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OFFICE OF
FOOD ADDITIVE SAFETY

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

GENERALLY RECOGNIZED AS SAFE *(GRAS)* NOTICE (Subpart E of Part 170)

Form Approved: OMB No. 0910-0342; Expiration Date: 09/30/2019
(See last page for OMB Statement)

FDA USE ONLY

GRN NUMBER	DATE OF RECEIPT
000842	
ESTIMATED DAILY INTAKE	INTENDED USE FOR INTERNET
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KEYWORDS	

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,5001 Campus Drive, College Park, MD 20740-3835.

SECTION A – 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)*

3 Most recent presubmission meeting *(if any)* with FDA on the subject substance *(yyyy/mm/dd)*: 2018-09-18

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

SECTION B – INFORMATION ABOUT THE NOTIFIER

1a. Notifier	Name of Contact Person Joanne Donoghue	Position or Title Director
	Organization *(if applicable)* Mascoma LLC	
	Mailing Address *(number and street)* 67 Etna Road, Suite 200	

City	State or Province	Zip Code/Postal Code	Country
Lebanon	New Hampshire	03766	United States of America

Telephone Number	Fax Number	E-Mail Address
603-676-3320		jdonoghue@lallemand.com

1b. Agent or Attorney *(if applicable)*	Name of Contact Person Martha Marrapese	Position or Title Attorney at Law
	Organization *(if applicable)* Wiley Rein LLP	
	Mailing Address *(number and street)* 1776 K Street NW	

City	State or Province	Zip Code/Postal Code	Country
Washington	District of Columbia	20006	United States of America

Telephone Number	Fax Number	E-Mail Address
202-719-7156		mmarrapese@wileyrein.com

SECTION C – GENERAL ADMINISTRATIVE INFORMATION

1. Name of notified substance, using an appropriately descriptive term

Maltogenic α-amylase enzyme

2. Submission Format: *(Check appropriate box(es))*

☒ Electronic Submission Gateway

☐ Electronic files on physical media

☐ Paper

If applicable give number and type of physical media

3. For paper submissions only:

Number of volumes _____

Total number of pages _____

4. Does this submission incorporate any information in CFSAN's files? *(Check one)*

☒ Yes *(Proceed to Item 5)* ☐ No *(Proceed to Item 6)*

5. The submission incorporates information from a previous submission to FDA as indicated below *(Check all that apply)*

☒ a) GRAS Notice No. GRN 746

☐ 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)* GRN Nos. 88, 120, 175, 350, 405, 422, 594, 626, 744

6. Statutory basis for conclusions of GRAS status *(Check one)*

☒ Scientific procedures *(21 CFR 170.30(a) and (b))* ☐ Experience based on common use in food *(21 CFR 170.30(a) and (c))*

7. Does the submission (including information that you are incorporating) contain information that you view as trade secret or as confidential commercial or financial information? (see 21 CFR 170.225(c)(8))

☐ Yes *(Proceed to Item 8*

☒ No *(Proceed to Section D)*

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, 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

SECTION D – INTENDED USE

1. Describe the intended conditions of use of the notified substance, including the foods in which the substance will be used, the levels of use in such foods, and the purposes for which the substance will be used, including, when appropriate, a description of a subpopulation expected to consume the notified substance.

The maltogenic α-amylase enzyme is used as a food additive during manufacturing of baked goods. The enzyme should be used in baking at levels to achieve the desired technical effect and according to current good manufacturing practices (cGMP). The amount of enzyme used will vary with the food manufacturer and will be optimized for their process. It can provide a better softness, moistness and resilience of the final product by breaking down amylopectin, which delays staling. No special subpopulation is expected to consume the enzyme.

2. Does the intended use of the notified substance include any use in product(s) subject to regulation by the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture?

(Check one)

☐ Yes ☒ No

3. If your submission contains trade secrets, do you authorize FDA to provide this information to the Food Safety and Inspection Service of the U.S. Department of Agriculture?

(Check one)

☒ Yes ☒ No , you ask us to exclude trade secrets from the information FDA will send to FSIS.

SECTION E – PARTS 2 -7 OF YOUR GRAS NOTICE

(check list to help ensure your submission is complete – PART 1 is addressed in other sections of this form)

☒ PART 2 of a GRAS notice: Identity, method of manufacture, specifications, and physical or technical effect (170.230).

☒ PART 3 of a GRAS notice: Dietary exposure (170.235).

☒ PART 4 of a GRAS notice: Self-limiting levels of use (170.240).

☒ PART 5 of a GRAS notice: Experience based on common use in foods before 1958 (170.245).

☒ PART 6 of a GRAS notice: Narrative (170.250).

☒ PART 7 of a GRAS notice: List of supporting data and information in your GRAS notice (170.255)

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

SECTION F – SIGNATURE AND CERTIFICATION STATEMENTS

1. The undersigned is informing FDA that Mascoma LLC

(name of notifier)

has concluded that the intended use*(s)* of Maltogenic α-amylase enzyme

(name of notified substance)

described on this form, as discussed in the attached notice, is (are) not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on your conclusion that the substance is generally recognized as safe recognized as safe under the conditions of its intended use in accordance with § 170.30.

2. Mascoma LLC agrees to make the data and information that are the basis for the conclusion of GRAS status available to FDA if FDA asks to see them; 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; agrees to send these data and information to FDA if FDA asks to do so.

(name of notifier)

67 Etna Road, Lebanon, NH 03766

(address of notifier or other location)

The notifying party certifies that this GRAS notice is a complete, representative, and balanced submission that includes unfavorable, as well as favorable information, pertinent to the evaluation of the safety and GRAS status of the use of the substance.The notifying party certifies that the information provided herein is accurate and complete to the best or his/her knowledge. Any knowing and willful misinterpretation is subject to criminal penalty pursuant to 18 U.S.C. 1001.

3. Signature of Responsible Official, Agent, or Attorney	**Printed Name and Title**	**Date (mm/dd/yyyy)**
jdonoghue@lallemand.com Digitally signed by jdonoghue@lallemand.com Date: 2019.02.01 10:15:04 -05'00'	Joanne Donoghue	02/01/2019

SECTION G – 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	Folder Location (select from menu) (Page Number(s) for paper Copy Only)
	GRASNotice_MaltogenicAlphaAmylaseproducedbyScerevisiae_2019-01-15.pdf	Submission
	APPENDIX1AminoAcidSequence_MaltogenicAlphaAmylase_2019-01-15.pdf	Submission
	APPENDIX2SafetyDecisionTree_MaltogenicAlphaAmylase_2019-01-15.pdf	Submission
	APPENDIX3ProductionProcess_MaltogenicAlphaAmylase_2019-01-15.pdf	Submission

#GRP2

GRAS Conclusion

A Maltogenic α-amylase from *Geobacillus stearothermophilus* produced by *Saccharomyces cerevisiae*

is Generally Recognized As Safe

for Use in Baking

January 15, 2019

Contents

1. Signed Statements and Certifications

1.1 Exemption from Premarket Approval

Mascoma LLC has determined that its maltogenic α-amylase enzyme produced by *Saccharomyces cerevisiae* expressing the gene encoding a sequence of maltogenic α-amylase from *Geobacillus stearothermophilus* is a Generally Recognized as Safe ("GRAS") substance for the intended food application and is, therefore, exempt from the requirement for premarket approval.

1.2 Basis for GRAS Determination

The determination of the GRAS status is based on scientific procedures and conforms to the regulations in accordance with 21 CFR § 170.30(a) and (b).

1.3 Name and Address of Notifier

Mascoma LLC
67 Etna Road, Suite 200
Lebanon, New Hampshire, 03766

1.4 Common Name of the Notified Substance

Maltogenic α-amylase enzyme

1.5 Intended Conditions of Use

The maltogenic α-amylase enzyme is used in baking to reduce crumb firmness and prevent staling in bread. The maltogenic α-amylase enzyme will be denatured during the baking process and will be present in insignificant quantities as inactive residue. This product is intended to replace other maltogenic α-amylases currently in commercial use for this application that are produced in other microorganisms, including *Bacillus subtilis.*

1.6 Availability of Information for FDA Review

A notification package providing a summary of the information that supports this GRAS conclusion is enclosed with this notice. The package includes a safety evaluation of the production strain, the enzyme and the manufacturing process, as well as an evaluation of dietary exposure. The complete data and information that are the basis for this GRAS conclusion are available for review and copying at 67 Etna Rd, Suite 200, Lebanon, NH 03766 or will be sent to the Food and Drug Administration upon request.

1.7 Disclosure and Certification

Parts 2 through 7 of this notification do not contain any data and or information that is exempt from disclosure under the Freedom of Information Act.

Mascoma LLC certifies to the best of our knowledge that this GRAS notice is complete, representative and balanced and includes unfavorable information as well as favorable information known to us and pertinent to the evaluation of the safety and GRAS status of the use of the notified substance.

Signature of Authorized Official

Joanne Donoghue
Director, EHS & Operations
Mascoma LLC

2. Identity, Method of Manufacture, Specifications, and Technical Effect

2.1 Enzyme Identity

IUB Name: glucan 1,4-α-maltohydrolase

Other name(s): maltogenic α-amylase; 1,4-α-D-glucan α-maltohydrolase

Systematic name: 4-α-D-glucan α-maltohydrolase

IUBMB Number: 3.2.1.133

CAS registry number: 160611-47-2

Reaction: hydrolysis of (1→4)-α-D-glucosidic linkages in polysaccharides so as to remove successive α-maltose residues from the non-reducing ends of the chains. It catalyzes the linkages in amylose, amylopectin and related glucose polymers. Maltose units are successively removed from the non-reducing end of the polymer chain until the molecules are degraded, or in the case of amylopectin, the branch-point is reached.

The amino acid sequence of the mature maltogenic α-amylase enzyme produced by our *Saccharomyces cerevisiae* strain is shown in **Appendix 1**. This sequence has 100% identity to maltogenic amylase amyM of *Geobacillus stearothermophilus* (UniProt Accession P19531 AMYM_GEOSE).

2.2 Production Organism and Construction

2.2.1 Production Strain

The production organism is a strain of *Saccharomyces cerevisiae* that is encoded with a maltogenic α-amylase enzyme gene that is native to *Geobacillus stearothermophilus*. The gene was amplified by polymerase chain reaction (PCR) from an artificially synthesized gene based on the Genbank sequence, which negates the possibility of donor DNA transfer to the strain.

The genetically modified production organism complies with OECD (Organization for Economic Cooperation) and criteria for GILSP (Good Industrial Large Scale Practice) microorganisms and meets the criteria for a safe production microorganism as described by various experts (Pariza & Foster, 1983; IFBC, 1990; OECD, 1993; Pariza & Johnson, 2001; JECFA 2001, 2006).

The production strain has been confirmed to belong to the genus *Saccharomyces* using the large subunit ribosomal rRNA (LSU) region as a marker for genus identification, as these regions are highly conserved. For species identification, the divergence regions of this rDNA LSU, D1 and D2, were further compared to confirm the species as *cerevisiae*. In addition, whole genome sequencing was completed for the strain.

