LALLEMAND
BAKING SOLUTIONS

Application to amend Schedule 18 of the Australian New Zealand Food Standards Code to include maltogenic α-amylase enzyme from a modified strain of *Saccharomyces cerevisiae* as a Processing Aid

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Executive Summary

This application dossier concerns the enzyme processing aid maltogenic α-amylase (4-α-D-glucan α-maltohydrolase; EC 3.2.1.133), produced by a genetically modified strain of *Saccharomyces cerevisiae* (traditional baker's yeast) that has been engineered to express an optimized variant of the maltogenic α-amylase gene from *Geobacillus stearothermophilus*..

The recipient *S. cerevisiae* strain has an extensive history of use in the food industry in the production of baked goods and in the production of food enzymes. It is recognised as a safe microorganism by various regulatory agencies worldwide. For example, it meets the required qualifications to be considered as a Qualified Presumption of Safety (QPS) organism by the European Food Safety Agency (EFSA) and is therefore presumed to be safe (EFSA BIOHAZ Panel, 2020).). Moreover, *S. cerevisiae* is an approved source organism for β-fructofuranosidase in the Australia New Zealand Food Standards Code, Schedule 18.

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 *G. stearothermophilus* as a gene donor. *Geobacillus stearothermophilus* is already listed as an accepted gene donor for both α-amylase and maltogenic α-amylase in the Australia New Zealand Food Standards Code, Schedule 18.

A whole genome sequencing of the production strain (source organism) has been performed, to characterize the strain and to demonstrate the absence of toxigenic potential and antibiotic resistance genes.
Also, the source organism has been 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 from the *S. cerevisiae* production strain by fermentation, isolation and formulation. All the production steps are achieved in accordance with current good manufacturing practices (cGMP) and the principles of hazard analysis and critical control points (HACCP).

The enzyme is intended to be used as a technological aid in baking processes to reduce crumb firmness and staling in bread and other bakery products and is intended to substitute the use of other commercially available maltogenic α-amylase already evaluated and recognized as safe by various regulatory agencies and authoritative bodies all over the world.

The enzyme is added to the raw materials during the preparation of the dough and performs its technological function during baking. It is then expected to be inactivated and has no further technological effect after baking. The technological action of the enzyme processing aid is achieved by catalysing the hydrolysis of the starch polysaccharides in smaller molecules during baking. These molecules become too short to crystallise, and the formation of a permanent network is largely prevented leading to a reduction of bread staling.

The Total Maximum Daily Intake (TMDI) calculated for the maltogenic α-amylase enzyme processing aid using the Budget Method is 0.358 mg TOS/kg body weight per day based on the maximum intended level of use and the intended food uses. This TMDI, calculated using a conservative approach, is 889 times lower than the dose for which an adverse effect has been observed in an animal model with an equivalent maltogenic α-amylase (318.4 mg TOS/kg bw per day).

Based on the demonstration of the safety of the production strain and the absence of any hazards from the whole production process, we are confident that the maltogenic α-amylase enzyme processing aid from the modified strain of *saccharomyces cerevisiae* does not raise safety concerns for the intended use.

References

EFSA BIOHAZ Panel. Scientific Opinion on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA (2017-2019). *EFSA Journal* (2020a), 18(2):5966. Available at: https://doi.org/10.2903/j.efsa.2020.5966

Pariza M.W., Johnson E.A. . Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing: Update for a New Century. *Regulatory Toxicology and Pharmacology* (2001), 33(2), 173-186. Available at: https://doi.org/10.1006/rtph.2001.1466

3.1.1 General Requirements

B Applicant Details

(a) Applicant

Lallemand Baking Solutions

(b) Name of Contact person responsible for the dossier

[redacted]

(f) Nature of applicant's business

Lallemand Baking Solutions is a division of Lallemand Inc., a privately-held Canadian company, founded at the end of the 19th century, specializing in the development, production, and marketing of yeasts, bacteria, and their derivatives for the food, fermented beverages, health and other agricultural industries.

[redacted]

C Purpose of the Application

The purpose of this application is to request for the addition to Schedule 18 of the Australia New Zealand Food Standards Code of a maltogenic α-amylase enzyme produced from a *Saccharomyces cerevisiae* strain (traditional baker's yeast) engineered to express an optimized variant of the maltogenic α-amylase gene from *Geobacillus stearothermophilus*.

