal source)*, and include any known toxicants that could be in the source.

The ß-galactosidase enzyme preparation is a powdered enzyme preparation that contains 33-43% ß-galactosidase enzyme and 57-67% dextrin (or maltodextrin).

The ß-galactosidase enzyme component is an extracted and purified enzyme product obtained from the fermentation and processing of genetically modified *B. subtilis* expressing a ß-galactosidase gene from *B. circulans*. The chemical name of the enzyme is ß-D-galactoside galactohydrolase. It has a calculated molecular mass of 155977.22 Da.

3. Synonyms

Provide as available or relevant:

1	lactase
2	ß-D-galactosidase
3	ß-gal

PART V – IDENTITY

1. Information about the Identity of the Substance

	Name of Substance[1]	Registry Used *(CAS, EC)*	Registry No.[2]	Biological Source *(if applicable)*	Substance Category *(FOR FDA USE ONLY)*
4					
5					

[1] Include chemical name or common name. Put synonyms *(whether chemical name, other scientific name, or common name)* for each respective item (4 - 6) in Item 3 of Part V *(synonyms)*

[2] Registry used e.g., CAS *(Chemical Abstracts Service)* and EC *(Refers to Enzyme Commission of the International Union of Biochemistry (IUB), now carried out by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB))*

2. Description

Provide additional information to identify the notified substance*(s)*, which may include chemical formula*(s)*, empirical formula*(s)*, structural formula*(s)*, quantitative composition, characteristic properties *(such as molecular weight(s))*, and general composition of the substance. For substances from biological sources, you should include scientific information sufficient to identify the source *(e.g., genus, species, variety, strain, part of a plant source (such as roots or leaves), and organ or tissue of an animal source)*, and include any known toxicants that could be in the source.

3. Synonyms

Provide as available or relevant:

4	
5	

PART VI – OTHER ELEMENTS IN YOUR GRAS NOTICE

(check list to help ensure your submission is complete – check all that apply)

- ☒ Any additional information about identity not covered in Part V of this form
- ☐ Method of Manufacture
- ☒ Specifications for food-grade material
- ☒ Information about dietary exposure
- ☒ Information about any self-limiting levels of use *(which may include a statement that the intended use of the notified substance is not-self-limiting)*
- ☐ Use in food before 1958 *(which may include a statement that there is no information about use of the notified substance in food prior to 1958)*
- ☒ Comprehensive discussion of the basis for the determination of GRAS status
- ☐ Bibliography

Other Information

Did you include any other information that you want FDA to consider in evaluating your GRAS notice?

☐ Yes ☒ No

Did you include this other information in the list of attachments?

☐ Yes ☐ No

PART VII – SIGNATURE

1. The undersigned is informing FDA that GenoFocus, Inc.
(name of notifier)

has concluded that the intended use(s) of β-galactosidase enzyme preparation
(name of notified substance)

described on this form, as discussed in the attached notice, is (are) exempt from the premarket approval requirements of section 409 of the federal Food, Drug, and Cosmetic Act because the intended use(s) is (are) generally recognized as safe.

2. ☒ GenoFocus, Inc. *(name of notifier)* agrees to make the data and information that are the basis for the determination of GRAS status available to FDA if FDA asks to see them.

GenoFocus, Inc. *(name of notifier)* agrees to allow FDA to review and copy these data and information during customary business hours at the following location if FDA asks to do so.

Keller and Heckman LLP, 1001 G St. NW, Suite 500W, Washington, DC 20001
(address of notifier or other location)

GenoFocus, Inc. *(name of notifier)* agrees to send these data and information to FDA if FDA asks to do so.

OR

☐ The complete record that supports the determination of GRAS status is available to FDA in the submitted notice and in GRP No. ----------
(GRAS Affirmation Petition No.)

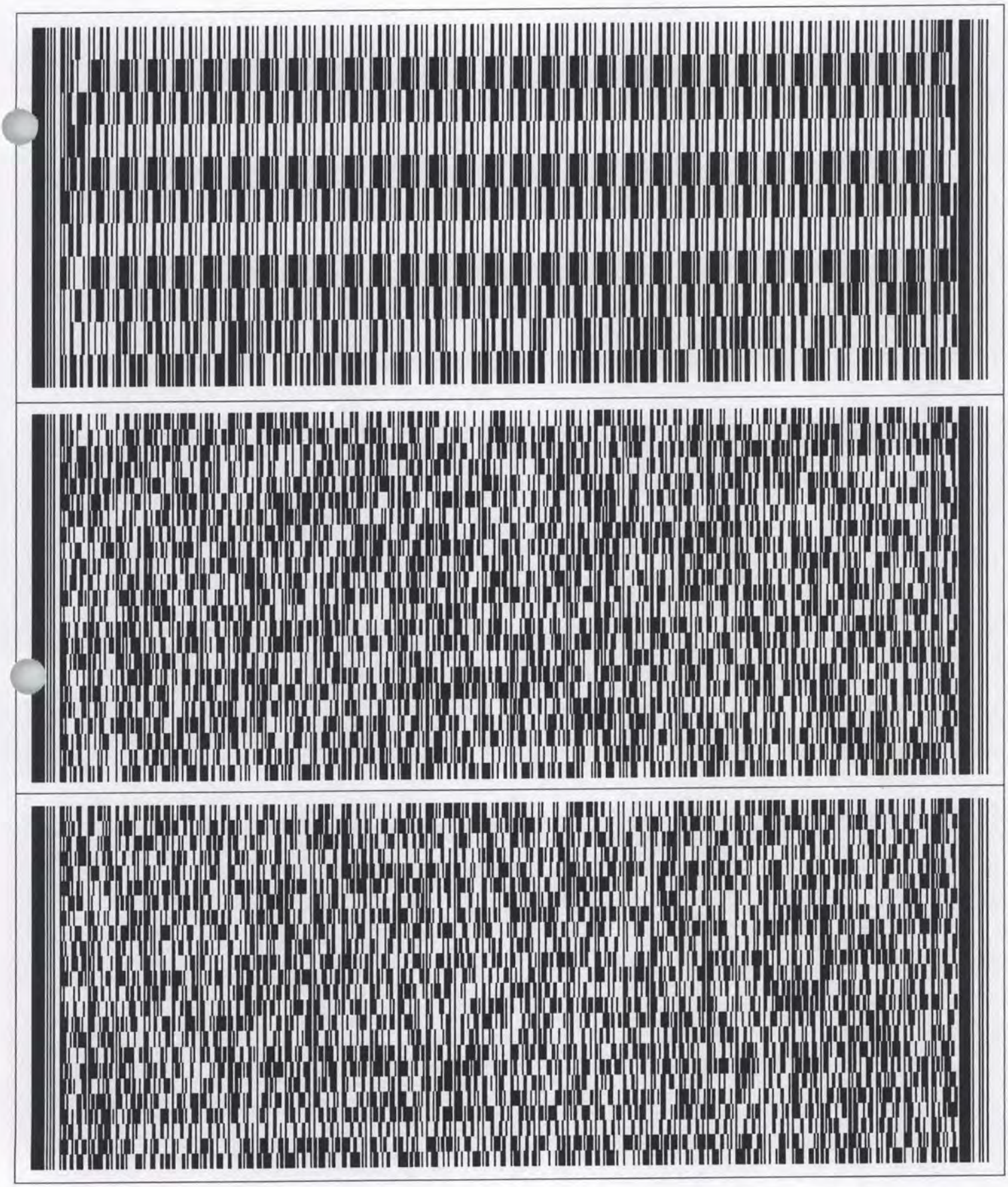
3. Signature of Responsible Official, Agent, or Attorney	Printed Name and Title	Date (mm/dd/yyyy)
(b) (6)	Richard F. Mann	04/25/2016