Taxonomic characteristics of the parent yeast:

Name:	*Saccharomyces cerevisiae*
Class:	*Saccharomyces*
Order:	*Saccharomycetales*
Genus:	*Saccharomyces*
Species:	*cerevisiae*

In addition to other commonly used names associated with *Saccharomyces cerevisiae* (e.g. yeast, baker's yeast, brewer's yeast, and lager beer yeast), the taxonomic literature lists other synonyms such as *Saccharomyces bayanus*, *Saccharomyces carlsbergensis*, *Saccharomyces uvarum*, *Saccharomyces sake*, and *Saccharomyces vini* because the classification has undergone many changes over the years (Lodder & Kreger-van-Rij, 1952; Lodder, 1970; Demain *et al.*, 1998; Barnett *et al.*, 1983).

The *S. cerevisiae* strain has been genetically modified to express a maltogenic α-amylase enzyme which catalyzes the linkages in amylose, amylopectin and related glucose polymers, thereby reducing staling in bread. The maltogenic α-amylase is native to *Geobacillus stearothermophilus*.

2.2.2 Host Microorganism

The *Saccharomyces cerevisiae* parent yeast was isolated from a commercial sample of baker's yeast used in baking. The parental strain has been used for over 20 years in the baking industry.

2.2.3 Maltogenic α-amylase from Geobacillus stearothermophilus

The maltogenic α-amylase gene encoded in the *S. cerevisiae* was amplified by PCR from an artificially synthesized gene based on the Genbank sequence.

Table 1. Source of the Introduced Genes

Gene	Enzyme	EC/TC number	Donor Organism	Source of inserted genetic material
Maltogenic α-amylase	*amyM*	3.2.1.133	*Geobacillus stearothermophilus*	Synthesized and codon optimized for *S. cerevisiae*

Information on the source of the inserted genetic material is provided in **Table 1**.

2.2.4 Construction

The production strain was constructed using one genetic modification. The molecular tools and practices used during the construction of the production strains are standard to the field of biotechnology and yeast genetics. The genetic modification techniques utilized to develop these modified strains relies upon directed integration to insert the genes at specific and known sites within the yeast chromosome. The direct integration approach creates strains with integration

events that are stable and easy to characterize. Chromosomal integration, by its very nature, reduces the probability of any mobilization of the heterologous DNA and enhances strain stability relative to other approaches.

The expression cassette for the maltogenic α-amylase was directly integrated into the chromosome of the host strain by homologous recombination using one step integration. The PCR products used to transform the host included the *Geobacillus stearothermophilus amyM* gene encoding maltogenic α-amylase under the regulation of the native *S. cerevisiae* TDH1 and HOR7 promoters; and DIT1 and IDP1 terminators. The genetic construction was confirmed by PCR analyses and phenotypic characterizations. No genes encoding for virulence factors, protein toxins or enzymes involved in the synthesis of mycotoxins or any other toxic or undesirable substances are expected based on our knowledge of the strain, the maltogenic α-amylase sequence and the promoters and terminators.

The DNA insert contains two copies of the maltogenic amylase gene. PCR genotyping and whole genome sequencing confirmed that this insert was integrated into the yeast genome at the intended locus. The host strain has three copies of the chromosome into which we integrated the insert, therefore our modified strain has multiple copies of maltogenic amylase present in the genome.

2.2.5 Stability & Genetic Transfer Capability of Introduced DNA Sequences

The inserted DNA is integrated into the *Saccharomyces cerevisiae* chromosome resulting in transformants that are mitotically stable. Genetic transfer of the inserted DNA to other organisms is poor because the chromosomal integration severely limits the mobility of the inserted DNA.

The genetic stability of our strain has been confirmed from stock to end of fermentation by using three methods: quantitative PCR measurement of maltogenic amylase gene copy number, qualitative PCR genotyping of the entire inserted genetic construct, and measurement of enzyme activity. For all analyses, stock and end of fermentation were observed to be the same.

2.2.6 Antibiotic Resistance Genes

During construction of the engineered strain, only a single plasmid was used during the transformation step, which contained the hygromycin resistance gene. This plasmid was only used as a co-transformation aid and no plasmid DNA was integrated into the yeast genome. The plasmid was cured with passaging of the transformant. Absence of antibiotic markers was confirmed by whole genome sequencing and by assessing growth on selective media. Therefore, confirmation of removal of any antibiotic resistance genes was confirmed and no antibiotic resistance was conferred to the modified strain.

2.2.7 Absence of the Production Organism in the Final Product

Absence of the production microorganism in one gram of material is our established specification for the commercial product. The production organism does not end up in food and therefore, the first step in the safety assessment as described by IFBC (1990) is adequately addressed.

2.3 Manufacture of the Production Organism

The maltogenic α-amylase enzyme is produced by fermentation and subsequent concentration, cell breakage, solid/liquid separation, concentration and finally polish and germ filtration of *Saccharomyces cerevisiae* encoding for the wild-type maltogenic α-amylase gene from *Geobacillus stearothermophilus*. The use of the enzyme increases the ability to reduce retrogradation of starch.

2.3.1 Manufacturing in the Enzyme Production Plant

The manufacturing process for the production of modified *Saccharomyces cerevisiae* strain containing maltogenic α-amylase starts with a traditional baker's yeast process (Reed, 1982; Chen & Chiger, 1985; Rose & Vijayalakshmi, 1993; Plomp, 1999). The genetically modified yeast product is produced in accordance with current good manufacturing practices for food (cGMP). When production is run in the EU, it is also subject to the Food Hygiene Regulation (852/2004).

A HACCP (Hazard Analysis Critical Control Points) plan, which includes ensuring microbiological purity, is employed during the entire production process. The production is conducted at a fermentation facility with established procedures and equipment suitable for Good Industrial Large-Scale Practice (GISLP) and meets the criteria for safe production organism as described in Pariza and Johnson (2001). Physical inspection and the appropriate microbiological and fermentation analyses are conducted to confirm strain identity and functionality in application, ensuring that the product meets the finished product specifications. These methods are based on generally available and accepted methods used for the production of microbial production organisms and the production of microbial enzymes (Stanbury & Whitaker, 1984).

The culture stocks are sent to the yeast plant (as frozen vials or as slants) from the location of the master cell bank. The plant keeps a record of all stocks received and used in production. A unique sequential number is assigned to each stock to ensure traceability during all steps of production. During production, many parameters are checked according to the Quality Plans and Inspection Plans in place. Inspection Plans are developed to ensure testing during critical steps of the production process from beginning to end. Many parameters are followed such as physical-chemical analysis (solids, color, pH, *etc*.), microbiological analysis and processing activities.

2.3.2 Raw Materials

The raw materials used in the fermentation and recovery processes for the yeast product are standard food grade ingredients used in traditional baker's yeast production. The raw materials include a source of carbon, which are typically molasses, sugar or glucose, a nitrogen source typically ammonia, and other nutrients (essential elements and vitamins). For the recovery process, filter-aids, foam control agents and flocculants might be used. The raw materials conform to either specifications set out in the Food Chemical Codex, 10th edition, 2016 or The Council Regulation 93/315/EEC, setting the basic principles of EU legislation on contaminants and food, and Commission Regulation (EC) No 1881/2006 setting maximum limits for certain contaminants in food. For those that do not appear in FCC specifications, suitable ingredients are

used and internal specifications are established to meet the ones set forth by the FCC requirements.

2.3.3 Lab Stage

Yeast propagation is initiated from frozen master stocks of pure culture maintained at -80°C in glycerol. The strain may be struck from the master cell bank to a sterile agar slant, and the slant may be used to inoculate a flask of 5-10L of sterile medium (autoclaved) under strict sterile conditions. Alternatively, a working stock culture derived from the master cell bank is used to start the propagation. The frozen working stock culture is first inoculated under strict sterile conditions into a flask of 5 – 10 L of sterile medium (autoclaved). This flask is cultivated in the laboratory to increase the numbers of growing cells prior to inoculating the culture into the production vessels.

2.3.4 Fermentation

The yeast from the flask is inoculated into a propagation tank of 0.1 – 3 m^3 working volume. The culture is sequentially transferred into increasing fermenter volumes up to 100 – 250 m^3. The final fermentation is fed with carefully controlled amounts of sugar and air to achieve the maximum output of yeast product.

To prevent contamination of foreign microorganisms, all equipment is carefully operated, cleaned, and maintained including steam sterilization of primary ingredients. The fermentation vessels are cleaned in place (CIP) with acid and base, and then rinsed with water until a neutral pH is reached before production batches. Throughout the fermentation steps, key control parameters are monitored to confirm proper growth and ensure consistent production. Temperature, pH, and aeration rate are monitored and controlled. The fermenter off-gas is monitored for ethanol production, and the feeding rate of carbon source is adjusted to provide the optimal growth with minimal ethanol production.

2.3.5 Recovery and Formulation of the Finished Product

During fermentation, the enzyme protein is being produced intracellularly in the yeast. The recovery process is initiated upon completion of fermentation. The purpose of the recovery process is to:

- extract the enzyme from the yeast cell;
- separate the extract containing the enzyme from the yeast cell debris; and
- concentrate the desired enzyme protein and to improve the ratio enzyme activity/Total Organic Substance (TOS).

This section first describes the materials used during recovery (downstream processing), followed by a description of the different recovery process steps:

- Yeast concentration
- Cell breakage
- Primary solid/ liquid separation

- Concentration
- Polish and germ filtration

The nature, number and sequence of the different types of unit operations described below may vary, depending on the specific enzyme production plant.

2.3.5.1 Yeast concentration

Yeast cells may be concentrated by centrifuging from the fermentation broth. The yeast is then washed to remove remaining non-yeast soluble solids, leading to a liquid yeast cream with 15 - 24% solids.

The specifications for yeast cream are measured on every batch prior to QC release before further processing.

2.3.5.2 Cell breakage

Cell disruption techniques for recovery of intracellular compounds from yeast including mechanical (bead mill, high-pressure homogenization, ultrasonication), and non-mechanical (physical, chemical and/or enzymatic) techniques.

2.3.5.3 Primary solid/liquid separation

The purpose of the primary separation is to remove the solids from the enzyme containing liquid. The primary separation is performed at a defined pH and a specific temperature range in order to minimize loss of enzyme activity.

The separation process may vary, depending on the specific enzyme production plant. This can be achieved by different operations like centrifugation or filtration.

2.3.5.4 Concentration

The liquid containing the enzyme protein may be concentrated in order to achieve the desired enzyme activity and potentially diafiltrated to increase the ratio enzyme activity/TOS before formulation. Concentration is done using ultrafiltration or evaporation.

Temperature and pH are controlled during the concentration step, which is performed until the desired concentration has been obtained. The filtrate containing the enzyme protein is collected for further recovery and formulation.

2.3.5.5 Polish and germ filtration

After concentration, for removal of residual cells of the production strain and as a general precaution against microbial contamination, filtration on dedicated germ filters can be applied at various stages during the recovery process. Pre-filtration (polish filtration) is included if needed to remove insoluble substances and facilitate the germ filtration. The final polish and germ

filtration at the end of the recovery process results in a concentrated enzyme solution free of the production strain and insoluble substances.

2.3.5.6 Formulation and packaging

Subsequently, the enzyme is formulated as a dried solid product. The resulting product is defined as a 'food enzyme preparation'.

Maltogenic α-amylase enzyme preparations are sold mainly as solid preparations. Drying can be done using various technologies in order to deliver the preferred particle properties. Carriers, typical salt, starch or dextrin, can be added to improve the drying process. All carriers are GRAS. The food enzyme is adjusted to a declared activity, standardized and preserved if needed.

The food enzyme preparation is tested by Quality Control for all quality related aspects, like expected enzyme activity and the general JECFA Specification for Food Enzyme Preparations, and released by Quality Assurance.