Maltogenic α-amylase (EC 3.2.1.133) is already listed as a permitted enzyme in Schedule 18, the source being *Bacillus subtilis* containing the gene for maltogenic α-amylase isolated from *Geobacillus stearothermophilus*.

The maltogenic α-amylase enzyme described is protein engineered. It is produced by a strain of Saccharomyces cerevisiae expressing an optimized variant of the gene encoding for maltogenic α-amylase protein from Geobacillus stearothermophilus..

D Justification for the Application

Maltogenic α-amylase (EC 3.2.1.133) is already listed as a permitted enzyme in Schedule 18, the source being Bacillus subtilis containing the gene for maltogenic α-amylase isolated from Geobacillus stearothermophilus. This application is for a new source organism of maltogenic α-amylase.

The amino acid sequence of the modified enzyme from this source is near 100% homologous to the native maltogenic α-amylase enzyme from *G. stearothermophilus*. The modification confers improved thermostability for baking applications.

D.1 Regulatory Impact Information

D.1.1 Costs and Benefits of the Application

(a) Cost and benefit to consumers

It is not anticipated that the inclusion of this enzyme processing aid in Schedule 18 will have any cost impact on consumers. Our application relates to an equivalent enzyme, in terms of technological effect in baking, to an enzyme already listed in Schedule 18, but expressed in another microorganism. In fact, maltogenic amylase (EC 3.2.1.133) is already listed as a permitted enzyme in Schedule 18, the source being *Bacillus subtilis* containing the gene for maltogenic α-amylase isolated from *Geobacillus stearothermophilus*. The addition of the enzyme described in the dossier provides an alternative source of maltogenic amylase.

(b) Cost and benefit to industry and business

It is not anticipated that the inclusion of this enzyme processing aid in Schedule 18 will have any cost impact on industry. If anything, the impact on industry will be a cost reduction due to increased competition for maltogenic amylase.

Our application relates to an equivalent enzyme, in terms of technological effect in baking, to an enzyme already listed in Schedule 18, but expressed in another microorganism. In fact, maltogenic amylase (EC 3.2.1.133) is already listed as a permitted enzyme in Schedule 18, the source being *Bacillus subtilis* containing the gene for maltogenic α-amylase isolated from *Geobacillus stearothermophilus*. The inclusion of maltogenic amylase from another source will provide industry with an alternative option and hence more competition.

(c) Cost and benefit to government

The inclusion of this enzyme processing aid in Schedule 18 will not have any cost impact on government. Our application relates to an equivalent enzyme, in terms of technological effect in baking, to an enzyme already listed in Schedule 18, but expressed in another microorganism. In fact, maltogenic amylase (EC 3.2.1.133) is already listed as a permitted enzyme in Schedule 18, the source being Bacillus subtilis containing the gene for maltogenic α-amylase isolated from Geobacillus stearothermophilus.

D.1.2 Impact on International Trade

The inclusion of maltogenic α-amylase enzyme produced from a *Saccharomyces cerevisiae* strain engineered to express an optimized variant of the maltogenic α-amylase gene from *Geobacillus stearothermophilus* in the Australia New Zealand Food Standards Code as a processing aid may promote international trade on products using this enzyme as a processing aid, and reduce technical barriers to trade.

E Information to Support the Application

This application is based on Chapter 3.3.2 (Guidelines for applications for substances added to food – Processing aids) of the Food Standards Australia New Zealand Application Handbook. It relates to an enzyme processing aid.

E.1 Data Requirements

E.1.1 Data related to safety Studies

Please refer to Section 3.3.2 Processing Aids, C Information Related to the Safety of an Enzyme Processing Aid.

E.1.2 Data related to surveys on chemicals and other substances in food

Please refer to section 3.3.2 Processing Aids, F Information Related to Dietary Exposure of the Processing Aid

E.1.3 Data related to epidemiological / intervention studies in human

No data resulting from epidemiological or intervention studies in human is provided to support this application.

F Assessment Procedure

The applicant considers the appropriate assessment procedure for the application to add maltogenic α-amylase produced from *saccharomyces cerevisiae* containing an optimized gene isolated from *Geobacillus stearothermophilus* coding for maltogenic α-amylase to Schedule 18 of the Australia New Zealand Food Standards Code to be the General Procedure, Level 1 (maximum 240 variable hours). This is based on the fact that maltogenic α-amylase (EC 3.2.1.133) is already listed as a permitted enzyme in Schedule 18, the source being *Bacillus subtilis* containing the gene for maltogenic α-amylase isolated from *Geobacillus stearothermophilus.* This application is for a new source organism of maltogenic α-amylase.