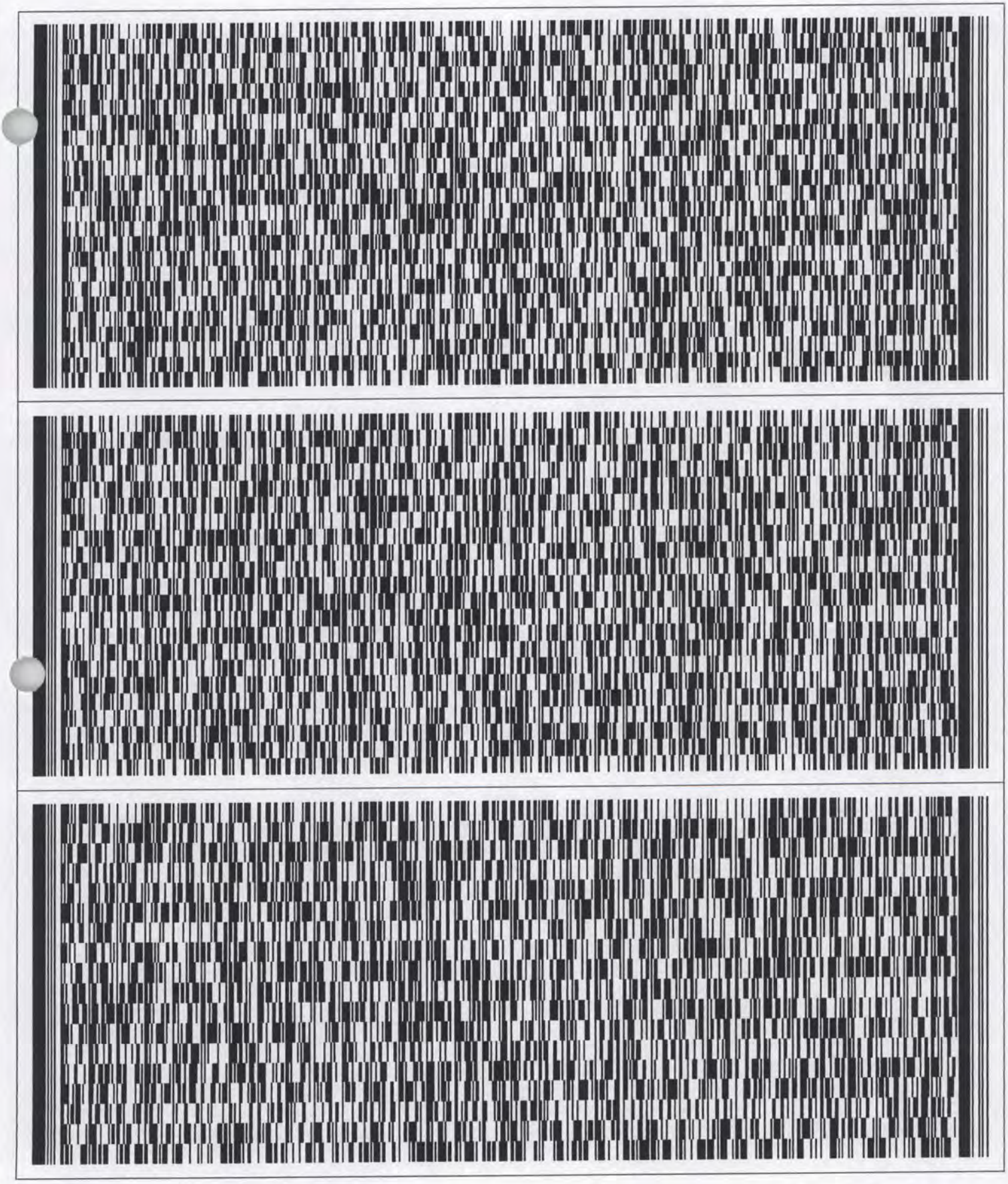
PART VIII – LIST OF ATTACHMENTS

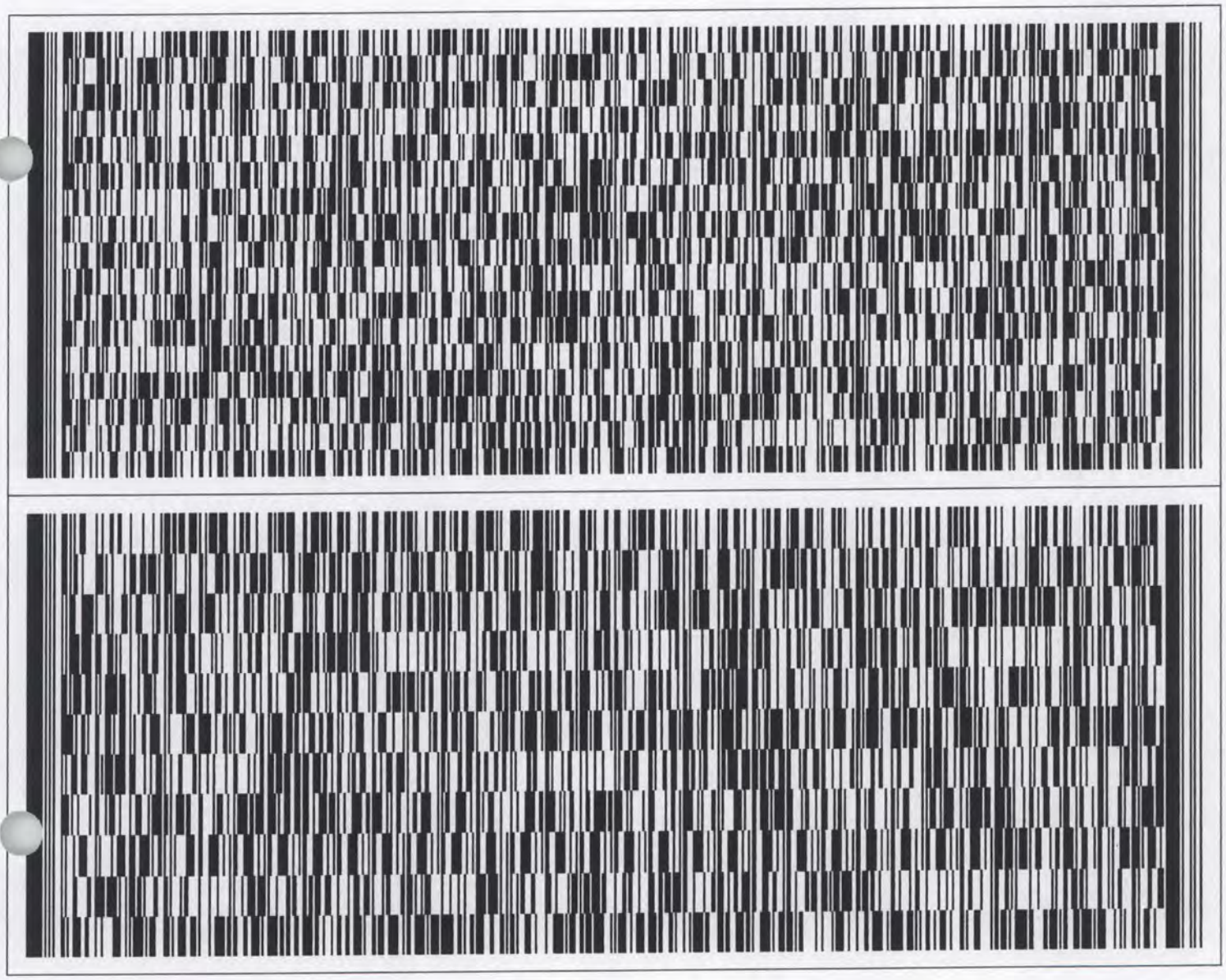
List your attached files or documents containing your submission, forms, amendments or supplements, and other pertinent information. Clearly identify the attachment with appropriate descriptive file names (or titles for paper documents), preferably as suggested in the guidance associated with this form. Number your attachments consecutively. When submitting paper documents, enter the inclusive page numbers of each portion of the document below.

Attachment Number	Attachment Name	[redacted]
	Cover Letter to Dr. Antonia Mattia	N/A
	Designation of Confidential Information	N/A
	GRAS Notification for ß-galactosidase enzyme preparation (COMPLETE)	1-25
	[redacted]	[redacted]

[redacted] **Statement:** Public reporting burden for this collection of information is estimated to average 150 hours per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to: Department of Health and Human Services,Food and Drug Administration, Office of Chief Information Officer, 1350 Piccard Drive, Room 400, Rockville, MD 20850. (Please do NOT return the form to this address.). An agency may not conduct or sponsor, and a person is not required to respond to, a collection of information unless it displays a currently valid OMB control number.







DESIGNATION OF CONFIDENTIAL BUSINESS INFORMATION (CBI) in GRAS Notification for β-galactosidase Enzyme Preparation derived from *Bacillus circulans* expressed in *Bacillus subtilis*

The above-referenced GRAS Notification includes confidential and proprietary commercial information. We respectfully request that FDA treat designated portions of this GRAS Notification as confidential business information (CBI) exempt from disclosure under 5 U.S.C. § 552(b)(4). Should the Agency have any questions or concerns about the information that GenoFocus has designated as CBI, we respectfully request that you contact us to discuss these issues prior to filing the Notification.