The final product is packed in suitable food packaging material before storage. Warehousing and transportation are performed according to specified conditions mentioned on the accordant product label for food enzyme preparations.

2.3.6 General Production Controls and Specifications

In order to comply with cGMPs and HACCP principles for food production, the following potential hazards in food enzyme production are taken into account and controlled during production as described below:

2.3.6.1 Identity and purity of the producing microorganism

The assurance that the production microorganism, which is described in Section 2.2, efficiently produces the desired enzyme protein is of utmost importance to the food enzyme producer. Therefore, it is essential that the identity and purity of the microorganism is controlled. Production of the required enzyme protein is based on a well-defined Master Cell Bank and Working Stock Culture. The cell line history and the production of a Cell Bank, propagation, preservation and storage is monitored and controlled. A stock culture is only accepted for production runs if its quality meets the required standards. This is determined by checking identity, viability, microbial purity and productivity of the culture.

2.3.6.2 Microbiological hygiene

For optimal enzyme production, it is important that hygienic conditions are maintained throughout the entire fermentation process. Measures utilized to guarantee microbiological hygiene and prevent contamination with microorganisms ubiquitously present in the environment (water, air, raw materials) are as follows:

- Hygienic design of equipment:
    - All equipment is designed, constructed and used to prevent contamination by foreign micro-organisms

- Cleaning and sterilization:
    - Validated standard cleaning and sterilization procedures of the production area and equipment: all fermentors, vessels and pipelines are washed after use with a CIP-system (Cleaning in Place), where hot caustic soda and nitric acid are used as cleaning agents. After cleaning, the vessels are inspected.
    - Sterilization of all fermentation media: all the media are sterilized with steam injection in fermentors or media tanks
    - Use of sterile air for aeration of the fermentors: Air and ammonia water are sterilized by filtration.

- Hygienic processing:
    - Aseptical transfer from the lab stage and between fermentation steps.
    - Temperature and pH controlled to minimize growth

- Germ filtration
    - Filtration used to keep level of microorganisms under control during recovery

In parallel, hygienic conditions in production are furthermore ensured by:

- Training of staff, all the procedures are executed by trained staff according to documented procedures complying with the requirements of the quality system.
- Procedures for the control of personal hygiene
- Pest control
- Inspection and release by independent quality organization according to version-controlled specifications
- Procedures for cleaning of equipment including procedures for check of cleaning efficiency (inspections, flush water samples etc.) and master cleaning schedules for the areas where production take place
- Procedures for identification and implementation of applicable legal requirements
- Control of labelling
- Requirements to storage and transportation

2.3.6.3 In-process controls

In addition to these control measures in-process testing and monitoring is performed to guarantee an optimal and efficient enzyme production process and a high quality product (cGMPs). The whole process is controlled with a computer control system, which reduces the probability of human errors in critical process steps.

These in-process controls comprise:

- Microbial controls:
    - Absence of significant microbial contamination is analyzed by microscopy or plate counts before inoculation of both the seed and main fermentation, at regular intervals, and at critical process steps during fermentation and recovery.

- Monitoring of fermentation parameters may include:
    - pH
    - Temperature
    - Aeration conditions

The measured values of these parameters are constantly monitored during the fermentation process. The values indicate whether sufficient biomass or enzyme protein has been developed and the fermentation process evolves according to plan. Deviations from the pre-defined values lead to adjustment, ensuring an optimal and consistent process.

Enzyme activity and other relevant analyses (like dry matter, refraction index or viscosity); this is monitored at regular intervals and at critical steps during the whole food enzyme production process.

2.3.7 Stability of the enzyme during storage and prior to use

Food enzymes are formulated into various enzyme preparations in order to obtain standardized and stable products. Therefore, the stability depends on the type of formulation, not on the food enzyme as such.

The date of minimum durability or use-by-date is specified on the label of the food enzyme preparation. If necessary, special conditions of storage and/or use will also be stated on the label.

2.4 Product Specifications

2.4.1 Typical Quantitative Composition

The maltogenic α-amylase enzyme preparation is produced in a solid form. The enzyme preparation does not contain any major food allergens from the fermentation media. **Table 2** provides typical compositions two formats: a concentrated product and a product standardized to a specific level of activity. **Table 2** identifies the substances that are considered diluents and stabilizers used in the enzyme preparations. **Table 3** provides analytical data of four test production batches. Of the four batches, two had no diluents or stabilizers included in the formulation, while the other two batches were formulated with maltodextrin and are more typical of a concentrated (but not standardized) product format.

Table 2. Typical compositions of the enzyme preparations

Component	Concentrated format	Standardized format
Enzyme activity	40,000-60,000 U/g	11,000 U/g
Enzyme solids (TOS*)	25-60%	4-18%
Maltodextrin	40-75%	8-90%
Sodium chloride	0-10%	0-2%
Flour	0	0-80%
Water	remainder	remainder

*Total Organic Solids, defined as: 100% - water – ash – diluents.

Table 3. Compositional analysis of four production batches

Component				
Amylase activity (U/g)	64,012	84,518	51,778	61,739
Maltodextrin (%)	0	0	45.9	47.6
Water (%)	5.8	3.8	8.3	4.9
Ash (%)	8.9	6.8	3.4	3.3
TOS (%)	85.3	89.4	42.5	44.3

*Total Organic Solids, defined as: 100% - water – ash – diluents.

2.4.2 Specifications

The maltogenic α-amylase enzyme preparation complies with the recommended purity criteria for enzyme preparations as described in the *Food Chemicals Codex* (FCC, 2016). In addition, the maltogenic α-amylase enzyme preparation also conforms to the General Specifications for Enzyme Preparations Used in Food Processing as proposed by the Joint FAO/WHO Expert Committee on Food Additives in Compendium of Food Additive Specifications (JECFA, 2006).

Table 4 includes analytical data of the four production batches, demonstrating compliance with FCC and JECFA.

Table 4. Analytical data for four food enzyme batches

Parameter	Specification	[redacted]	[redacted]	[redacted]	[redacted]
Lead (mg/kg)	<5	< 0.061	< 0.061	< 0.061	< 0.061
Total aerobic plate count (CFU/g)	<50,000	10,000	41,000	22,000	28,000
Coliforms (CFU/g)	<30	<10	<10	<10	10
E. coli (in 25g)	Not detected	ND	ND	ND	ND
Salmonella (in 25g)	Not detected	ND	ND	ND	ND
Production organism (in 1g)	Not detected	ND	ND	ND	ND
Antimicrobial activity	Not detected	ND	ND	ND	ND
Mycotoxins	Not detected	ND		ND	ND

For mycotoxin testing, lots [redacted] and [redacted] were combined 1:1 by weight to provide sufficient material. Toxins tested (detection limits): 15-Acetyl Deoxynivalenol (0.1ppm), 3-Acetyl Deoxynivalenol (0.1ppm), Aflatoxin B1 (1ppb), Aflatoxin B2 (1ppb), Aflatoxin G1 (1ppb), Aflatoxin G2 (1ppb), Citrinin (50bbp), Diacetoxyscirpenol (20ppb), Deoxynivalenol (0.1ppm), Fumonisin B1 (0.1ppm), Fumonisin B2 (0.1ppm), Fumonisin B3 (0.1ppm), Fusarenon X (0.1ppm), HT-2 Toxin (5ppb), Neosolaniol (20ppb), Nivalenol (0.1ppm), Ochratoxin A (1ppb), Sterigmatocystin (10ppb), T-2 Toxin (5ppb), Zearalenone (12.5ppb).

2.5 Application and Use Levels

2.5.1 Mode of Action

Maltogenic α-amylase catalyzes the hydrolysis of α-1,4-glucosidic linkages in amylose, amylopectin and related glucose polymers. It has been reported to have exo activity, hydrolyzing maltose from the non-reducing end of a polymer chain (Outtrup & Norman, 1984), but it has also been shown to act without a reducing end and can reduce the molecular weight of amylose (Christophersen, 1998), suggesting endo activity. The maltogenic α-amaylase enzyme produced from the modified *S. cerevisiae* can be used to improve the freshness of the final baked product. It can provide a better softness, moistness and resilience of the final product by breaking down amylopectin, which delays staling.

2.5.2 Use Levels

The maltogenic α-amylase enzyme should be used in baking at levels to achieve the desired technical effect and according to current good manufacturing practices (cGMP). The recommended use rate depends on the application. **Table 5** provides enzyme activity doses commonly used by the baking industry in the manufacturing of baked goods, as well as the corresponding TOS dose. This TOS dose is calculated using the lowest anticipated enzyme activity per gram of TOS from **Table 2**.

Table 5. Typical dosage rates of enzyme in baked goods.

Type of food	Typical dose of enzyme in flour (MAU/kg flour)	Typical dose of enzyme in final food (MAU/kg food)	TOS dose in final food (mg TOS/kg food)*
Total white bread	1000	675	11.0
Total whole grain and wheat bread	1000	667	10.9
Rolls	2000	1350	22.1
Tortillas	5000	3350	54.8
Quickbreads and muffins	10000	3000	49.1
Cake	10000	3000	49.1

* TOS dose is calculated based on lowest anticipated enzyme activity per gram TOS.

2.5.3 Enzyme Residues in the Final Food

The potential exposure of humans to the maltogenic α-amylase enzyme is limited by the baked foods production process itself, whereby baking denatures the enzyme. In addition, enzymatic activity will be halted by the depletion of the substrate during the process. The enzyme does not exert a function in the final product.

3. Dietary Exposure

3.1 Estimates of Human Consumption and Margin of Safety

The maltogenic α-amylase enzyme presents no safety risk to humans and is intended for use as a food additive for baking and does not impart any nutritional or safety effects on baked products consumed by humans.

"Worst case" residues were calculated assuming that the product is retained in bread. However, as mentioned previously, the enzyme is expected to be denatured during the baking process.

Theoretical maximum daily intake (TMDI) is calculated based on the Budget Method (Hansen, 1966; Douglass *et al.*, 1997), which assumes all foods contributing to energy intake will contain the additive at permitted use levels. For solid foods, the average consumption over the course of a lifetime is **0.025 kg food per kg body weight per day**.

Assuming processed foods are 50% of total solid food, the average consumption over a lifetime is **0.0125 kg food per kg body weight per day**. For TMDI calculations, we chose to make the extremely conservative assumption that all processed foods consumed use the maltogenic α-amylase enzyme described in this notification, and that all of those foods use the maximum dosage rate of enzyme noted in Table 5 (49.1 mg TOS/kg food).

The calculated TMDI is:

49.1 mg TOS/kg food × 0.0125 kg food per kg body weight per day =

0.614 mg TOS/kg body weight/day

A recent GRAS notice (GRN 746) for a *Geobacillus stearothermophilus* maltogenic α-amylase enzyme preparation produced by *Bacillus subtilis* reported the results of a 13-week oral toxicity study in rodents. The sequence of enzyme in this GRAS notice has 100% identity to the maltogenic amylase enzyme produced by our *Saccharomyces cerevisiae* production host. No observed adverse effect was reported at the highest dose of 1,000 mg TOS/kg body weight (bw)/day in a 13-week oral toxicity study.