G Confidential Commercial Information (CCI)

The following sections of the dossier contain information that is claimed confidential in this submission:

Appendix 3: Technological effect of the Enzyme Processing Aid

Appendix 4: Manufacturing Process Flow Chart

Appendix 5: Manufacturing Process – List of Raw Materials and Processing Aids

Appendix 6: Certificates of Analysis

Appendix 7: Maltogenic Amylase Activity Determination Method
Appendix 13: Whole Genome Sequencing Analysis Report
Appendix 14: Absence of GMO DNA
Appendix 15: Genetic Stability of the Source Organism
Appendix 16: Genetic Modification of the Source Organism
Annex 2- M17906 WGS phylogenetic and AMR analysis - v2020-03-09

These sections contain information related to the genetic engineering of the strain used to produce the maltogenic α-amylase processing aid, the production process of the enzyme processing aid, and its technological effect.

Lallemand Baking Solutions has expended a considerable amount of resources in research and development, production process improvement, technological support and business development for the enzyme processing aid that is the object of the application.

The specificity of the enzyme processing aid, its production strain and manufacturing process, distinguishes this enzyme processing aid from more conventional products. Disclosing related information would allow competitors to develop similar products without the same expenditure of resources. Maintaining this information as confidential is therefore required to reduce the likelihood of a competitor manufacturing a similar product without investing time in conducting the necessary research and development required to develop such a product.

Consequently, the disclosure of this confidential information would be expected to cause substantial harm to the Lallemand Baking Solutions competitive position and could result in a material financial loss, and a material financial gain to its competitors. If this confidential information was made known, competitors would require significantly less capital investment to duplicate this organism, thereby allowing competitors to realize a profit in much less time than could Lallemand Baking Solutions. As such, competitors could also provide products containing the enzyme at lower market prices. Consequently, this would confer to Lallemand Baking Solutions a severe competitive disadvantage in the market place.

A summary of the confidential information is presented under Section 3.3.2 of this dossier.

H Other Confidential Information

Not applicable.

I Exclusive Capturable Commercial Benefit (ECCB)

Lallemand is not claiming ECCB. This application relates to an equivalent enzyme, in terms of technological effect in baking, to an enzyme already listed in Schedule 18, but expressed in another microorganism. In fact, maltogenic amylase (EC 3.2.1.133) is already listed as a permitted enzyme in Schedule 18, the source being Bacillus subtilis containing the gene for maltogenic α-amylase isolated from Geobacillus stearothermophilus. The inclusion of maltogenic amylase from another source will provide industry with an alternative option and hence more competition.

J International and Other National Standards

J.1 International Standards

Maltogenic α-amylase from *Geobacillus stearothermophilus* produced by *Saccharomyces cerevisiae* has not been reviewed by JECFA; there is no specific Codex Standard relevant to this application.

J.2 Other National Standards or Regulations

United States:

The safety of the enzyme processing aid subject of this application has been independently and collectively, critically evaluated by a panel of scientific experts, and was determined to be Generally Recognized as Safe (GRAS) for use as a food enzyme in the in the production of baked goods in the U.S. The GRAS notice has been filed with the U.S. Food and Drug Administration (GRN 842) and recognised as GRAS by the FDA ("FDA has no questions") (Cf. Appendix 12: FDA No Question Letter).

European Union:

While a union list of authorised food enzymes has not been published yet in the European Union, an application for authorisation of the enzyme processing aid has been submitted in April 2020 to the European Commission for evaluation by the European Food Safety Agency Panel on Food Contact Materials, Enzymes and Processing Aids (EFSA CEP Panel).

Canada:

An application for the approval of the enzyme processing aid for use as a food enzyme in Canada has been submitted in April 2020 to the Health Products and Food Branch (HPFB) of Health Canada.