We designate the following specific information as CBI and have marked it accordingly in both the complete and redacted copies of the GRAS Notification submitted to FDA:

Page Number	Type of Information Designated as CBI
7	Conditions of incubation in main fermentation step of manufacturing process
7	Conditions of agitation in main fermentation step of manufacturing process
7	Conditions of aeration in main fermentation step of manufacturing process
7	Specific level of addition of $CaCl_2$ $2H_2O$ in separation step of manufacturing process
7	Specific level of addition of K_2HPO_4 in separation step of manufacturing process
7	Pore size of membrane used in microfiltration in separation step of manufacturing process
7	Size of molecules removed during ultrafiltration as part of the concentration & partial purification step of manufacturing process
7	Specific level of addition of bulking agent in sterile filtration step of manufacturing process
7	Pore size of membrane used in microfiltration in sterile filtration step of manufacturing process
9	Pore size of membrane used in filtration steps of manufacturing process

GRAS Notification for β-galactosidase Enzyme Preparation derived from *Bacillus circulans* expressed in *Bacillus subtilis*

[redacted]

[redacted]

[redacted]

[redacted]

[redacted] 2016

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Appendices

Appendix 1	Collection Receipt Indicating Taxonomy of Production Strain GFBSL201
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I. Introduction

Keller and Heckman LLP submits the enclosed information on behalf of our client, GenoFocus, Inc. (GenoFocus), in support of this notification that a β-galactosidase enzyme preparation derived from *Bacillus circulans* (*B. circulans*) expressed in *Bacillus subtilis* (*B. subtilis*) is generally recognized as safe (GRAS) for use in the production of the food ingredient, galacto-oligosaccharides (GOS). GOS, in turn, may be used in a variety of food applications.

We submit information in the following areas:

- The identity and specifications for the β-galactosidase enzyme preparation;
- The manufacturing process for the β-galactosidase enzyme preparation;
- The intended use of the β-galactosidase enzyme;
- Supportive evidence regarding the GRAS status of other β-galactosidase enzyme preparations, including some derived from *B. circulans*, used in the production of GOS; and
- Safety information for the production strains, the donor organism (*B. circulans*), and the production organism (*B. subtilis*), as well as supportive evidence from their widespread applications in food production.

It is our expectation that FDA will concur that the information presented fully supports the determination that a β-galactosidase preparation from *B. circulans* expressed in *B. subtilis* is GRAS for use as a processing aid in the production of the food ingredient, GOS.

II. Administrative Information

A. Claim Regarding GRAS Status

GenoFocus has determined that a β-galactosidase preparation derived from *B. circulans* expressed in *B. subtilis* is GRAS for the production of GOS based on scientific procedures in accordance with 21 C.F.R. § 170.30(b) and in conformance with the guidance issued by FDA under proposed 21 C.F.R. § 170.36, 62 Fed. Reg. 18938 (Apr. 17, 1997). The analytical data, published studies, and information that are the basis for this GRAS determination are available for FDA review and copying at reasonable times at Keller and Heckman LLP, 1001 G Street, NW, Suite 500W, Washington, DC 20001, or will be sent to FDA upon request.

B. Name and Address of the Notifier

GenoFocus, Inc.
65, Techno 1-ro, Yuseong-gu, Daejeon
34014, Korea

All communications on this matter are to be sent to Counsel for the Notifier:

Richard F. Mann
Keller and Heckman LLP
1001 G Street, NW
Suite 500 West
Washington, DC 20001
Telephone: (202) 434-4229
Facsimile: (202) 434-4646
Email: mann@khlaw.com

C. Common or Usual Name of GRAS Substance

The common or usual name for the GRAS ingredient is "β-galactosidase enzyme preparation." GenoFocus's product will be marketed under the trade name, "GF lactase B2."

D. Conditions of Use

The β-galactosidase enzyme preparation will be used as a processing aid in the production of GOS, which, in turn, may be used in a variety of food applications.[1] GOS produced with the use of the GenoFocus β-galactosidase enzyme preparation will meet the specifications provided in GRN No. 236[2] and is intended to be used in the same food applications covered in that GRAS Notice.[3] The inclusion rate for the β-galactosidase enzyme preparation is up to 0.3% (by percentage of the lactose starting material that is used to produce GOS).

E. Self-Limiting Levels of Use

The use of the β-galactosidase enzyme preparation to produce GOS is limited by the level that can economically be added to maximize enzyme efficiency in the production of the

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1 FDA has issued letters indicating that the Agency has "no questions" about the GRAS status of GOS for use in a variety of food applications. *See, e.g.,* GRN No. 236: GOS produced from β-galactosidase derived from *B. circulans* (Friesland Foods Domo) ("No questions" letter issued July 28, 2008); GRN No. 285: GOS produced from β-galactosidase derived from *B. circulans* (GTC Nutrition) ("No questions" letter issued September 4, 2009); GRN No. 286: GOS produced from β-galactosidase derived from *B. circulans* (GTC Nutrition) ("No questions" letter issued September 4, 2009); GRN No. 334: GOS produced from β-galactosidases from *Sporobolomyces singularis and Kluyveromyces lactis* (Yakult Pharmaceutical Industry) ("No questions" letter issued October 27, 2010); GRN No. 484: GOS produced from β-galactosidase derived from B. *bifidum* produced in an *E. coli* strain (Clasado) ("No questions" letter issued May 8, 2014); GRN No. 489: GOS produced from β-galactosidase derived from *Aspergillus oryza*e (International Dairy Ingredients) ("No questions" letter issued May 22, 2014); GRN No. 495: GOS produced from β-galactosidase derived from *B. bifidum* produced in an *E. coli* strain (Clasado) ("No questions" letter issued May 30, 2014); GRN No. 518: GOS produced from β-galactosidase derived from *B. circulans* (New Francisco Biotechnology Company) ("No questions" letter issued December 22, 2014); GRN No. 569: GOS produced from β-galactosidase derived from *B. circulans* (New Francisco Biotechnology Company) ("No questions" letter issued November 25, 2015).

2 GRN No. 236, at Tables II-4 and II-5 on pages 000025-000026.

3 *Id.* at Table 1 on page 000004.

ingredient. As noted above, the intended inclusion rate is up to 0.3% of the lactose starting material to which the enzyme preparation is added.

III. Product Identity and Specifications

A. Product Identification

The β-galactosidase enzyme preparation is a powdered enzyme preparation that contains 33-43% β-galactosidase enzyme and 57-67% dextrin. The β-galactosidase enzyme component is an extracted and purified enzyme product obtained from the fermentation and processing of genetically modified *B. subtilis* expressing a β-galactosidase gene from *B. circulans*. Dextrin may appropriately be used as a carrier for a powdered enzyme preparation.[4] Maltodextrin also may be used as an alternative to dextrin in the production process and as a carrier for the finished preparation.[5]

The β-galactosidase enzyme is a hydrolase that can transfer non-reducing β-D-galactose residues from β-D-galactosides (*e.g.*, lactose) to water. Under high lactose concentration and specific processing conditions, β-galactosidase can utilize lactose as an alternative acceptor instead of water, which results in the formation of GOS. Detailed information regarding the product's identity is summarized in **Table 1** below:

Table 1: Enzyme Information

Common or Usual Name	Beta-galactosidase
Chemical Name	β-D-galactoside galactohydrolase
Synonyms	Lactase; β-galactosidase; β-D-galactosidase; β-gal
Trade name	GF lactase B2
International Union of Biochemistry and Molecular Biology (IUBMB) Enzyme Nomenclature	beta-galactosidase
IUBMB Number:	3.2.1.23

[4] 21 C.F.R. § 184.1277 ("Dextrin") (affirming the GRAS status of dextrin for use as a formulation aid, a processing aid, a stabilizer and thickener, and a surface-finishing agent with no limitation other than good manufacturing practice).

[5] See 21 C.F.R. § 184.1444 ("Maltodextrin") (affirming the GRAS status of maltodextrin for use in food with no limitation other than good manufacturing practice).