If we use the no observed adverse effect level (NOAEL) of 1,000 mg TOS/kg bw/day, the margin of safety is:

1,000 mg TOS/kg bw/day NOAEL ÷ 0.614 mg TOS/kg bw/day intake = **1629**

Since our production host organism (*Saccharomyces cerevisiae*) differs from the production host organism of GRN 746, it is relevant to also consider toxicity testing performed on yeast material. Schauss, *et al.* (2012) reported on a safety evaluation of a food-grade, dried fermentate (EpiCor) of *Saccharomyces cerevisiae*. Several studies were performed, including 90-day subchronic and 1-year chronic oral toxicity studies in rodents. The NOAEL was 1500 mg/kg bw/day for the 90-day study and 800 mg/kg bw/day for the 1-year study, the highest doses tested.

If we use the NOAEL of 800 mg/kg bw/day from the 1-year study, the margin of safety is:

800 mg/kg bw/day NOAEL ÷ 0.614 mg TOS/kg bw/day intake = **1303**

3.2 Dietary Exposure to Any Other Substance Formed in or on Food

The maltogenic α-amylase enzyme acts on the linkages in amylose, amylopectin and related glucose polymers, breaking them down. When this enzyme acts on starch, the products are maltose, glucose, and some maltodextrins (Christophersen, 1998). These products are contained in many foods, such as honey, pasta, and cereals and are not expected to have any adverse effects on humans consuming these products.

3.3 Dietary Exposure to Contaminants or Byproducts

Monitoring of fermentation parameters may include pH, aeration, temperature, and off-gas production. The measured values of these parameters are constantly monitored during the fermentation process. The values indicate whether sufficient biomass develops and the fermentation process evolves according to plan. Deviations from the pre-defined values lead to adjustment, ensuring an optimal and consistent process. Therefore, no harmful contaminants or by-products are expected.

4. Self-Limiting Levels of Use

There are no proposed restrictions for the use of the maltogenic α-amylase enzyme because the enzyme should be used in accordance with good manufacturing practices. See Section 2.5 for use levels.

5. Experience Based on Common Use in Food Before 1958

This part is not applicable to the notified substance.

6. Narrative

This safety assessment of our maltogenic α-amylase enzyme used in baking includes an evaluation of the safety of the production organism, the host organism, the enzyme, the donor, the manufacturing process and consideration of the dietary exposure. Each of these topics is addressed below.

6.1 Safety of the Production Organism *Saccharomyces cerevisiae*

The safety of the production organism is a prime consideration in assessing the probable degree of safety of an enzyme used in food (Pariza & Foster, 1983; Pariza & Johnson, 2001). The host strain used for modified *Saccharomyces cerevisiae* strain producing the maltogenic α-amylase is a non-modified baker's yeast. This *Saccharomyces cerevisiae* strain was selected because of its use as a commercial strain in baker's yeast production and similarity to other baking yeast strains.

Saccharomyces cerevisiae has an extensive history of safe use for over thousands of years in connection with food and feed, primarily the fermentation and preservation of foods. *Saccharomyces cerevisiae* yeast has been used by the ancient Egyptians, Romans, Hebrews and Greeks in fermentation processes for the production of wine, bread, and beer. Commercialized yeast cell preparations and associated nutrients such as proteins, amino acids, vitamins, minerals and trace elements are used as food supplements or in the production of medical products (Moyad, 2007; Moyad, 2008).

Saccharomyces cerevisiae is ubiquitous, is commonly found in our daily lives as it is in the air we breathe, and grows naturally on foods, such as fruits and vegetables especially ones with high fermentable sugars that we consume daily. *Saccharomyces cerevisiae* is a common colonizer of mucosal surfaces and part of the normal flora of the gastrointestinal tract, the respiratory tract, and the vagina (Salonen et al., 2000; Munoz et al., 2005). A summary of the extensive benefits of *S. cerevisiae* on human health has been reviewed (Moslehi-Jenabian et al., 2010). Fleet notes that humans consume large quantities of yeasts without adverse impact on human health, which is unlike bacteria and viruses (2007). Recent studies, such as the acute and subacute toxicity testing of yeast hydrolysate from *Saccharomyces cerevisiae*, show very low toxicity providing additional support of the safety of the yeast as a probiotic (Jung *et al.*, 2010). This further supports the conclusion that *Saccharomyces cerevisiae* yeast is non-pathogenic and non-toxigenic.

Over 2.5 million tons of yeasts are commercially produced each year worldwide making *Saccharomyces cerevisiae* the most widely used microorganism (Halász & Lásztity, 1991; Boekhout & Robert, 2003; Fleet, 2006). About 150 different wine yeast strains, mainly S. cerevisiae, are commercially available (Branduardi *et al.*, 2008). The genome of *Saccharomyces cerevisiae* has been completely sequenced disclosing about 6,000 genes that are identical or

similar to human genes (Goffeau *et al.*, 1996; Branduardi *et al.*, 2008). *Saccharomyces cerevisiae* is the microorganism of choice for research and industrial use as it is easy to manipulate and grow with the capability of producing high, predictable yield that can be well controlled and scaled for industrial use (Ostergaard *et al.*, 2000).

6.2 Regulatory Overview of *S. cerevisiae*

Extensive regulatory approvals support the safety of *Saccharomyces cerevisiae* for diverse uses including food, feed, and pharmaceutical applications.

6.2.1 US Regulatory Overview

6.2.1.1 Code of Federal Regulations (CFR)

Listings of *Saccharomyces cerevisiae* in the Code of Federal Regulations (C.F.R.) are extensive and include:

- Baker's yeast extract (21 C.F.R. § 184.1983)
- Baker's yeast protein (21 C.F.R. § 172.325);
- Yeast-malt sprout extract (21 C.F.R. § 172.590);
- Dried yeast as an ingredient in food (21 C.F.R. § 172.896);
- Baker's yeast glycan (21 C.F.R. § 172.898);
- Direct addition of food grade baker's yeast (*S. cerevisiae*) in
    - Eggs (dried eggs – 21 C.F.R. § 160.105
    - Dried egg whites – 21 C.F.R. § 160.145
    - Dried egg yolks – 21 C.F.R. § 160.185
- Since 1902, autolyzed yeast and cell membranes of yeast have been used for treatment of wine (27 C.F.R. § 24.246).

According to the European Food Safety Agency *(*EFSA), yeasts used in food production, particularly bakers/brewer's yeast, are considered among the safest of microorganisms (EFSA, 2007, 2013). *Saccharomyces cerevisiae* has been designated Qualified Presumption as Safe (QPS) status in Europe, which indicates that no additional safety assessment is needed according to established guidelines (EFSA, 2007, 2008).

6.2.1.2 GRAS

In addition to the common use of *Saccharomyces cerevisiae* in human food, FDA has had no questions on GRAS Notifications for a number of modified *Saccharomyces cerevisiae* for the direct addition to human food. These include:

- GRN 744 Steviol Glycosides with a High Rebaudioside M Content Produced by Microbial Fermentation

- GRN 626 Steviol glycosides produced in *Saccharomyces cerevisiae* strain S288C as a general use sweetener in foods and beverages

- GRN 422 *Saccharomyces cerevisiae* transformed with three copies of the *S. cerevisiae* ASP3 gene encoding for asparaginase
- GRN 350 *Saccharomyces cerevisiae* strain P1Y0 for use as a starter culture for alcoholic beverage fermentation
- GRN 175 *Saccharomyces cerevisiae* strain ECMo01 with enhanced expression of urea amidolyase—for use in fermented beverages
- GRN 120 *Saccharomyces cerevisiae* strain ML01 carrying a gene encoding the malolactic enzyme from *Oenococcus oeni* and a gene encoding malate permease from *Schizosaccharomycespombe*—for use in winemaking as a yeast starter culture for grape must fermentation
- GRN 88 Invertase enzyme preparation from *Saccharomyces cerevisiae* and lactase enzyme preparation from *Kluyveromyces marxianus*—for use in foods in general as an enzyme

6.2.1.3 National Institutes of Health (NIH)

The NIH Guidelines for Research Involving Recombinant DNA Molecules considers *Saccharomyces cerevisiae* a safe host organism and qualifies as a Risk Group 1 agent as it is not associated with disease in healthy adult humans under its Basis for the Classification of Biohazardous Agents by Risk Group (U.S. DHHS, 2016– Appendix C-III).

As EPA recognized in its Final Risk Assessment of *Saccharomyces cerevisiae* (February 1997; U.S. EPA, 1997 - p. 9), "[m]any scientists believe that under appropriate conditions any microorganism could serve as an opportunistic pathogen." The Agency concluded that *S. cerevisiae* has an extensive history in food processing and neither it nor other closely related species "has been associated with pathogenicity toward humans or has been shown to have adverse effects on the environment" (p.2). Specifically, with respect to human exposure, EPA concluded on p. 3 of the Final Risk Assessment that:

> "There are individuals who may ingest large quantities of *S. cerevisiae* every day, for example, people who take the yeast as part of a "health food" regimen. Therefore, studies were conducted to ascertain whether the ingestion of large numbers of these yeasts might result in either colonization, or colonization and secondary spread to other organs of the body. It was found that the installation of very large numbers of *S. cerevisiae* into the colons of animals would result in both colonization and passage of the yeasts to draining lymph nodes. It required up to 10^{10} *S. cerevisiae* in a single oral treatment to rats to achieve a detectable passage from the intestine to the lymph nodes (Wolochow *et al.*, 1961). The concentrations of *S. cerevisiae* required were well beyond those that would be encountered through normal human daily exposure."

EPA concluded that: "*Saccharomyces*, as a genus, present low risk to human health or the environment. Criteria used to differentiate between species are based on their ability to utilize specific carbohydrates without relevance to pathogenicity. Nonetheless, this risk assessment applies to those organisms that fall under the classical definition of *S. cerevisiae* as described by

van der Walt (1971)." The modified *S. cerevisiae* strain falls under the classical definition described by van der Walt (1971).

Thus, FDA, NIH, and EPA have concluded the safety of *Saccharomyces cerevisiae* as a non-pathogenic microorganism.

6.2.2 European Food Safety Agency (EFSA) Regulatory Overview

According to EFSA, yeasts used in food production, particularly bakers/brewer's yeast, are considered among the safest of microorganisms (EFSA, 2007, 2018). *Saccharomyces cerevisiae* is one of the safest microorganisms used in food and feed production and has been designated Qualified Presumption as Safe (QPS) status in Europe, which indicates that no additional safety assessment is needed according to established guidelines (EFSA, 2007, 2008). A recent safety review by EFSA continues to support the QPS status of *S. cerevisiae* (EFSA, 2018). One example of a feed approval is the inactivated and dried selenized yeast produced by *S. cerevisiae* providing selenium, an essential trace element, in an organic form as a nutritional additive for use in poultry, pigs, and bovines (EFSA, 2006.) EFSA reviewed the safety and efficacy of selenium-enriched yeast (*Saccharomyces cerevisae* CNCM I-3399) for all animal species (EFSA, 2009).

EFSA notes that "[r]are opportunistic infections have been caused by *Saccharomyces cerevisiae*," and EFSA maintains its QPS (Qualified Presumption as Safe) status (EFSA, 2008). EFSA provides additional clarification stating, "*Saccharomyces cerevisiae*, subtype *boulardii* is contraindicated for patients of fragile health, as well as patients with a central venous catheter in place. A specific protocol concerning the use of probiotics should be formulated" (EFSA, 2008, Table 4, pp.21, 43). Even with the infrequent cases of fungemia associated with *S. boulardii*, McFarland discusses contraindications and precautions and recommends closely monitoring adult immuno-compromised patients and catheter use, especially with unexplained fever and notes that some recommend not giving *S. boulardii* to immuno-suppressed patients or those with central catheters to reduce the risk of fungemia (Buts, 2009; McFarland, 2010).