K Statutory Declaration

Please see Appendix 1

L Checklists

This application concerns an enzyme product intended to be used as a processing aid. Therefore, the applicable checklists are:

- Section 3.1.1 – General requirements
- Section 3.3.2 – Processing aids, subsections A, C, D, E, F

Checklist can be found in Appendix 2

3.3.2 Processing Aids

A. Technical Information on the Processing Aid

A.1 Information on the type of processing aid

The processing aid subjects to this application is a protein engineered maltogenic α-amylase enzyme produced from a *Saccharomyces cerevisiae* strain (traditional baker's yeast) engineered to express an optimized variant of the maltogenic α-amylase gene from *Geobacillus stearothermophilus*.

A list of already permitted enzymes is available in the Australia New Zealand Food Standards Code, Schedule 18 (FSANZ, processing aids), under section 18-4 (permitted enzymes). Permitted enzymes of microbial origin are listed under subsection 5.5. Maltogenic amylase (EC 3.2.1.133) is listed as a permitted enzyme, the source being *Bacillus subtilis* containing the gene for maltogenic α-amylase isolated from *Geobacillus stearothermophilus*.

A.2 Information on the identity of the processing aid:

IUBMB Name	glucan 1,4-α-maltohydrolase
Common/Accepted names	maltogenic α-amylase; 1,4-α-D-glucan α-maltohydrolase
Systematic name	4-α-D-glucan α-maltohydrolase
IUBMB No	EC 3.2.1.133
CAS registry No	160611-47-2

The maltogenic α-amylase enzyme assessed in this dossier is produced by a strain of *Saccharomyces cerevisiae* expressing an optimized variant of the gene encoding for maltogenic α-amylase protein from *Geobacillus stearothermophilus*. Therefore, this enzyme has been protein engineered.

Information on the Recipient (Host) Organism

The *S. cerevisiae* parent yeast is a commercial baking strain (with no prior genetic modifications) that has been used for more than 20 years in the baking industry. *S. cerevisiae* is a non-pathogenic and non-toxigenic species that has been demonstrated to be suitable for food production through an extensive history of safe use in food applications and is not anticipated to produce any toxin secondary metabolites or have antibiotic activity. The taxonomic classification is presented in Table 1 below. Commonly used names associated with *S. cerevisiae* include yeast, baker's yeast, brewer's yeast, and lager beer yeast.

Kingdom	Fungi
Division	Ascomycota
Class	Saccharomycetes
Order	Saccharomycetales
Family	Saccharomycetaceae
Genus	*Saccharomyces*
Species	*S. cerevisiae*

Table 1: Taxonomic Identity of Recipient Strain *S.cerevisiae*

Several safety assessments have been conducted by authoritative bodies for *S. cerevisiae*. The National Institute of Health (NIH) currently classifies *S. cerevisiae* as a safe host organism belonging Risk Group 1 "*Agents that are not associated with disease in healthy adult humans*" according to their established guidelines (NIH, 2016).

According to EFSA, yeasts used in food production, particularly bakers/brewer's yeast, are considered among the safest of microorganisms (EFSA Scientific Committee, 2007). *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. Recent safety reviews by EFSA continue to support the QPS status of *S. cerevisiae* (EFSA BIOHAZ Panel, 2020a and 2020b).

Moreover, *S. cerevisiae* is an approved source organism for β-fructofuranosidase in the Australia New Zealand Food Standards Code, Schedule 18.

Information on the Donor Organism

Geobacillus stearothermophilus (formerly classified as *Bacillus stearothermophilus*) is a non-pathogenic and non-toxigenic species that has been demonstrated to be suitable for food production through an extensive history of safe use in food applications. *G. stearothermophilus* has been designated QPS status in Europe by the EFSA (EFSA BIOHAZ Panel, 2020a).

Geobacillus stearothermophilus is already listed as an accepted gene donor for both α-amylase and maltogenic α-amylase in the Australia New Zealand Food Standards Code, Schedule 18.

The taxonomy of the strain is provided in Table 2 below.

Kingdom	Bacteria
Phylum	Firmicutes
Class	Bacilli
Order	Bacillales
Family	Bacillaceae
Genus	*Geobacillus*
Species	*G. stearothermophilus*

Table 2: Taxonomic Identity of Source (Donor Organism)

No material from the donor organism was used in the construction of the modified yeast strain. A publicly available sequence of *G. stearothermophilus* maltogenic amylase was used to prepare a synthetic DNA sequence (Dauter *et al.*, 1999). This sequence was engineered into the recipient *s. cerevisiae* strain to avoid any possible carryover of donor strain genetic material.