[Enzyme Commission (EC) Number]	
Chemical Abstracts Service (CAS) Number	9031-11-2
Specificity	The β-galactosidase enzyme is a hydrolase that can transfer non-reducing β-D-galactose residues from β-D-galactosides (*e.g.*, lactose) to water. Under high lactose concentration and specific processing conditions, the enzyme can utilize lactose as an alternative acceptor instead of water, resulting in the formation of GOS.
Calculated Molecular Mass	155977.22 Da
Amino acid sequence	MRRINFNDNWRFQREISTSLREAQKPSFNDHSWRQLSLPHDWSI ELDFNKDSLATHEGGYLDGGVGWYRKTFTVPSAMEGKRISLDF DGVYMNSTTYLNGEELGTYPFGYNAFSYDITDKLFMDGRENVL AVKVDNTQPSSRWYSGSGIYRNVYLTVTNPVHVARYGTFVTTP DLESAYAARKAEVNIKTKINNDSDAAVQVKVKSTIYDTDGKEV ASVVSQEKTAAAGTTAHFEDNTVIENPELWSLDNPYRYKLVTD VLIGGETVDTYETRFGARFFKFDANEGFSLNGKPMKLYGVSMH HDLGALGAATNARAVERQLQIMKDMGVNAIRGTHNPVSPEFLE AVNNLGLLLIEEAFDCWSQSKKTYDYGRFFTRWAEHDVKEMVD RGKNEPSIIMWSIGNEIYDTTSPSGVETARNLVRWIKEIDTTRPTTI GEDKTRGDKVNVTPIDPNILEIFHTVDVVGLNYSENNYVGYHEQ HPNWKLYGSETSSATRSRGVYTHPYEYNLGTKYDDLQQSSYDN DYVPWGRTAEDAWKSDRDLKHFAGQFIWTGFDYIGEPTPYYDS YPAKSSYFGAVDTAGFPKDIFYYYQSQWKKEPMVHLLPHWNW TEGEPVRVLAYTNAHQVELFLNGKSLGVRGYENKKTSWGAPYK ETKDGKTYLEWAVPFKAGTLEAVAMDENGKEIARDQVTTAGAP AAVKLTADRKVIKADGTDLSFITAEIVDSKGNVVPNADHLIQFHL SGHGELAGVDNGDAASVERYKDNKRKAFSGKALAIVQSNKLDG NITLHASAEGLSSGNVTIFTTASADQNSITIAGIDEVNVLVDFNVV PELPSQIKVYYSDSTVEMKPVTWDAVDPNLLNTAGKIIVEGTVE GTDKKAKALLIVKGNGQENSEYRIDLFSPDPKLISTELTVEKTNI MEDDFIDIKVIGQLENKEVVDLSNFMPIYEFDCDIIKIEGNKLYAL EEGLVKVTAAVTYKGRTVTSPEMMLKITKNPVPKTITHIDSITVV AGKGEAPVLPATAVAHFDRGMPRDVKVKWEIVNPALYQNLGEF TVSGDVEGTEIKAQAKVMVRSALAIETISMAVLPNQKPELPQKV TVYYSDGTEEQADVDWDAMPSAELKSEGVVKVKGSVKGVDLK ATAQIRVTSEVGGVQNISRAKNGYEYPKAEASFTNTGPGSNDRIE AINDDVISYDAEPHNRWTNWQPTPRPGDWVSITFGDSKPRKYDI DSMEIHWYEDLGTSSPAYFRIQYKSGDEWKDVSGLKTNPSNTVL RQANVYTFDKVRTSAIRVDMTAKTGKSLAITEIKVFSKWAKAHT HPMVTDIKLGDLSILDDFSKKGDNNELTFQVKDPRDIPEIKVKAE

	DNTSITIIPTFTAPSTAKIIAKSEDGMKVEIYNIRFTE

B. Product Specifications

Tables 2 and 3 provide the typical composition and the chemical, lead, and microbiological specifications for the β-galactosidase enzyme preparation, as well as their associated analytical methods. **Table 4** provides the results of a five-batch analysis of the β-galactosidase enzyme preparation.

Table 2: Typical Composition

Ingredient	**Contents (%)**	**Function**
Lactase preparation	33 ~ 43	Active ingredient
Dextrin	57 ~ 67	Carrier
Total	100	
- *Total Organic Solids (%)*	*25 to 35*	
- *Water content (%)*	*<10*	

Table 3: Product Specifications

CHEMICAL SPECIFICATIONS:	**Test Procedure**	**Spec Limit**
* Activity	FCC[6]	≥1000 LAU/g
* Arsenic (As)	JECFA	≤4 ppm
* Lead (Pb)	JECFA	≤5 ppm
MICROBIOLOGICAL SPECIFICATIONS:		
* Standard Plate Count	3M petrifilm[7]	≤10,000 CFU/g
* Coliforms	3M petrifilm	≤10 CFU/g
* *E. coli*	3M petrifilm	Negative/g
* *Salmonella*	JECFA	Negative/25g
* *Staphylococcus*	3M petrifilm	<10 CFU/g
* Yeast and Mold	3M petrifilm	<10 CFU/g
* *Listeria* spp.	3M petrifilm	Negative/25g
PHYSICAL SPECIFICATIONS:		

[6] GenoFocus modified the FCC method by adding 100 µg/ml of BSA (bovine serum albumin) to improve reproducibility of the assay. The method was validated, and it was concluded that the addition of BSA did not affect the validity of the method. If FDA would like additional data regarding GenoFocus' validation of the addition of BSA, we will provide that information upon request.

[7] 3M Petrifilm has been internationally recognized as a valid method for use in microbial testing of food products, and its utility has been recognized by AOAC International, the American Public Health Association, USDA and FDA. See http://multimedia.3m.com/mws/media/241188O/3m-petrifilm-plate-certificates-recognitions-validations.pdf?fn=70-2008-5431-6_Rev-1014.pdf for more detailed references on this point.

Appearance/color	Visual	Yellow to light brown powder

Table 4: Batch Analysis for β-galactosidase Enzyme Preparation

Test Item	Specification	Lot Number				
		B150511	**B150520**	**B150526**	**B150920**	**B151005**
Color	Yellow to light brown	Conforms	Conforms	Conforms	Conforms	Conforms
Appearance	Powder	Conforms	Conforms	Conforms	Conforms	Conforms
Activity of Lactase	Min. 1,000 LAU/g	1,280 LAU/g	1,120 LAU/g	1,180 LAU/g	1,143 LAU/g	1,296 LAU/g
Heavy Metal Content (As)	Max. 4 ppm	ND*	ND	ND	ND	ND
Heavy Metal Content (Pb)	Max. 5 ppm	ND	ND	ND	ND	ND
Standard Plate Count	Max. 10,000 CFU/g	Negative	Negative	125 CFU/g	Negative	Negative
Coliforms	Max. 10 CFU/g	Negative	Negative	Negative	Negative	Negative
E. coli	Negative/g	Negative	Negative	Negative	Negative	Negative
Salmonella	Negative/25g	Negative	Negative	Negative	Negative	Negative
Staphylococcus	Max. 10 CFU/g	Negative	Negative	Negative	Negative	Negative
Yeast and Mold	Max. 10 CFU/g	Negative	Negative	Negative	Negative	Negative
Listeria spp.	Negative/25g	Negative	Negative	Negative	Negative	Negative

* ND: Not detected

IV. Manufacturing Process

Table 5 below provides a detailed description of the manufacturing process for the β-galactosidase enzyme preparation, which is performed in accordance with ISO9001:2008 and FSSC22000.

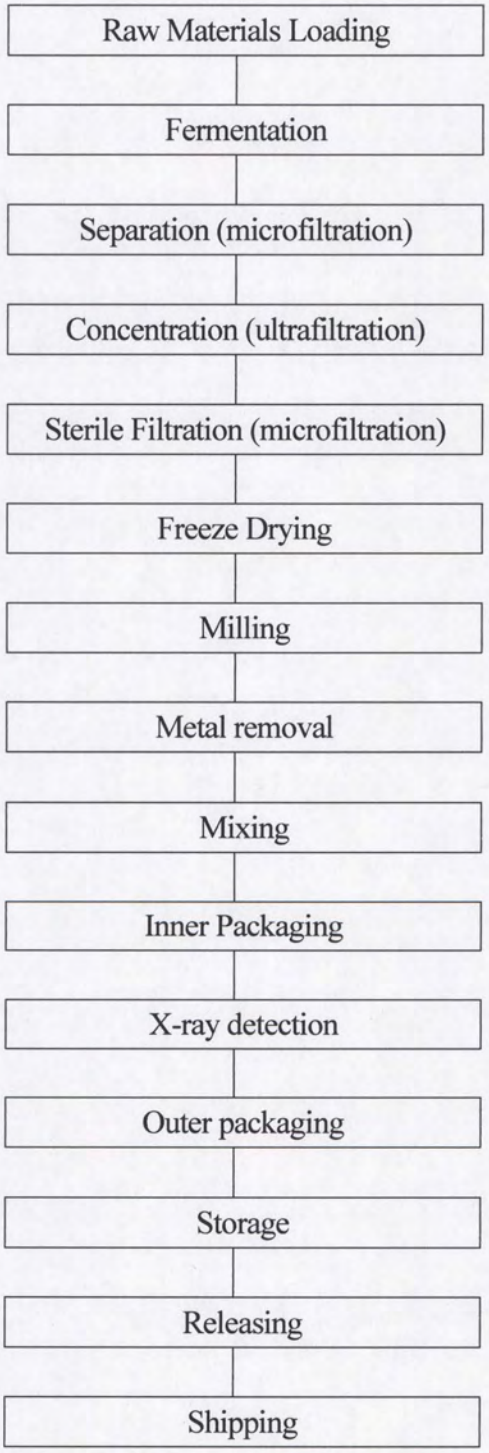
Table 5: Detailed Description of the Manufacturing Process