6.2.3 Food Standards Australia New Zealand

Saccharomyces cerevisiae is recognized as a safe microorganism for processing aids (Schedule 18)[1].

[1] Available at http://www.foodstandards.gov.au/code/Documents/Sched%2018%20Processing%20aids%20v159.pdf.

6.2.4 Health Canada

Saccharomyces spp. is listed as a source microorganism for the production of invertase and lactase[2].

6.2.5 Regulatory Overview of Pharmaceuticals

As of January 2009, twenty-eight of the 151 protein-based recombinant pharmaceuticals that have been approved by the FDA and EMEA (European Medicines Agency) were produced in *Saccharomyces cerevisiae* (Ferrer-Miralles *et al.*, 2009; Huang *et al.*, 2010). The first vaccine effective against hepatitis B was produced intracellularly in recombinant *S. cerevisiae* (McAleer *et al.*, 1984; Çelik & Çalık, 2012). Insulin, hepatitis B surface antigen, GM-CFS, hirudin, platelet-derived growth factor are among other pharmaceuticals produced by *S. cerevisiae* (Demain & Vaishnav, 2009).

6.2.6 Safety Studies

Pineton de Chambrun, *et al.* (2015) conducted a randomized clinical trial of *Saccharomyces cerevisiae* versus a placebo in the irritable bowel syndrome. 179 adults with irritable bowel syndrome were randomized to receive once daily 500 mg of *Saccharomyces cerevisiae*, delivered by one capsule (n = 86, F: 84%, age: 42.5 ± 12.5), or placebo (n = 93, F: 88%, age: 45.4 ± 14) for 8 weeks followed by a 3-week washout period. After a 2-week run-in period, cardinal symptoms (abdominal pain/discomfort, bloating/distension, bowel movement difficulty) and changes in stool frequency and consistency were recorded daily and assessed each week. A safety assessment was carried out throughout the study. The proportion of responders, defined by an improvement of abdominal pain/discomfort, was significantly higher (p = 0.04) in the treated group than the placebo group (63% vs 47%, OR = 1.88, 95%, CI: 0.99-3.57) in the last 4 weeks of treatment. A non-significant trend of improvement was observed with *Saccharomyces cerevisiae* for the other symptoms. *Saccharomyces cerevisiae* was well tolerated and did not affect stool frequency and consistency.

Schauss, *et al.* (2012) reported on a safety evaluation of a food-grade, dried fermentate (EpiCor) of *Saccharomyces cerevisiae*. Studies included the following assays: bacterial reverse mutation, mouse lymphoma cell mutagenicity, mitogenicity assay in human peripheral lymphocytes, and a cytochrome P450 ([CYP] CYP1A2 and CYP3A4) induction assessment as well as 14-day acute, 90-day subchronic, and 1-year chronic oral toxicity studies in rats. No evidence of genotoxicity or mitogenicity was seen in any of the in vitro or in vivo studies. The CYP assessment showed no interactions or inductions. No toxic clinical symptoms or histopathological lesions were observed in the acute, subchronic, or chronic oral toxicity studies in the rat. Results of the studies performed indicate that EpiCor does not possess genotoxic activity and has a low order of

[2] Invertase, http://webprod.hc-sc.gc.ca/nhpid-bdipsn/ingredReq.do?id=4588&lang=eng; Lactase, http://webprod.hc-sc.gc.ca/nhpid-bdipsn/ingredReq.do?id=7307&lang=eng last accessed October 9, 2018.

toxicity that is well tolerated when administered orally. The no observable adverse effect level (NOAEL) was 1500 mg/kg body weight (bw)/d for the 90-day study and 800 mg/kg bw/d for the 1-year study, for the highest doses tested.

Pereyra *et al.* (2014) reports on the probiotic *Saccharomyces cerevisiae* RC016 and test its ability to reduce genotoxicity caused by dietary aflatoxins (AFs). The probiotic was orally administered to Wistar rats. Six groups (n = 6) were arranged: feed and probiotic controls, two levels of AFs-contaminated feed and two treatments including both the probiotic and the toxin. Genotoxicity and cytotoxicity were evaluated with the bone marrow micronuclei assay and the comet assay and internal organs were macroscopically and microscopically examined. The tested *Saccharomyces cerevisiae* strain did not cause genotoxicity or cytotoxicity in vivo, and it was able to attenuate AFs-caused genotoxicity. *Saccharomyces cerevisiae* RC016 did not cause any impairment on the rats' health and it showed no negative impact on the weight gain. Moreover, RC016 improved zootechnical parameters in AFs-treated animals. The beneficial effects were likely to be caused by adsorption of AFs to the yeast cell wall in the intestine and the consequent reduction in the toxin's bioavailability. It was concluded that dietary administration of RC016 does not induce genotoxicity or cytotoxicity to rats.

Jung *et al.* (2010) showed that yeast hydrolysate from *Saccharomyces cerevisiae* had very low toxicity in rat studies. This study was designed to test yeast hydrolysate in 10-30 kDa molecular weight for use as a dietary supplement by assessing its acute and subacute oral toxicity in female and male Sprague-Dawley (SD) rats. The single oral dose of the hydrolysate at 5000 mg/kg did not produce mortality or significant changes in the general behavior and gross appearance of the internal organs of rats. In subacute toxicity study, the hydrolysate was administered orally at a dose of 1000 mg/kg/day for a period of 14 days. The satellite group was treated with the hydrolysate at the same dose and the same period and kept for another 14 days after treatment. There were no significant differences in organ weights between control and treated group of both sexes. Hematological analysis and blood chemistry revealed no toxicity effects of *Saccharomyces cerevisiae* hydrolysate. Pathologically, neither gross abnormalities nor histopathological changes were observed. It was concluded that results showed that the hydrolysate has very low toxicity in the SD rat model.

Ardiani *et al.* reviews preclinical and clinical studies supporting the use of heat-killed whole recombinant *Saccharomyces cerevisiae* cells as therapeutic vaccines to treat cancer and infectious diseases (2010). Wansley *et al.* further notes that ‘one of the reasons for interest in recombinant *Saccharomyces cerevisiae* as a vaccine vehicle is its lack of toxicity. Besides being inherently nonpathogenic, this particular species of yeast can be heat-killed before administration and has been shown to be safe in humans in several clinical trials, with maximum tolerated dose not reached (2008; Franzusoff *et al.*, 2005).

6.2.7 Conclusions

As summarized above, modern biotechnology delivers a wide range of safe products derived from *Saccharomyces cerevisiae* including food, beverages, feed, pharmaceuticals, enzymes, lipids and vitamins (Stewart & Russell, 1985; Bigelis, 1985; Gerngross, 2004; Redwan, 2007).

Based on the safety assessment, Mascoma concludes that there is reasonable certainty of no harm to humans using the *Saccharomyces cerevisiae* as a production strain.

6.3 Safety of the Donor *Geobacillus stearothermophilus*

The gene from *Geobacillus stearothermophilus* (previously called *Bacillus stearothermophilus*) was PCR amplified from an artificially synthesized gene based on publicly available sequences and codon-optimized for expression in the *S. cerevisiae* host. Use of synthetic DNA for the engineering of the production strain negates the possibility of donor DNA transfer to the production strain. The *Geobacillus* genus comprises obligate thermophiles containing vegetative cells that are gram-positive, motile rod-shaped, spore-forming bacteria that occur singly or in short chains and have an optimum temperature of 55-65°C (Nazina, 2001). *Geobacillus* spp. are closely related with a 96.5-99.5% 16S rDNA sequence similarity (Nazina, 2001) and have been isolated from temperate areas as well as hot springs, oilfields, deep sea sediments, sugar refineries, canned foods, raw and dehydrated vegetables and dairy factories (Nazina, 2001; Burgess, 2010). The species *Geobacillus stearothermophilus* was first described in 1920 (Donk) and was isolated from canned cream-style corn. Diderichsen cloned maltogenic alpha amylase from *Bacillus stearothermophilus* in 1988 (Diderichsen & Christiansen, 1988). The *Geobacillus* genus is especially useful in the biotechnology industry for its production of thermostable enzymes (Burgess, 2017).

Based on 16S rRNA, fatty acid composition, DNA-DNA hybridization analysis and other phenotypic analysis, *Bacillus stearothermophilus* underwent a reclassification to a new genus and its name was emended to *Geobacillus stearothermophilus* (Nazina, 2001; Coorevits *et al.*, 2012; Vos *et al.*, 2009). The *Geobacillus* genus includes the thermophilic species that were previously classified in rRNA Group 5 of the Bacillus genus (Ash, 1991). Notification that new names and new combinations have appeared in the International Journal of Systematic and Evolutionary Microbiology in 2001.

Geobacillus stearothermophilus is used in transglycosylation reactions that benefit the food and pharmaceutical industries (Cha *et al.*, 1992; Derde *et al.*, 2012; Hwa Park *et al.*, 1998; (Kolcuoğlu *et al.*, 2010; van der Maarel & Leemhuis, 2013; Lee *et al.*, 2002b). Erythritol, a sugar alcohol, was modified by transglycosylation with *Geobacillus stearothermophilus* maltogenic amylase to generate maltosyl-erythritol, a major transglycosylation product (Yoon *et al.*, 2003). In medicine, puerarin (daidzein 8-C-glucoside) can be used to treat coronary heart disease, cardiac infarction, problems with ocular blood flow, sudden deafness, and alcoholism. Puerarin cannot be given by injection due to its low solubility in water, however. To increase its solubility, puerarin is transglycosylated *in vitro* using *Geobacillus stearothermophilus* maltogenic α-amylase to generate puerarin glycosides—two major transfer products are α-D-glucosyl-(1,6)-puerarin and α-D-maltosyl-(1,6)-puerarin. The solubility of the transfer products is 14 and 168 times higher than that of puerarin, respectively (Li *et al.*, 2004).

A branching enzyme from *Geobacillus stearothermophilus* showed the enzyme was non-mutagenic and did not result in death or acute toxicity when orally administered to rates by gavage at a dose of 2000 mg/kg body weight/day. Thus, these studies support the safety of an enzyme from native *Geobacillus stearothermophilus* (An-Pyo Center, 1999a & b, Cited in: Choi, 2009b)

Choi extended the An-Pyo study further by evaluating the safety of branching enzymes from genetically modified organisms. Choi provided a safety evaluation of the branching enzyme (EC

2.4.1.18) gene from *Geobacillus stearothermophilus* strain TRBE14 and *Aquifex aeolicus* strain VF5. Two branching enzymes were tested in two- 90-day sub-chronic toxicity studies conducted in rats. Genotoxicity was assessed in the Ames reverse mutation assay and in the in vitro chromosomal aberration assay. This standard toxicological testing supported the safe used of these enzymes (Choi, 2009b).

Choi conducted a safety evaluation of a highly-branched cyclic dextrin (HBCD) and a 1,4-α-glucan branching enzyme from *Geobacillus stearothermophilus*. Standard toxicological studies were conducted, including an acute oral toxicity study in rats and mutagenicity studies in the mouse lymphoma assay, an in vitro microbial mutagenicity assay, and an in vitro digestion assay; it was concluded that the enzymes were safe for human consumption. (Choi, 2009a). HBCD is used as a food ingredient in Japan, which includes its carbohydrate supplementation of sports drinks.