A.3 Information on the chemical and physical properties of the processing aid:

The enzyme is intended to be used as a processing aid in baking processes to reduce crumb firmness and staling in bread and other bakery products, therefore improving bread shelf-life.

Maltogenic α-amylase derived from *G. stearothermophilus* is widely used as bread crumb anti-firming enzyme (Goesaert *et al*., 2009). It catalyses the hydrolysis of 1-4-*α*-glucosidic linkages in polysaccharides to remove successive α-maltose residues from the non-reducing ends of the chains. As a result of the catalytic activity of maltogenic α-amylase, mainly maltose is formed (Goesaert *et al*., 2009). Maltose is naturally present in spelt, kamut and sweet potatoes and in general is found in germinating cereal seeds (e.g. wheat, barley, rye, oat, and triticale) as they break down their starch stores to use for food, which is why it was named after malt.

Moreover, when starchy foods such as cereal grains, corn, potatoes, legumes, nuts and some fruits and vegetables are digested, maltose results. Maltose is as well created in the malting process when making beer and when distilling malt alcohol. During beer production, grains such as barley are germinated and dried to encourage the breakdown of starch into sugars, including maltose. The use of malted cereal products (e.g. malt flour) is a common practice for the production of certain bakery products.

Staling process is highly complex with firming being the most well-known and important phenomenon (Gray and Bemiller, 2003). During baking, the flour starch (composed of amylose and amylopectin) undergo a gelatinisation process, absorbing water. After baking, as the bread cools, the solubilized amylose retrogrades or recrystallizes within few hours. This is an intermolecular association in which the amylose chains bond together to form an ordered, very stable array. After this initial rapid retrogradation of the amylose, a much slower rate of retrogradation of the amylopectin occurs. During storage, an extensive, partially crystalline, permanent amylopectin network is formed, with junction zones formed by intermolecular recrystallisation of amylopectin branches. This network further matures during storage, thereby increasing size and number of both inter-and intramolecular crystalline zones and, hence contributes to increased crumb firmness (Goesaert *et al*. 2009).

The anti-staling action of the enzyme processing aid is achieved by catalysing the hydrolysis of the starch polysaccharides in smaller molecules during baking (mainly maltose). The obtained molecules become too short to crystallise, and the formation of a permanent network is largely prevented leading to a reduction of bread staling.

The enzyme processing aid is added to the dough and will perform its technological function during the baking process. The optimal pH for the process is 5.5 and the enzyme reaches his maximum level of activity at 80°C. The enzyme is then expected to be inactivated by heat at higher temperature (95°C for 10 minutes), and has no further technological effect after baking (See also section F.2 The levels of residues of the processing aid or its metabolites for each food or food group). Internal tests have demonstrated the technical effect of the enzyme processing aid (See Appendix 3: Technological effect of the Enzyme Processing Aid (CONFIDENTIAL)).

The maltogenic α-amylase enzyme is specifically characterized by its maltogenic amylase activity, which is determined using an internal method. It is measured using a water insoluble blue-dyed cross-linked starch as substrate. The substrate is hydrolysed by the amylase, releasing the blue dye which dissolves. After terminating the reaction and centrifuging, the absorbance of the solution is measured spectrophotometrically and is a measure for the enzyme activity. (Cf. Appendix 7: Maltogenic Amylase Activity Determination Method (CONFIDENTIAL) for details of analytical method). The amount of dye released is proportional to the activity present and is related to a standard curve produced with known activities from an internal standard.

The enzyme activity is expressed in Lallemand Baking JUN Units/g (LBJU/g). One LBJU is defined as the amount of enzyme estimated to hydrolyze 0.175 micromoles of glucosidic linkages per minute in a starch substrate (Phadebas) under the conditions of the assay and is equivalent to the amount of a reference maltogenic amylase that hydrolyses 0.1 micromoles of maltotriose per minute at 37 Celsius and pH = 5.5.

A.4 Manufacturing Process

The maltogenic α-amylase enzyme is produced by fermentation and subsequent concentration, cell breakage, solid/liquid separation, concentration, polish and germ filtration, and formulation from the engineered production strain of *Saccharomyces cerevisiae*.

The maltogenic α-amylase is manufactured in accordance with current good manufacturing practices (cGMP) and the principles of hazard analysis and critical control points (HACCP), within certified manufacturing facilities.