1) Production of Glycerol stock	A single colony of *Bacillus subtilis* production strain isolated on LB agar plate (Enzymatic hydrolysate casein, yeast extract, NaCl, Agar) is suspended in 20% glycerol and stored at -72°C.
2) 1st and 2nd Seed Fermentation	2 ml of the glycerol stock of the *Bacillus subtilis* production strain is inoculated into 2 L of LB media (enzymatic hydrolysate casein, yeast extract, NaCl) and cultured at 37°C 200 rpm for 5 hr. 2L of culture broth is inoculated into 200 L of LB media in 500 L seed fermentor and incubated at 37°C, 200 rpm for 5 hr.
3) Main fermentation	An antifoam agent is added at the start of fermentation at levels ranging

	from 0.01 to 0.1%. The 200 L of culture broth is inoculated into 10 tonne main fermentation media in a 20 tonne fermentor and incubated at [redacted] During the fermentation process, various physical and chemical parameters — agitation [redacted] aeration [redacted], dissolved oxygen concentration, pH, exhausted O_2/CO_2 concentration, glucose concentration, lactase activity — are measured at regular intervals.
4) Separation	After fermentation, $CaCl_2$ $2H_2O$ is added up to [redacted] and then K_2HPO_4 is subsequently added up to [redacted] in the fermentation broth without pH adjustment for flocculation purposes. The fermentation broth is subjected to the centrifugation process for removal of the production microorganism. For the complete removal of residual production microorganism, the centrifuged medium is subject to microfiltration using a [redacted] membrane.
5) Concentration & partial purification	The filtrate from the microfiltration step is subjected to ultrafiltration. [redacted] and β-galactosidase enzyme is concentrated until the lactase activity meets the defined specification of the finished product.
6) Sterile filtration	For the complete removal of the residual production microorganism, the ultrafiltration concentrate and bulking agent (dextrin) [redacted] are subject to microfiltration using a [redacted] membrane.
7) Lyophilisation	Filtered enzyme solution is freeze-dried from -40°C to 25°C under vacuum for 50 hours.
8) Mixing & packaging	The freeze-dried β-galactosidase enzyme concentrate is mixed with dextrin, which acts as a bulking agent/carrier, to produce the final food enzyme preparation that is marketed.

Figure 1 below provides a step-by-step illustration of the manufacturing process.

Figure 1: Schematic Overview of the Manufacturing Process for the β-Galactosidase Enzyme Preparation



All raw materials and processing aids used in the production of the β-galactosidase enzyme preparation are listed in **Table 6** below. Each raw material has an appropriate FDA regulatory status for its intended use, and where applicable, conforms to Food Chemicals Codex (FCC) specifications. In the future, should GenoFocus choose to modify the raw materials or processing aids used in the production of the β-galactosidase enzyme preparation, the Company will ensure that all such components are safe and suitable for their intended use.

Table 6. Materials Currently Used in Production of the β-galactosidase Enzyme Preparation

Name of Process	**Raw Material or Processing Aid**
Production of glycerol stock	LB agar plate (enzymatic hydrolysate casein, yeast extract, sodium chloride, agar) 20% glycerol
1st and 2nd Seed Fermentation	LB media (enzymatic hydrolysate casein, yeast extract, sodium chloride)
Main fermentation	Maltose, $MgSO_4$ $7H_2O$, peptone, yeast extract, $(NH_4)_2SO_4$, K_2HPO_4, NaCl, $CaCl_2$ $2H_2O$, and an antifoam agent[8]
Cell separation (flocculation)	$CaCl_2$ $2H_2O$ and K_2HPO_4
Mixing & packaging	Dextrin

V. Consideration of Potential Toxins and Contaminants

A. Absence of Source Microorganism from the Enzyme Preparation

As described in **Table 5** above, there are two filtration steps with a [redacted] filter. These filtration steps are expected to filter out the source microorganism as the vegetative *B. subtilis* is approximately 3-4 μm in length and about 1 μm in width (Sargent, 1975). This strain has lost the ability to form spores, which can be smaller than the vegetative cells, and therefore the vegetative cells are the only microbial size to consider.

[8] The antifoam agent will be used in accordance with current good manufacturing practice per FDA's correspondence with the Enzyme Technical Association, dated September 11, 2003. This correspondence has been cited as the basis for the appropriate regulatory status of antifoam agents used in the production of other GRAS-Notified food enzymes. *See, e.g.,* Lactase from *Bifidobacterium bifidum* produced in *Bacillus subtilis* (GRN 579, at page 000010; "no questions" letter issued October 22, 2015) and *Beta*-galactosidase enzyme preparation (GRN 485, at page 8 (internal citation omitted); "no questions" letter issued April 15, 2014).

B. Absence of Enzymatic Activity in GOS

During the production of GOS using the GenoFocus β-galactosidase enzyme preparation, the manufacturer will take steps to ensure the absence of enzyme activity. There is a pH adjustment step that will inactivate the enzyme, followed by an enzyme removal step using ultrafiltration. The manufacturer also will perform sporadic testing for the presence of protein and enzyme activity.

C. Absence of Toxins from Enzyme Preparation

The source microorganism is not known to produce toxins, and the manufacturing process is tightly controlled to prevent microbial contamination from other sources.

D. Absence of Antibiotics from Enzyme Preparation

No antibiotics are used during the manufacturing of the enzyme preparation; thus, there is no possibility that antibiotics are present in the enzyme preparation.

VI. Safety of β-galactosidase Enzyme Preparation

A. Safety of β-galactosidase Enzyme in Food Production

The enzyme, β-galactosidase, has a history of safe use in food production, and even more specifically, in the production of GOS. As relevant examples, FDA has issued several letters stating that the Agency has "no questions" about the GRAS status of GOS produced from β-galactosidase enzyme preparations:

- GRN No. 236: GOS produced from β-galactosidase derived from *B. circulans* (Friesland Foods Domo) ("No questions" letter issued July 28, 2008)
- GRN No. 285: GOS produced from β-galactosidase derived from *B. circulans* (GTC Nutrition) ("No questions" letter issued September 4, 2009)
- GRN No. 286: GOS produced from β-galactosidase derived from *B. circulans* (GTC Nutrition) ("No questions" letter issued September 4, 2009)
- GRN No. 334: GOS produced from β-galactosidases from *Sporobolomyces singularis and Kluyveromyces lactis* (Yakult Pharmaceutical Industry) ("No questions" letter issued October 27, 2010)
- GRN No. 484: GOS produced from β-galactosidase derived from *B. bifidum* produced in an *E. coli* strain (Clasado) ("No questions" letter issued May 8, 2014)
- GRN No. 489: GOS produced from β-galactosidase derived from *Aspergillus oryzae* (International Dairy Ingredients) ("No questions" letter issued May 22, 2014)

- GRN No. 495: GOS produced from β-galactosidase derived from *B. bifidum* produced in an *E. coli* strain (Clasado) ("No questions" letter issued May 30, 2014)

- GRN No. 518: GOS produced from β-galactosidase derived from *B. circulans* (New Francisco Biotechnology Company) ("No questions" letter issued December 22, 2014)

- GRN No. 569: GOS produced from β-galactosidase derived from *B. circulans* (New Francisco Biotechnology Company) ("No questions" letter issued November 25, 2015)

Active β-galactosidases of microbial and human origin are naturally present in the gastrointestinal tract. Pariza and Foster have noted that the results of exhaustive literature reviews, conducted by FDA, relating 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). Two successful GRAS Notifications for GOS produced from β-galactosidase enzymes – GRN Nos. 285 and 334 – included the results of 90-day repeat dose oral gavage toxicity studies on whole cell biomass and enzyme preparations containing β-galactosidase, and these studies demonstrated a No Observed Adverse Effects Level (NOAEL) greater than 25,000 mU of β-galactosidase activity per kg body weight per day in both male and female Sprague-Dawley rats.