Ke *et al.* conducted an extensive safety evaluation of a thermolysin enzyme produced from *Geobacillus stearothermophilus* since this no previous toxicological evaluation had been conducted on thermolysin. The standard toxicological studies included acute toxicity studies in rats and mice showing no toxicity with an oral dose of 10,000 mg/kg in rats and 24,000 mg/kg in mice. Subchronic feeding studies in rats for 91 day oral gavage with doses up to 1000 mg/kg showed no significant differences between experimental and control groups with a No Observed Effect Level (NOEL) of 1000 mg/kg per day being established. Genotoxicity testing (in vitro chromosomal aberration assay; in vivo mouse micronucleus) were negative. Allergenicity sequence analysis was negative. It was concluded that the safety studies conducted support the safe use of thermolysin derived from *Geobacillus stearothermophilus* for use in food production and their findings are consistent with the history of safety consumption of enzymes derived from *Geobacillus stearothermophilus*. (Ke *et al.*, 2013).

Geobacillus spp. are common spoilage organisms in food manufacturing plants and products (Lücking *et al.*, 2013; Postollec *et al.*, 2012). Thermophilic bacilli, such as *Geobacillus stearothermophilus*, can cause spoilage in foods, especially in dairy products and canned foods, because of the persistence of non-pathogenic thermophilic and highly heat-resistant spore-forming bacteria that have the ability to form endospores and biofilms (Burgess *et al.*, 2010; Rigaux *et al.*, 2014). As potential spoilage organisms, enzymes and acids produced by these bacteria may cause off-flavors in the final products. *Geobacillus stearothermophilus* is recognized as a major source of spoilage in canned foods. It can be detected in cans presenting defects after a 7-day incubation at 55°C (Andre *et al.*, 2013). Good hygiene and sterilization processing under GMP are critical for spore inactivation and spoilage control leading to product stability, meeting quality standards and consumer acceptance, which leads to economic benefits to the food industry. Also, since *Geobacillus stearothermophilus* can grow and sporulate at temperatures between 40°C and 70°C, it was recommended that the food industry reassess heat-treatment process settings and maintain processing line temperatures above 70°C or to decrease the pH to 5.0 to hinder the growth of most strains (Durand *et al.*, 2015). *Geobacillus stearothermophilus* is commonly used as a challenge organism for sterilization validation studies and periodic check of sterilization cycles.

Geobacillus stearothermophilus has a history of extensive US regulatory approvals for a multitude of uses. A summary of some of the regulatory activities are as follows:

- FDA has affirmed alpha-amylase from *Geobacillus stearothermophilus* as GRAS (21 CFR § 184.1012) for the production of maltodextrins and nutritive carbohydrate sweeteners. FDA concluded that the organism is a non-pathogenic and non-toxicogenic strain of *Geobacillus stearothermophilus* (1995).

FDA issued Agency letters with no objections for four GRAS notifications using *Geobacillus stearothermophilus*: Alpha amylase from *Geobacillus stearothermophilus* produced by *Bacillus licheniformis* (GRN 594); 1,4-alpha-glucan branching enzyme preparation from *Geobacillus stearothermophilus* strain TRBE 14 (GRN 405); thermolysin enzyme preparation derived from *Geobacillus stearothermophilus* (GRN 598); and maltogenic amylase enzyme from *Geobacillus stearothermophilus* produced in *Bacillus subtilis* (GRN 746).

Geobacillus stearothermophilus has been designated Qualified Presumption as Safe (QPS) status in Europe by the European Food Safety Agency (EFSA), which indicates that no additional safety assessment is needed according to established guidelines (EFSA, 2018).

Geobacillus stearothermophilus, as a non-pathogenic and non-toxigenic gram-positive bacterium, has a long history of safe use in the food processing industry and has been identified as a safe source of enzymes as both a host and a donor organism (Pariza & Johnson, 2001; Olempska-Beer *et al.*, 2006). *Geobacillus stearothermophilus* is a safe donor organism for the maltogenic α-amylase. A search of the scientific literature provided no evidence connecting strains of *Geobacillus* to pathogenicity in humans or animals and there are no reports we were able to identify of secondary metabolites with toxicity.

As mentioned previously, the maltogenic α-amylase enzyme gene from *Geobacillus stearothermophilus* was amplified by PCR from an artificially synthesized gene based on the Genbank sequence, which negates the possibility of donor DNA transfer to the strain.

In conclusion, we were unable to identify any risk factors for using *Geobacillus stearothermophilus* as a gene donor for a maltogenic α-amylase enzyme.

6.4 Safety of the Maltogenic α -amylase Enzyme

Maltogenic amylase is used in the baking and starch industry. Maltogenic α-amylase derived from *Geobacillus stearothermophilus* has been widely used in baking since the mid-1990s as a bread crumb anti-staling enzyme (Derde *et al.*, 2012; Goesaert *et al.*, 2009). Maltogenic α-amylase is an important enzyme in the dairy industry catalyzing the conversion of starch into maltose, an important sugar in food and pharmaceutical industries (Derde *et al.*, 2012; Straksys *et al.*, 2016).

The α-amylase family of enzymes can be divided into endo- and exo-type enzymes, which catalyze the hydrolysis of amylose, amylopectin, and other carbohydrates. This maltogenic α-amylase is an exo-acting enzyme, which catalyzes the hydrolysis of α-1,4-glucosidic linkages in amylose, amylopectin and related glucose polymers. Maltose units are successively removed from the non-reducing end of the polymer chain until the molecule is degraded, or in the case of amylopectin, a branch point is reached (Christophersen *et al.*, 1998; Goesaert *et al.*, 2009).

Most α-amylase are extracellular enzymes and enable the bacteria to utilize starch and carbohydrates outside the cell. However, α-amylase also include enzymes that are intracellular, such as maltogenic α-amylase, cyclomaltodextrinases, and neopullulanases. These enzymes are members of the glycoside hydrolase family 13 (GH13) and have been classified under a common subfamily, GH13_20 (subfamily 20 of glycoside hydrolase family; at least 29 members have been characterized covering different substrates and linkage specificities. In contrast to other α-amylase, members of GH13_20 efficiently hydrolyze cyclodextrins.

Cyclomaltodextrinases and neopullulanses have an extra domain either at the N-terminal or at the C-terminal, while maltogenic α-amylases have extra domains at both ends. In addition to four conserved regions found in the members of α-amylase, maltogenic α-amylase include a characteristic sequence within the MPKLN region (or MPKIN and MPKLR) that is unable to bind calcium ions due to the Asp to Lys substitution (Nasrollahi *et al.*, 2013). Van der Maarel & Leemhuis provides an overview of the properties and applications of microbial alpha amylases, including a comparison of the four regions of conserved catalytic amino acid residues from ten alpha amylase enzymes, including maltogenic amylase (2002, Table 1).

Like other amylolytic enzymes, maltogenic α-amylase share the characteristics of being able to hydrolyze multiple substrates, such as starch, pullulan and cyclodextrins, and to simultaneously transfer the hydrolyzed sugar moiety to another sugar moiety making them useful for the preparation of branched oligosaccharide mixtures and novel carbohydrates (Lee *et al.*, 2002a, Lee *et al.*, 2002b, Lee *et al.*, 2003, Kim *et al.*, 2002; Kim *et al.*, 2003, Hwa Park *et al.*, 1998). Crystallographic analysis of enzymes in this subfamily reveal that they possess the $(\alpha/\beta)_8$ barrel and the C domain are common to amylolytic enzymes as well as an extra 124-residue N domain that is involved in domain-swapped homodimer formation (Lee *et al.*, 2002a; Lee *et al.*, 2002b).

Maltogenic α-amylase is distinguishable from typical α-amylases by its extensive transglycosylation activity, which is in addition to its hydrolysis activity. Maltogenic α-amylase transfers a donor molecular released by the hydrolyzing activity of the enzyme to an acceptor molecule by mainly forming an α-(1,6)-glycosidic linkage between the donor and the acceptor molecules. These combined activities of maltogenic α-amylase have been used to produce branched oligosaccharides from liquefied starch (Lee et al, 2002a; Lee *et al.*, 2002b; Lee *et al.*, 2003). The crystal structure of a maltogenic α-amylase shows the enzyme has catalytic versatility (Kim *et al.*, 1999).

6.4.1 Regulatory Approvals

Extensive regulatory approvals support the safety of the maltogenic α-amylase enzyme, including FDA, JECFA, Food standards Australia New Zealand (FSANZ), Health Canada and the American Association of Feed Control Officials (AAFCO):

6.4.1.1 GRAS

FDA had no questions on a GRAS Notice (GRN 746) for maltogenic amylase enzyme from *Geobacillus stearothermophilus* produced in *Bacillus subtilis*.

6.4.1.2 Joint FAO/WHO Expert Committee on Food Additives (JECFA)

JECFA evaluated its first maltogenic amylase in 1998. The maltogenic amylase contained the *amyM* gene from *B. stearothermophilus* that was produced by submerged fermentation of a non-pathogenic and non-toxicogenic strain of *Bacillus subtilis*. The scientific evaluation included an assessment of toxicological studies, including an acute toxicity study in rats, a short-term toxicity study in rats, skin genotoxicity studies (Ames test and chromosomal aberrations), skin irritation study using rabbits, eye irritation study in rabbits, and an immune response study using guinea pigs. (JECFA, 1998). The Committee noted that the *B. stearothermophilus* was a well-documented, non-pathogenic and non-toxigenic source of maltogenic alpha amylase in the literature and concluded that the human intake of maltogenic amylase resulting from its intended use as a processing aid in the baking and starch industry would be low and that the material consumed would not be active maltogenic amylase, but a heated, denatured material. The ADI that was designated as "temporary" at the 49th meeting because the specifications were tentative were upgraded in its final review in 2000. At the 51st meeting, the "tentative" designation was deleted and replaced with ADI "not specified" as is with other enzymes (JECFA, 2000).

Before JECFA's initial review in 1998, Anderson published a safety evaluation of a maltogenic amylase derived from *B. stearothermophilus* that was produced in *B. subtilis*. The recipient *(B. subtilis)* and the donor strain *(B. stearothermophilus)* are "non-pathogenic and non-toxicogenic, is accepted a harmless contaminant present in food and is used as a source for producing commercial food enzymes." The authors concluded that no adverse effects were associated with the administration of the maltogenic amylase (90-day and 28-day oral toxicity studies, inhalation, mutagenicity, and irritation studies) (Andersen *et al.*, 1987).

6.4.1.3 Food Standards Australia New Zealand (FSANZ):

Proposal P276 is a review of enzyme processing aids that began with an initial assessment in December 2003, which was followed by a draft assessment in October 2006 and, finally a final assessment report in August 2007.

The 2006 assessment provided a safety review of a preparation of maltogenic amylase produced by submerged fermentation of a strain of *B. subtilis*, which has been genetically modified to contain the *amyM* gene from *B. stearothermophilus* coding for maltogenic amylase (pages 32-34). (http://www.foodstandards.gov.au/code/proposals/pages/proposalp276reviewof2315.aspx, last accessed July 30, 2018). The short-term toxicity and genotoxicity study results are provided below:

Short-term toxicity studies:

- The product tested in the toxicological studies was a concentrated material (enzyme activity 35,900 maltogenic amylase units/g). Groups of 20 male and 20 female CD rats received the equivalent of 0, 390, 1200 or 4000 mg maltogenic amylase/kg bw/day for males and 0, 440, 1300 or 4300 mg maltogenic amylase/kg bw/day for females for 13 weeks.