A HACCP (Hazard Analysis Critical Control Points) plan is employed during the entire production process. The production is conducted at fermentation facilities with established procedures and meets the criteria for safe production organism as described in Pariza and Johnson (2001). Physical inspection and the appropriate microbiological and chemical analyses, as well as other necessary analyses are conducted to ensure 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, 1995).

A schematic overview of the manufacturing process for maltogenic α-amylase processing aid is presented in Appendix 4: Manufacturing Process Flow Chart (CONFIDENTIAL) and all the steps are described in the following sections.

A list of the raw materials and processing aids used in the production of the maltogenic α-amylase enzyme processing aid at plant level is provided in Appendix 5: Manufacturing Process – List of Raw Materials and Processing Aids (CONFIDENTIAL). All raw materials and processing aids used in the manufacture of the enzyme are of high quality and acceptable for use in the manufacture of food enzymes, and are commonly used in food industrial processes. All processing aids and raw materials conform to FCC, 11th edition, 2018 quality standards where available or established internal specifications (where monographs are not available) aligned with the FCC.

Fermentation Process

i) Fermentation at Laboratory Stage

Yeast propagation is initiated from frozen master stocks of pure culture maintained at -80°C in glycerol. The assurance that the production microorganism efficiently produces the desired enzyme protein is key during the production process. Therefore, it is essential that the identity and purity of the production strain 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 following procedures. 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.

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 to 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 to 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.

ii) Fermentation at Plant Stage

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. The yeast from the flask obtained from the laboratory is inoculated into a propagation tank. The culture is sequentially transferred into increasing fermenter volumes. The first fermentation after the laboratory one is called Pure Culture, followed by one or more Fed Batch(s), based on the amount of yeast cream needed.

During the fermentation steps the nutrients feeding rate, as well as the temperature and pH are controlled, according to the fermentation recipe, to provide the optimal growth with minimal ethanol production. The aeration is kept constant. At the end of the fermentation (duration is based on the recipe) the feeding is stopped to end the fermentation sequence. Yeast cells are separated from the fermentation broth and concentrated by centrifugation. The yeast is then washed to remove remaining non-yeast soluble solids, leading to a liquid yeast cream. The specifications for yeast cream are controlled on every batch prior to release, including, but not limited to, microbial control, as well as protein and phosphate content. The yeast cream is kept at 0-4°C before further processing.

The genotypic stability of the production strain during the propagation procedure has been demonstrated by PCR genotyping comparison of DNA isolated from the cells used for seeding the yeast propagation, and from the final yeast cream, for 3 commercial batches. (Cf. section D.3 Information on the genetic stability of the source microorganism)

Recovery and Formulation of the Enzyme Processing Aid (Downstream Processing)

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 mainly 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 to improve the ratio enzyme activity/TOS.

Finally, the enzyme is formulated to desired specifications.

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

i) Autolysis

The intracellular produced maltogenic α-amylase is usually extracted from the cell by autolysis (lysis of cells by own yeast enzymes). During autolysis, temperature, pH, solid content and release of the maltogenic α-

amylase are monitored. The final autolysate is cooled before further downstream processing. The pH may be adjusted to minimize the build-up of microbial charge during intermediate storage and further downstream processing.

Alternatively, other techniques may be used to achieve cell disruption for recovery of the intracellular maltogenic α-amylase from yeast including mechanical (bead mill, high-pressure homogenization, ultrasonication), and non-mechanical (physical, chemical and/or enzymatic) techniques.

ii) Primary Solid/Liquid Separation

The purpose of the primary separation is to free the soluble fraction containing the enzyme from the insoluble cell debris. In order to minimize loss of enzyme activity the separation is performed at a defined pH and a specific temperature range. Depending on the scale of the process and the site of operation, separation may either be conducted by centrifugation or filtration. The solid debris are then washed with water to ensure optimal enzyme recovery. The duration of the separation step is defined by the initial volume to be separated, the throughput of the separators (centrifugation or filtration) and the wash factor applied.

iii) Concentration

The enzyme solution can be quite diluted due to the applied wash volumes during primary separation. Solid content can be increased during previous filtration step (when applied), but further increase may be required in order to improve the further steps. Increase of solid content can be achieved by evaporation and/or ultrafiltration. The use of ultrafiltration can also improve the ratio enzyme activity/TOS. Temperature and pH are controlled to minimize the loss of enzymatic activity 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 steps.