B. Safety of the Production Strains

The production strain, GFBSL210 was created from *B. subtilis* KCTC 3135, identical strain ATCC 6051, which is the recipient strain described in GRN No. 205.[9] The collection receipt to support that taxonomy of the production strain is provided in **Appendix 1**. The genetic modifications, screening, and verifications as described in this notification are described in further detail in Section VI. All introduced genetic material is well characterized, limited in size, stably integrated, and free of toxin or allergen encoding sequences. Safety evaluations of the recipient (*B. subtilis*) and donor (*B. circulans*) microorganisms are provided in the following sections.

The production strain was evaluated and meet the criteria for safe genetically modified production microorganisms as outlined by Pariza and Johnson, 2001 (see **Appendix 2** for detailed analysis).

C. Safety of the Recipient Strain

B. subtilis is a non-pathogenic and non-toxigenic Gram positive bacterium that is well characterized and widely used in the production of food-grade enzymes. Additionally, *B. subtilis* is a saprophytic microorganism that is ubiquitous in nature and is commonly found in water, soil,

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[9] GRN No. 205 (Novozymes North America Inc., Pullulanase enzyme preparation from *Bacillus subtilis* expressing the pullulanase gene from *B. acidopullulyticus*).

air, and decomposing plant residues. *B. subtilis* is considered to be "the best characterized member of the Gram-positive bacteria" and its genome was sequenced as early as 1997 (Kunst *et al.*, 1997). It is a common contaminant in foods eaten by both man and animals.

According to the European Food Safety Authority (EFSA), the primary concern associated with the *Bacillus* genus is the potential for producing *Bacillus cereus* enterotoxins, *i.e.*, cereulide peptide synthetase of *B. cereus*, and enterotoxin, haemolysin BL, cytotoxin and bacteriocin of *B. cereus* G9241 (EFSA, 2014). However, *B. subtilis* has been shown not to contain genes with homology to those that encode for known *B. cereus* enterotoxin and not to produce such toxins (de Boer and Diderichsen, 1991; Pedersen *et al.*, 2002; Olempska-Beer *et al.*, 2006). There are no reports in the public literature that any strains of *B. subtilis*, including the notified *B. subtilis* ATCC 6051, are capable of producing any of these toxins.

The regulatory history of *B. subtilis* further confirms its safety. In 1999, FDA affirmed the GRAS status of carbohydrase and protease enzyme preparations from either *B. subtilis* or *B. amyloliquefaciens* as direct food ingredients.[10] FDA stated that nontoxigenic and nonpathogenic strains of *B. subtilis* are widely available and had have been safely used in a variety of food applications, including the documented consumption of *B. subtilis* in the Japanese food, natto, a fermented soy bean food. FDA concluded that carbohydrase and protease enzyme preparations derived from *B. subtilis* were in common use in food prior to January 1, 1958. Additionally, published articles have reported the use of *B. subtilis* in traditionally-fermented food from Asia and Africa (Isu and Ofuya, 2000; Omafuvbe *et al.*, 2000; Kiers *et al.*, 2000). These articles support the safe use of the source, *B. subtilis* in food applications.

The U.S. Environmental Protection Agency (EPA) classifies *B. subtilis* as one of the Tier 1 exempt microorganisms, which means that genetic modifications to *B. subtilis* that meet certain introduced genetic material criteria do not require pre-manufacture notification and review from EPA, due to the established safety of *B. subtilis*. EPA concluded that *B. subtilis* was "not considered pathogenic or toxigenic to humans, animals, or plants."[11]

Since in our final production host, the *bgaII* expression module for expression of the β-galactosidase is the only inserted coding region, it can be concluded that our production host, *i.e.*, *B. subtilis* producing the β-galactosidase, is non-toxic and non-allergenic.

D. Safety of the Donor Strain

Like *B. subtilis*, *B. circulans* is a member of the *Bacillus* genus that contains a large number of bacterial strains used in the production of food-grade enzymes (Schallmey *et al.*, 2004). A literature search revealed limited data linking *B. circulans* to pathogenic or toxigenic effects. Although foodborne illness from *Bacillus spp.* in general is rare (with the exception of *B. cereus*), isolates of *B. circulans* have been obtained from the blood samples of patients with

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[10] 64 Fed. Reg. 19886 (Apr. 23, 1999).

[11] EPA, Final Risk Assessment of Bacillus subtilis (February 1997), available at: http://www2.epa.gov/regulation-biotechnology-under-tsca-and-fifra/tsca-biotechnology-regulatory-and-policy-related.

sepsis (Rowan *et al.*, 2001). Also, certain strains of *B. circulans* isolated from whole milk expressed the enterotoxigenic proteins hblC, hblD, MA; nheA, and nheB (Phelps and McKillip, 2002). As the only coding region taken from *B. circulans* encodes for a well characterized β-galactosidase and not any of these identified enterotoxigenic proteins describe above, it can be concluded that the production strain does not produce toxins. In addition, the *B. circulans* ATCC 31382 was isolated from the soil and not from milk, and therefore is likely to be a different strain than the one described in Phelps and McKillip publication.[12]

In terms of its regulatory history, *B. circulans* has been used as a production organism for the enzyme cycloglycosyltransferase used to produce beta-cyclodextrin in the European Union (EU).[13] As noted above, FDA also has issued "no questions" letters in response to multiple GRAS Notifications for GOS produced from β-galactosidase enzymes derived from *B. circulans*.[14]

A literature search did not identify any articles that indicated that β-galactosidase is involved in pathogenicity or virulence, and therefore the expression of a well characterized β-galactosidase II gene (*bgaII*) from a safe donor strain into a safe production strain is expected to be safe for its intended use.

The β-galactosidase II from *B. circulans* is not homologous with any known toxins or allergens described as in "product description" section. A dossier submitted to the EU for the authorization of β-galactosidase from *B. circulans* Strain M3-1 as a Food Enzyme, the β-galactosidase enzyme concentrate from *B. circulans* strain M3-1 was non-clastogenic when tested using an in vitro chromosomal aberration test conducted in Chinese Hamster Lung (CHL) cells as well as non-genotoxic in the mouse lymphoma assay.

E. β-galactosidase Expression Module

A beta-galactosidase II expression module was constructed using an expression module that consists of a strong promoter, mRNA stabilizing element, and the coding sequence of the *bgaII* gene. All introduced genetic material is commonly used in molecular biology and is well characterized in the scientific literature. The expression module is flanked with two DNA fragments homologous to *B. subtilis thrC* gene coding for threonine synthase to replace the intervening DNA fragment between the chosen homologous regions with the expression module by double-crossover recombination (see **Figure 2** below).

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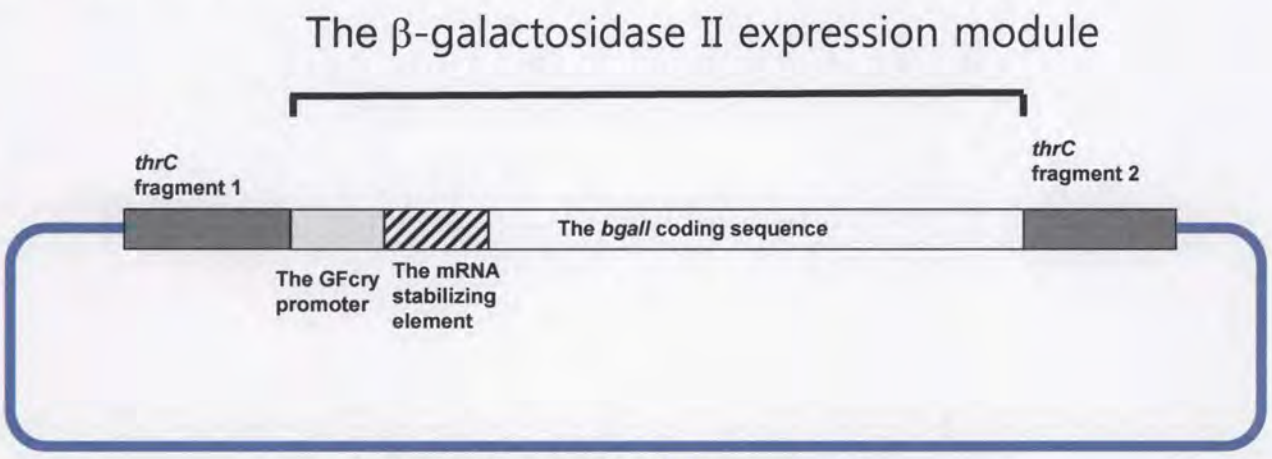
12 *See also Bacillus* sp. (ATCC 31382), available at: https://www.atcc.org/Products/All/31382.aspx.