- No mortality was seen and no clinical signs due to treatment were observed. Ophthalmoscopy did not show any abnormalities. A slight decrease in food intake of males and females given the highest dose was seen, accompanied by a significantly decreased body weight gain. Hematology did not reveal treatment related abnormalities nor were there any treatment related changes of toxicological significance to clinical chemistry parameters. Organ weights revealed significantly lower absolute and relative thyroid weights in males at the highest dose tested. A significantly lower absolute lung weight was also observed in females at the highest dose level. Macroscopy and microscopy did not reveal any treatment related abnormalities. The NOAEL for this study was 1200 mg/kg bw/day (equivalent to 1.5% of the diet).

Genotoxicity studies:

- Both *in vitro* and *in vivo* genotoxicity studies have been conducted on the maltogenic amylase enzyme preparation. Negative results were obtained for gene mutation studies in both bacterial and mammalian cells and chromosomal aberration tests in vitro and vivo were consistently negative.

FSANZ concluded that maltogenic amylase from *B. stearothermophilus* expressed in *B. subtilis* is an enzyme of low oral toxicity. Both the donor and production organisms are non-pathogenic and nontoxigenic to humans and can be regarded as a safe source of maltogenic amylase. There are no toxicological or other safety concerns with the use of maltogenic amylase from *Bacillus stearothermophilus* expressed in *Bacillus subtilis* as a processing aid.

6.4.1.4 Health Canada

Health Canada lists amylase (maltogenic) as a permitted enzyme for use with starch for the production of dextrin, glucose, maltose, bread and flour. *Geobacillus stearothermophilus* is a permitted source for the production of amylases[3].

6.4.1.5 AAFCO

In the United States, the American Association of Feed Control Officials (AAFCO) Official Publication lists enzymes and source organisms under Table 30.1 that are acceptable for use in animal feeds, including "*Bacillus subtilis* containing a *Bacillus stearothermophilus* gene for maltogenic alpha-amylase.

[3] https://www.canada.ca/en/health-canada/services/food-nutrition/food-safety/food-additives/lists-permitted/5-enzymes.html

6.4.2 Allergenicity & Toxigenic Potential

Enzymes are typically used as processing aids and have a long history of safe use in food, with no indication of adverse effects or reactions (Pariza & Foster, 1983). In 1998, the Working Group on Consumer Allergy Risk from Enzyme Residues in Food of the Association of Manufacturers of Fermentation Enzyme Products (AMFEP) conducted an in-depth analysis of the allergenicity of enzyme products. The study concluded that there are no scientific indications that small amounts of enzymes in bread and other foods can sensitize or induce allergy reactions in consumers and concluded that enzyme residue in bread and other foods do not represent any unacceptable risk to consumers. Exposure to enzymes via food is almost always low; generally, enzymes are added at the lowest level concentrations (parts per million) to obtain its reaction necessary for its application.

In addition, the enzyme is typically removed or denatured during food processing and denatured protein has been shown to be very susceptible to digestion in the gastro-intestinal system. A wide range of naturally-occurring food enzymes have been shown to be very labile in the gastro-intestinal system even in native unprocessed form.

According to the literature, the majority of proteins are not allergens; only 0.3% of all identified proteins are listed as allergens. A wide variety of enzyme classes and structures are naturally present in plant and animal-based foods. Based on enzymes long history of safe use in the production of foods, food enzymes are not homologous to known allergens and enzymes such as maltogenic α-amylase with a history of safe use have not raised safety concerns for food allergies (Bindslev-Jensen *et al.*, 2006).

Despite the general lack of concern for allergies by enzymes, potential allergenicity of the maltogenic α-amylase protein was evaluated using the full length FASTA and sliding 80 amino acid segments. The history of using exact 8 amino acid matching algorithms has indicated that the method is not predictive and is generally discounted as an approach to evaluating allergenicity. Therefore, this analysis should not be used (Ladics *et al.*, 2011; AllergenOnline, available at http://www.allergenonline.org/).

The protein's amino acid sequence was compared against known allergens using the Food Allergy Research and Resource Program (FARRP) Protein AllergenOnline Database (version 18B; released March 23, 2018; available at http://www.allergenonline.org/). This database includes a comprehensive list of putative allergenic proteins developed via a peer-reviewed process for the purpose of evaluating food safety. The *Geobacillus stearothermophilus* maltogenic α-amylase amino acid protein sequence expressed in *S. cerevisae* is shown in **Appendix 1** in FASTA format.

In accordance with the guidelines endorsed by Codex Alimentarius Commission (2009) and EFSA (2010) for the safety evaluation of newly expressed proteins from genetically modified plants and microorganisms, the database (AllergenOnline Database, version 18B; http://www.allergenonline.org/) was searched using a sliding window of 80-amino acid sequences derived from the full-length amino acid sequences. According to the approach adopted by the Codex Alimentarius Commission, significant homology is defined as an identity

match of greater than 35%, and in such instances, cross-reactivity with the known allergen should be considered a possibility. The 35% identity for 80 amino acid segments is a suggested guideline proposed by the Codex Alimentarius Commission for evaluating newly expressed proteins produced by recombinant-DNA plants (2009).

The sequence homology searches were performed and revealed greater than 35% identity matches with four proteins. These proteins are Taka-amylase A (Taa-G1) precursor from Aspergillus oryzae with 42% identity (NCBI gi|166531), Alpha-amylase A type 1/2 precursor with with 42% identity (NCBI gi|94706935), glycosidase hydrolase family 1 from Schizophyllum commue with 36% identity (NCBI gi|302681819), and probable maltase from Aedes aegypti with 36% identity (NCBI gi|126713). Ladics *et al.* (2007) indicates that using the 35% threshold for the sliding window of 80-amino acid sequence search is considered overly conservative and likely results in a number of false positive findings. In addition, Goodman and Teeteh (2011) indicate the threshold should be increased from 35% toward 50% to ensure that the bioinformatics search is relevant. Using this recommendation, the identity matches would be below the threshold for the sliding window of 80-amino acid sequence methodology.

According to recent analysis, FASTA or BLASTP searches may be the most predictive approach for allergeric reactions (Aalberse, 2000; Goodman & Teeteh, 2011; Goodman *et al.*, 2016) and according to Ladics *et al.* (2007) "resulted in identity matches that better reflected functional similarities between proteins." Ladics *et al.* (2011) suggests using the 35% threshold or greater shared amino acid sequence using this method. Using the FASTA alignment of the amino acid protein sequence with known allergens using the AllergenOnline Database (version 18B; http://www.allergenonline.org/) did not result in alignment with allergenic proteins at or above the 35% threshold of concern for allergenicity.

To assess if maltogenic amylase is a known toxin, the search term "maltogenic amylase" was used to query multiple databases using TOXNET (https://toxnet.nlm.nih.gov/) on November 28, 2018. 25 relevant records were identified in the TOXLINE database. Of these, 19 were research articles describing structure and function of wild type or protein-engineered maltogenic amylase; none identify the enzyme as a toxin. One record was a publication of safety studies of maltogenic amylase (Andersen et al., 1987), in which a 13-week oral toxicity study, bacterial mutagenic assay, in vivo cytogenetic study, acute inhalation study, and skin sensitization tests of maltogenic amylase showed no adverse effects at the levels tested; the overall conclusion was that the enzyme should be generally recognized as safe for use in the production of maltose syrups. The remaining five records refer to assessment of maltogenic amylase that took place at the 49th and 51st meetings of the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 1998 and JECFA, 2000). As described in section 6.4.1.2 of this document, the Committee concluded that stated the B. stearothermophilus was a well-documented, non-pathogenic and non-toxigenic source of maltogenic alpha amylase in the literature and concluded that the human intake of maltogenic amylase resulting from its intended use as a processing aid in the baking and starch industry would be low and that the material consumed would not be active maltogenic amylase, but a heated, denatured material.

A bioinformatics search for similarity of maltogenic amylase to known toxins was also performed. A custom FASTA database of known toxins was created by searching the UniProtKB

database (https://www.uniprot.org/) with the terms "keyword:toxin". This search was performed on November 28, 2018 and resulted in a list of 40,578 proteins from both the manually annotated and reviewed Swiss-Prot database and the computationally analyzed and unreviewed TrEMBL database. The amino acid sequence of maltogenic alpha amylase was queried against the custom toxin database using the BLAST function in Geneious software. The BLAST search used the BLOSUM62 matrix, gap cost (open extend) of 11 and 1, and word size 3. There were no hits with an E-value (the expectation of matching the sequence by random chance) below 1, indicating that similarity to any toxin sequence in the database is low and random.

As indicated above, enzymes are unlikely to be food allergens and the maltogenic α-amylase enzyme has a history of use in food with no indication of safety concerns. In addition, the enzyme is typically removed or denatured during the baking process. Therefore, it's concluded that the expressed maltogenic α-amylase enzyme is unlikely to be a concern with regard to food allergy or toxigenicity.

6.4.3 Safety Assessment Based on Decision Tree Analysis

An evaluation of the modified *Saccharomyces cerevisiae* strain based on criteria set forth by experts (Pariza & Foster, 1983; IFBC, 1990; EU SCF, 1991; OECD, 1992; FAO/WHO, 1996; Pariza & Johnson, 2001) demonstrates the safety of these genetically modified production strains. This evaluation includes the identity of the host strain, a description of the introduced DNA (the sources and functions of the introduced genetic material), an outline of the genetic construction of the production strain, and a characterization of the production strain.

Pariza and Foster base the decision tree concept on their 1983 publication that focused on the safety evaluation methodology of enzymes used in food processing, which was extended further by the International Food Biotechnology Council into the decision tree format (IFBC, 1990). In 2001, Pariza and Johnson published updated safety guidelines further building on the IFBC and other reports (Kessler et al., 1992) including considerations using rDNA technologies. The literature emphasizes that production strain safety is the primary consideration in evaluating enzymes derived from microorganisms, with particular focus on the toxigenic potential of the production strain. More specifically, the authors elaborate on the *safe strain lineage* concept and the elements critical to establish the safety of a production strain. "Thoroughly characterized non-pathogenic, non-toxigenic microbial strains, particularly those with a history of safe use in food enzyme manufacture, are logical candidates for generating safe strain lineage, through which improved strains may be derived via genetic modification by using either traditional/classical or rDNA strain improvement technologies." (Pariza & Foster, 1983). To establish safe strain lineage, the decision tree addresses elements such as "thoroughly characterizing the host organism, determining the safety of all new DNA that has been introduced into the host organism, and ensuring that the procedure(s) that have been used to modify the host organism are appropriate for food use" (Pariza & Johnson, 2001).

Pariza and Johnson (2001) outline a twelve-step decision tree for determining the safety of the production strain. In particular, by answering specific questions set forth in the decision tree, including whether the strain is non-pathogenic, free of antibiotics, and free of oral toxins (or below limits of concern), the production strain can be accepted as derived from a safe lineage at step 6 or step 11. Otherwise, step 12 concludes that there may be "an undesirable trait or

substance" present and the production strain may be 'unacceptable' in step 13. If the "genetic potential for producing the undesirable trait or substance can be permanently inactivated or deleted," the decision tree suggests that the "test material may be passed though the decision tree again."

Mascoma's decision tree analysis, based on the 2001 decision tree, is shown in **Appendix 2**. The production strain is genetically modified using standard recombinant DNA techniques, and the gene is integrated into a designated loci of the *Saccharomyces cerevisiae* parental strain. The production strain is free of transferable antibiotic resistance gene DNA. The introduced DNA is well-characterized and free of attributes that would render it unsafe for use in food products, such as bread.

6.5 Reports or Investigations Which May Appear to Be Inconsistent with the GRAS Determination

Discussion of scientific literature that claims Saccharomyces cerevisiae is a pathogen in immunocompromised individuals

The literature reports that *Saccharomyces cerevisiae* is an opportunistic pathogen. An extensive literature search on the safety of *Saccharomyces cerevisiae* reveals that for over the last fifty years, there have been reported cases of infections in mostly immunocompromised individuals (Eschete *et al.*, 1980; Eng *et al.*, 1984; Hazen, 1995; Murphy & Kavanagh, 1999; EFSA, 2008). McCusker (2006) provides a list of *S. cerevisiae* infections described in the literature. While the list includes infections in patients with AIDS; it does not identify which of the other patients were otherwise immunocompromised. Additionally, in a review of reported cases of invasive *S. cerevisiae* and *Saccharomyces boulardii* fungemia, Enache-Angoulvant *et al.* (2005), identified 92 reports, 76 of which were diagnosed between 1990 and 2005. These cases were frequently nosocomial in origin, primarily associated with central intravenous catheter (CVC) use or previous antibiotic therapy and each patient exhibited at least one underlying condition that might expedite the development of an invasive fungal infection.

Muñoz *et al.*, (2005) described 3 intensive care unit patients that had *Saccharomyces cerevisiae* fungemia at Hospital General Universitario. As part of the report, the authors searched MEDLINE for reports of *Saccharomyces cerevisiae* fungemia since 1966. Their search returned only 57 additional reported cases.

Since *Saccharomyces cerevisiae* is commonly used in the biotechnology industry, Murphy and Kavanagh (1999) also examined its potential pathogenicity. They also concluded that *Saccharomyces cerevisiae* can be regarded as an opportunistic pathogen for the immunocompromised, but one of low virulence.

Saccharomyces cerevisiae fungemia has been seen to manifest as unexplained fever, pneumonia, esophagitis, empyema, liver abscess, peritonitis, vaginitis, urinary tract infection, cellulitis, or septic shock (Lherm *et al.*, 2002; Williams *et al.*, 2007; Pfaller & Diekema, 2010; Kliemann *et al.*, 2011). A rare case was reported where a baker exhibited evidence of a *S. cerevisiae* induced lung nodule (Ren *et al.*, 2004), indicating that *S. cerevisiae* has some potential to colonise following inhalation exposure. However, even this route will carry a much greater risk in

individuals with pre-existing medical conditions that might predispose them to fungemia, such as hospital residents (Kelesidis & Pothoulakis, 2012). It is generally recognized that the main entry points for *S. cerevisiae* into the blood stream are enteral translocation following antibiotic induced yeast overgrowth or CVC hub/insertion site contamination (Enache-Angoulvant *et al.*, 2005; Pfaller & Diekema, 2010).

Despite these rare opportunistic infections, the FDA (and NIH), EPA, and EFSA maintain the safety of *Saccharomyces cerevisiae* as a nonpathogenic microorganism. EFSA notes that "[r]are opportunistic infections have been caused by *S. cerevisiae*," and EFSA maintains its QPS (Qualified Presumption as Safe) status (EFSA, 2008, p.27; EFSA, 2013, p.23). EFSA provides additional clarification stating, "*S. cerevisiae*, subtype boulardii is contraindicated for patients of fragile health, as well as patients with a central venous catheter in place. A specific protocol concerning the use of probiotics should be formulated" (EFSA, 2008, Table 4, pp.21, 43). Even with the infrequent cases of fungemia associated with *Saccharomyces boulardii*, McFarland (2010) discusses contraindications and precautions and recommends closely monitoring adult immunocompromised patients and catheter use, especially with unexplained fever and notes that some recommend not giving *Saccharomyces boulardii* to immunosuppressed patients or those with central catheters to reduce the risk of fungemia (Buts, 2009).

As EPA recognized in its Final Risk Assessment of *Saccharomyces cerevisiae* (February 1997) (p.9), "[m]any scientists believe that under appropriate conditions any microorganism could serve as an opportunistic pathogen." The Agency concluded that *Saccharomyces cerevisiae* has an extensive history in food processing and neither it nor other closely related species "has been associated with pathogenicity toward humans or has been shown to have adverse effects on the environment" (p.2).

6.6 Conclusions for GRAS determination

The following conclusions are made for the maltogenic α-amylase enzyme from *Geobacillus stearothermophilus* produced in a modified *Saccharomyces cerevisiae* for use in baking applications at the minimum level:

- A review of the published literature shows a long history of safe use of *Saccharomyces cerevisiae*, commonly known as bakers or brewer's yeast, for thousands of years of use in alcohol, brewing and baking. Individually, both *Saccharomyces cerevisiae* and *Saccharomyces cerevisiae*-derived products are approved food additives, affirmed as GRAS substances, used in the production of human pharmaceuticals and the subject of several previous GRAS Notifications.

- The modified *Saccharomyces cerevisiae* production strain is derived from a native *Saccharomyces cerevisiae* yeast that has been used in the baking industry for more than 20 years. The production strain has been determined to be substantially equivalent to the host strain with respect to overall performance such as growth and fermentation rates during propagation.

- The maltogenic α-amylase enzyme produced by a modified *Saccharomyces cerevisiae* strain is constructed via linear DNA transformation with synthetic genes to avoid any unintended transfer of genetic elements from the donor strain to the host strain. Thus, the modified yeast contains only a limited introduced sequence pertaining to the gene of interest.

- The maltogenic α-amylase donor organism *Geobacillus stearothermophilus* has a safe history of use in food and we were unable to identify any risk factors for using *Geobacillus stearothermophilus* as a gene donor.

- The modified *Saccharomyces cerevisiae* production strain was determined to meet the safe strain criteria, based on the decision tree analysis developed by Pariza and Johnson (2001) for evaluating the safety of microbial enzymes.

- The maltogenic α-amylase enzyme is produced according to the principles of cGMP, using food-grade ingredients or ingredients that are acceptable for general use in foods as specified under JECFA guidelines. Physical inspection and the appropriate chemical and microbiological analyses are conducted to confirm strain identity, no contamination, and to ensure the enzyme product meets the specifications set forth in Section 2.4.

- No viable amounts of maltogenic α-amylase enzyme remain in the bread products after baking.

- Extensive regulatory approvals support the safety of the maltogenic α-amylase enzyme, including FDA, JECFA, Food standards Australia New Zealand (FSANZ), Health Canada and the American Association of Feed Control Officials (AAFCO).

Based on this evaluation and a review of the scientific literature, it is concluded that maltogenic α-amylase enzyme from *Geobacillus stearothermophilus* produced in *Saccharomyces cerevisiae,* meeting appropriate food grade specifications and manufactured according to cGMP is GRAS for use in bread products and exempt from the premarket approval requirements based on scientific procedures.

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7. List of Supporting Data and Information

Appendix 1: The Amino Acid Sequence of the Maltogenic α-amylase
Appendix 2: Safety Decision Tree for Maltogenic α-amylase Enzyme
Appendix 3: Maltogenic α-amylase Production Process

APPENDIX 1: The Amino Acid Sequence of the Maltogenic α-Amylase

The host strain has three copies of the chromosome into which we integrated the insert, so our modified strain has multiple copies of maltogenic α amylase present in the genome. This gene was designed by creating a synthetic DNA sequence (codon optimized for *Saccharomyces cerevisiae*) based on the amino acid sequence of the wild type maltogenic α-amylase from *Geobacillus Stearothermophilus*, therefore neither the donor organism nor its DNA was actually used to modify the production organism yeast.

For expression in yeast, the N-terminal methionine is cleaved off in the enzyme. See **Table A1-1.**

Table A1-1. Amino Acid Sequence for maltogenic α-amylase from *Geobacillus stearothermophilus* expressed in *Saccharomyces cerevisiae*.

Gene	Native Sequence
Maltogenic α-amylase	SSSASVKGDVIYQIIIDRFYDGDTTNNNPAKSYGLYDPTKSKWKMYWGG DLEGVRQKLPYLKQLGVTTIWLSPVLDNLDTLAGTDNTGYHGYWTRDF KQIEEHFGNWTTFDTLVNDAHQNGIKVIVDFVPNHSTPFKANDSTFAEG GALYNNGTYMGNYFDDATKGYFHHNGDISNWDDRYEAQWKNFTDPA GFSLADLSQENGTIAQYLTDAAVQLVAHGADGLRIDAVKHFNSGFSKSL ADKLYQKKDIFLVGEWYGDDPGTANHLEKVRYANNSGVNVLDFDLNT VIRNVFGTFTQTMYDLNNMVNQTGNEYKYKENLITFIDNHDMSRFLSV NSNKANLHQALAFILTSRGTPSIYYGTEQYMAGGNDPYNRGMMPAFDT TTTAFKEVSTLAGLRRNNAAIQYGTTTQRWINNDVYIYERKFFNDVVLV AINRNTQSSYSISGLQTALPNGSYADYLSGLLGGNGISVSNGSVASFTLAP GAVSVWQYSTSASAPQIGSVAPNMGIPGNVVTIDGKGFGTTQGTVTFGG VTATVKSWTSNRIEVYVPNMAAGLTDVKVTAGGVSSNLYSYNILSGTQ TSVVFTVKSAPPTNLGDKIYLTGNIPELGNWSTDTSGAVNNAQGPLLAP NYPDWFYVFSVPAGKTIQFKFFIKRADGTIQWENGSNHVATTPTGATGN ITVTWQN

APPENDIX 2: Safety Decision Tree for Maltogenic α-Amylase Enzyme

1

- Is the production strain genetically modified? If yes, go to 2. ~~If no, go to 6.~~
- **Answer: Yes, the *S. cerevisiae* production strain is genetically modified.**

2

- Is the production strain modified using rDNA techniques? If yes, go to 3. ~~If no, go to 5.~~
- **Answer: Yes, the production strain was modified using standard recombinant DNA techniques, as described in Section 2.2.**

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.~~
- **Answer: The expressed enzyme product. maltogenic α-amylase, has a history of safe use in food. Further the donor organism *Geobacillus stearothermophilus* has a long history of safe use in baking. In addition, the enzyme will be inactivated during baking.**

3c

- Is the test article free of tansferable antibiotic resistance gene DNA? If yes, go to 3e. ~~If no, go to 3d~~
- **Answer: Yes, the test article is free of antibiotic resistance genes as stated in section 2.2.**

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.~~
- **Answer: Yes, the introduced DNA is well characterized and free of attributes that would render it unsafe for constructing microorganisms used to produce food products.**

4

- Is the introduced DNA randomly integrated into the chromosome? ~~If yes, go to 5~~. If no, go to 6.
- **Answer: No, the introduced DNA was integrated into the designated locus of the *Saccharomyces cerevisiae* strain.**

6

- Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure? If yes, the test article is ACCEPTED. ~~If no, go to 7.~~
- **Answer: Yes, the modified *Saccharomyces cerevisiae* production strain is derived from a safe lineage based on historical safety for the host strain that has been used in baking for more than 20 years. Thus, it is concluded that the modified *S. cerevisiae* strain expressing the maltogenic α-amylase enzyme is accepted under the decision tree guidelines as a safe strain lineage based on steps 1-6.**

Conclusion: ACCEPTED, under Decision Tree Guidelines

APPENDIX 3: Maltogenic α-amylase Production Process