13 Commission Directive 2003/95/EC, at L 283/77, available at: http://faolex.fao.org/docs/pdf/eur40476.pdf.

14 *See* GRN No. 236: GOS produced from β-galactosidase derived from *B. circulans* (Friesland Foods Domo) ("No questions" letter issued July 28, 2008); GRN No. 285: GOS produced from β-galactosidase derived from *B. circulans* (GTC Nutrition) ("No questions" letter issued September 4, 2009); GRN No. 286: GOS produced from β-galactosidase derived from *B. circulans* (GTC Nutrition) ("No questions" letter issued September 4, 2009); GRN No. 518: GOS produced from β-galactosidase derived from *B. circulans* (New Francisco Biotechnology Company) ("No questions" letter issued December 22, 2014); GRN No. 569: GOS produced from β-galactosidase derived from *B. circulans* (New Francisco Biotechnology Company) ("No questions" letter issued November 25, 2015).

Figure 2: Construction of the β-galactosidase II Expression Module



The promoter used is a derivative of the *cryIIIAa* promoter from *Bacillus thuringiensis*, designated pGFcry (DeSouza *et al.,* 1993). The pGFcry promoter contains the consensus sequence "TTGACA" at -35 region contrast to the wild type *cryIIIAa* promoter (Lee *et al.*, 2010). The mRNA stabilizing element was also obtained from 5'UTR of the *cryIIIAa* gene from *B. thuringiensis* (Agaisse and Lereclus, 1994). The amino acid sequence of the beta-galactosidase II is presented in **Table 1** above. A pair of DNA fragments was selected to introduce the expression module into the *thrC* locus on *B. subtilis* genome by double-crossover recombination.

All the components comprising the expression module and the homologous pair were cloned into an *E. coli-B. subtilis* shuttle plasmid. The shuttle vector contains two replication origins and a chloramphenicol (Cm)-resistant gene: the replication origin from pUC18 for *E. coli* (Yanisch-Perron *et al.*, 1985); the temperature sensitive replication origin from pIC333 for *B. subtilis* (Steinmetz and Richter, 1994); the Cm marker gene working both hosts (Guérout-Fleury *et al.*, 1996).

The constructed plasmid was transformed into *B. subtilis* and chromosomal integrates with single crossover were selected by growing the transformants in LB plate containing Cm (5 μg/ml) at non-permissive temperature (37°C). To select cells in which the plasmids are excised by second crossover and cured, the cells from the first screening were grown on LB plate without Cm. From cells from the second screening, cells susceptible to 5 μg/ml of Cm were selected. Among Cm-susceptible cells, the clones correctly replaced with the expression module were screened by PCR analysis; therefore there are no antibiotic resistance genes in the final production strain. The insertion site within the *thrC* gene, integrity of the expression module, and absence of the other plasmid sequence except the expression module in the resulting *B. subtilis* strain were verified by PCR analysis and subsequent nucleotide sequencing of the PCR product. Insertion within the *thrC* gene disrupts the threonine synthase function of the production microorganism (Malumbres *et al.*, 1995). In addition to the *bgalI* expression module, deletion was introduced in several genes encoding extracellular proteases involved in degrading the beta-galactosidase II and a gene involved in endospore formation by double-crossover

recombination. Plasmids used to introduce deletion were identical to the one used for inserting the ß-galactosidase expression module except that they contain homologous pairs chosen to delete target gene function instead of the *thrC* pair flanking the expression module. Deletion mutants were selected using the same procedure as described above. All the deletion sites were verified by PCR analyses with the appropriate primers. The list of the deleted genes is provided below in **Table 7**.

Table 7: Summary of Deleted Genes and Functions

Gene Name	Protein Name	Function	References
aprE	serine alkaline protease (subtilisin E)	Extracellular protease	Ogura, M *et al.*, 2003; Arai, A *et al.*, 2003; Connelly, M *et al.*, 2004; Lanigan-Gerdes, S *et al.* 2007; Ikemura, H *et al.*, 1987; Jain, S *et al.*, 1998.
nprE	extracellular neutral metalloprotease	Extracellular protease	Connelly, M *et al.*, 2004; Wang, L *et al.*, 1990; Yang, M *et al.*, 1984; Wang, L and Devenish, R, 1993.
nprB	extracellular neutral protease B	Extracellular protease	Chitlaru, T *et al.*, 2006; Tran, L *et al.*, 1991.
vpr	extracellular serine protease	Extracellular protease	Connelly, M *et al.*, 2004; Lanigan-Gerdes, S *et al.* 2007.
mpr	extracellular metalloprotease	Extracellular protease	Connelly, M *et al.*, 2004; Park, C *et al.*, 2004.
spoIIAC	RNA polymerase sporulation-specific sigma factor (sigma-F)	Sporulation	Connelly, M *et al.*, 2004; Hilbert, D and Piggot, P, 2004; Yudkin, M and Clarkson, J, 2005; Barák, I and Wilkinson, A, 2005; Hughes, K and Mathee, K, 1998; Coote, J., 1992; Waites, W *et al.*, 1970.
thrC	threonine synthase	Threonine synthesis	Malumbres *et al.*, 1995

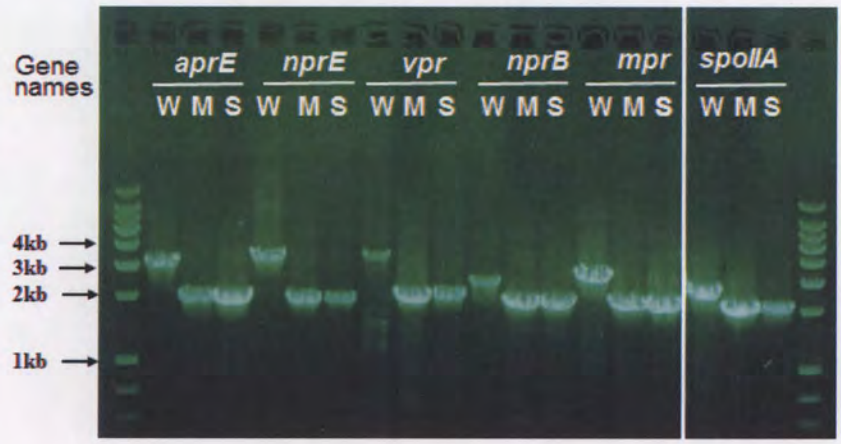
F. Genetic Construction

Bacillus circulans ATCC31382 is known to produce multiple isoforms of β-galactosidase enzyme which differ in substrate specificity and galactosyl transferring activity. (Mozaffar *et al.*, 1984). Of the isoforms, β-galactosidase II significantly contributed to the synthesis of GOS (*Id.*, Mozaffar *et al.*, 1986). GenoFocus cloned the gene encoding the β-galactosidase II (*bgaII*) from *B. circulans* ATCC31382 (WO2010098561A3). The coding region of the *bgaII* gene was codon-optimized to improve the expression level of the gene in *Bacillus subtilis*. The amino acid sequence from the codon-optimized coding sequence is identical to the amino acid sequence of the native *bgaII* gene.

G. Stability of Introduced Genetic Sequences

Double crossover recombination was utilized in the construction of the production strain to maximize the stability of the introduced or deleted sequences. The presence and integrity of the introduced expression module was determined by PCR analysis and sequencing of the PCR product to assess the stability and potential for transfer of genetic material as a component of the safety evaluation of the production microorganism. Cells from master glycerol stock of the production host were subcultured eight times to confirm genetic stability of the introduced DNA and the deleted regions. In each subculture (1% of inoculum), the cells were grown in LB broth until cell density was reached to about an OD_{600} of 7, which amounts to 6-7 generations per subculture. The cells obtained from last subculture were subjected to PCR verification. As for the deleted region, the size of the PCR products obtained from the last subculture was compared with the PCR products from the cells of the master stock and wild *B. subtilis* ATCC 6051 (see **Figure 3** below). The result shows that the deleted region is stable after about 50 generations.

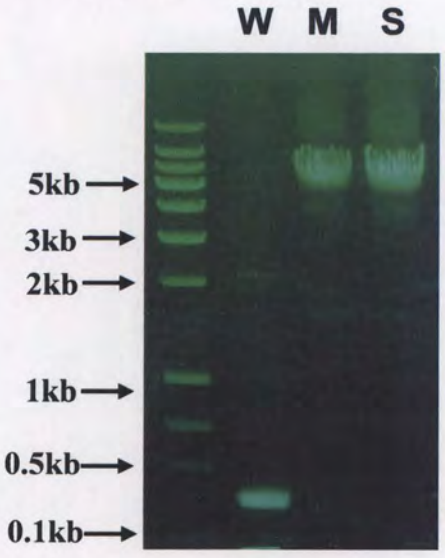
Figure 3: Genetic Stability of the Deleted Regions in the Production Host GFBSL210



The genes deleted from the production host are indicated. The letters W, M and S denote the PCR product obtained from the cells of wild type *B. subtilis* ATCC 6051, the master stock, and the last subculture, respectively.

As for the β-galactosidase expression module, the PCR product from the last subculture was subjected to size comparison using agarose gel electrophoresis (see **Figure 4** below) and nucleotide sequence determination (data not shown). We found no difference in the DNA sequences of the coding region of the *bgaII* gene between the master stock and the last subculture.

Figure 4: Genetic Stability of the β-galactosidase Expression Module in the Production Host GFBSL210



The letters W, M and S denote the PCR product obtained from the cells of wild type *B. subtilis* ATCC 6051 (W), the master stock (M), and the last subculture (S).

H. Antibiotic Resistance Genes

The final commercial production strain does not contain any antibiotic resistance markers. The plasmid-cured clones were obtained by growing in antibiotic-free medium and confirmed by antibiotics-susceptibility test; therefore there are no antibiotic resistance genes in the final production strain.

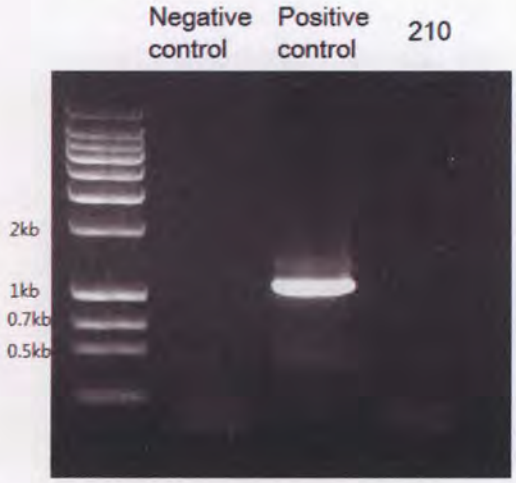
As described in Section VI.E. above, to introduce integration and deletions into the genome, GenoFocus used one kind of plasmid which contains a chloramphenicol (Cm) marker gene, which is the only antibiotics marker gene used during the genetic construction process. To select for plasmid-free cells (*i.e.*, plasmid-cured cells), GenoFocus picked colonies grown on LB media plates without antibiotics and transferred the colonies via replica plating to LB plates with 5 μg/ml Cm and LB plates without Cm. The colonies sensitive to Cm were reconfirmed as to their Cm-susceptibility by a second round of replica plating. To select for strains containing intended genetic modifications, GenoFocus confirmed the absence of the Cm-marker via PCR genotyping. Therefore, the final commercial strain was verified by through a phenotype and genotype analyses to be free of Cm resistance.

The absence of the Cm marker gene in the production host was verified by PCR analysis with primers specific to the Cm marker gene of the plasmid employed in the genetic modifications. The PCR conditions are provided in the **Table 8** and a representative gel is provided in **Figure 5**.

Table 8: PCR Conditions to Determine Absence of Antibiotic Resistance Marker in the Final Strain

Forward Primer	5' gtttgacagcttatcatcgg 3'
Reverse Primer	5' cgtagaggatctggagctg 3'
PCR conditions	95°C for 2 min 25 cycles: 95°C for 30 seconds; 50°C for 30 seconds; followed by 72°C for 1.5 min, and a final extension at 72°C for 10 min.

Figure 5: Absence of Cm Marker Gene in the Production Host GFBSL210



For **Figure 5**, the chromosomal DNA (100 ng) from *B. subtilis* ATCC 6051 (labeled "Negative control"), the plasmid DNA (10ng) employed in genomic modification (labeled "Positive control"), and the final commercial production host (labeled "210") were used as templates in separate PCR reactions and demonstrate that the final commercial production host is negative for the Cm marker. The absence or presence of PCR product with a length of 1059-bp was analyzed by 1.5% agarose gel electrophoresis. The PCR analysis was performed in triplicate to confirm the absence of the Cm marker.

I. Allergenicity Analysis

In addition to the toxicological studies described above, the amino acid sequence for β-galactosidase (see **Table 1**) was compared against the Food Allergy Research and Resource Program (FARRP) protein allergen database using AllergenOnline (http://www.allergenonline.org/databasefasta.shtml) to determine allergenicity potential. FARRP maintains and regularly updates a comprehensive database of proven or putative allergens (food, airway, venom/saliva, and contact). The search was performed on April 7, 2016 with version 16 of the database, which was updated on January 27, 2016. No positive matches were found (data not shown) when the amino acid sequence was subjected to a full-length Fast-Alignment (FASTA) search and an 80 amino acid sliding window and 8mer exact match analyses through the FARRP search algorithm.

J. Estimated Exposure

As described above in Section V.B., the β-galactosidase enzyme preparation is used as a processing aid in the production of GOS and is removed by ultrafiltration from the finished product. Thus, the GOS manufactured using the β-galactosidase enzyme preparation will not contain detectable concentrations of the enzyme protein. Accordingly, dietary exposure to the enzyme preparation from its intended use will not occur.

VII. Summary of Basis for GRAS Determination

GenoFocus has determined that its β-galactosidase enzyme preparation is GRAS based on the following:

- The identity and specifications for the β-galactosidase enzyme preparation;
- The manufacturing process for the β-galactosidase enzyme preparation;
- The intended use of the β-galactosidase enzyme;
- Supportive evidence regarding the GRAS status of other β-galactosidase enzyme preparations, including some derived from *B. circulans*, used in the production of GOS; and
- Safety information for the production strains, the donor organism (*B. circulans*), and the production organism (*B. subtilis*), as well as supportive evidence from their widespread applications in food production.

VIII. Conclusion

Based on the documentation provided in this GRAS Notification, and as discussed above, GenoFocus has concluded that its β-galactosidase enzyme preparation is GRAS via scientific procedures for use as a processing aid in the production of galacto-oligosaccharides.

IX. References

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X. Appendix 1

Collection Receipt Indicating Taxonomy of Production Strain GFBSL201

Korean Collection for Type Cultures (KCTC), Microbial Resource Center
Korea Research Institute of Bioscience and Biotechnology (KRIBB)
181 Ipsin-gil, Jeongeup-si, Jeonbuk 56212, Korea
Tel: 82-63-570-5603, FAX. 82-63-570-5609

To: Genofocus Co. Ltd

This letter is to confirm that Soo-Young Park from Genofocus Co., Ltd. purchased item KCTC 3135 from Korean Collection for Type Cultures (KCTC).

The rest of their contact information is follows:

Genofocus Co., Ltd.
Address: 65 Techno 1-ro, Yuseong-gu, Daejeon 305-509, Korea

82-41-862-4483 phone
82-42-862-4484 fax

The strain information is as follow

KCTC production No.	ATCC No.	Invoice No.	Date
3135	6051	35839	2015. 10. 15

23 February, 2016

Director, Doo-Sang Park, Ph.D

XI. Appendix 2

Pariza and Johnson Decision Tree

Taken from Pariza, Michael W., and Eric A. Johnson. "Evaluating the safety of microbial enzyme preparations used in food processing: update for a new century." *Regulatory Toxicology and Pharmacology* 33.2 (2001): 173-186.

1. Is the production strain genetically modified?
   Yes, go to 2.

2. Is the production strain modified using rDNA techniques?
   Yes, go to 3.

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?
   Yes go to 3c.

   3c. Is the test article free of transferable antibiotic resistance gene DNA?
   Yes, 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?
   Yes, go to 4.

4. Is the introduced DNA randomly integrated into the chromosome?
   No, go to 6.

6. Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via the evaluation procedure?
   Yes, the test article is ACCEPTED. If no, go to 7.

SUBMISSION END