iv) Polish Filtration

For removal of residual cells of the production strain and as a general precaution against microbial contamination, filtration on dedicated germ filters is applied during the recovery process. A filter aid is added into the liquid extract that helps control flow and solids removal. The filter aid forms a porous layer on the filter cloth and becomes the filtering medium. Alternatively to the use of a filter press, a cartridge filter can also be used, depending on the facility equipment. This final polish filtration at the end of the recovery process results in a concentrated enzyme solution free of the production strain and insoluble substances. This step can be performed before the formulation step (see below), or after the addition of maltodextrin, before drying (when applicable). The enzymatic activity is determined before formulation of the enzyme processing aid.

v) Formulation and Packaging

Drying can be performed using various technologies in order to deliver the preferred particle properties. Processing aids, including maltodextrin and canola oil may be added to improve the drying process. All processing aids are appropriate food-grade. More specifically, in the formulation process usually applied, maltodextrin is added to the maltogenic α-amylase extract. In the case where single stage spray drying might be the used drying technology, a dedusting step with canola oil can be needed.

The enzyme is tested by Quality Control for all quality related aspects before release, including expected enzyme activity and the general JECFA Specification for Food Enzyme Preparations.

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.

Production Controls

As mentioned above, the maltogenic α-amylase is manufactured at certified facilities, following established procedures, in accordance with cGMP and the principles of HACCP.

To confirm that the manufactured enzyme is of food-grade quality and meets international standards/specifications for food enzymes, the food enzyme is analysed for potential impurities and contaminants that may originate from the production strain or manufacturing process, and complies with the general JECFA specifications for food enzyme preparations.

To ensure that the enzyme processing aid meets these quality criteria, potential hazards are taken into account and controlled during the whole production process as described below:

i) Microbiological Hygiene

For optimal and qualitative enzyme production, it is important that hygienic conditions are maintained throughout the entire fermentation process. Actions in place 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:
    - o Validated standard cleaning and sterilization procedures of the production area and equipment: all fermenters, vessels and pipelines are washed after use with a CIP-system (Cleaning in Place). After cleaning, the vessels are inspected.
    - o Sterilization of fermentation media: the media may be sterilized with steam injection in fermenters or media tanks.
- Hygienic processing:
    - o Aseptical transfer from the lab stage and between fermentation steps.
    - o Use of sterile air for aeration of the fermenters.

During the downstream processing hygienic conditions are also ensured by careful cleaning of equipment and hygienic controls at each step of the process. A polish filtration is performed as additional safety measure to keep level of microorganisms in the enzyme processing aid within specifications.

All the production steps are achieved following procedures executed by staff trained according to documented procedures complying with the requirements of the quality system

ii) In-Process Controls

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

These in-process controls include, but may not be limited to:

• Microbial controls: Absence of significant microbial contamination is analysed 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 (pH, temperature, feeding, aeration conditions,…). The values of these parameters are constantly monitored during the fermentation process. Deviations from the pre-defined values lead to investigations and adjustment, ensuring an optimal and consistent process.

• Monitoring of operational parameters during recovery steps (pH, temperature, enzymatic activity,…) throughout the entire downstream processing.

A.5 Specification for Identity and Purity

Food-grade specifications for the maltogenic α-amylase are presented in Table 3 below. The specifications for the enzyme comply with the current purity and microbial limits established for enzyme preparations by the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2006) and the Food Chemicals Codex (FCC) (FCC, 2018).

Parameter	Specification	Analytical Method
Maltogenic amylase activity (LBJU/g)	> 25,000	Internal method
Total aerobic plate count (CFU/g)	< 50,000	BAM chapter 3
Coliforms (CFU/g)	< 30	BAM chapter 4
E. coli (/25g)	Absent	BAM chapter 4
Salmonella (/25g)	Absent	BAM chapter 5
Antimicrobial activity	Absent	Internal method following JECFA guidelines
Production organism (/g)	Absent	Internal method following EFSA guidelines
Lead (ppm)	≤ 5	NOM-117-SSA1-1994
Arsenic (ppm)	≤ 1	NOM-117-SSA1-1994
Cadmium (ppm)	≤ 0.5	NOM-117-SSA1-1994
Mercury (ppm)	≤ 0.5	NOM-117-SSA1-1994

Table 3: Product Specifications for Maltogenic α-Amylase from *S.cerevisiae*

Lallemand Baking Solutions confirms that the maltogenic α-amylase processing aid is free from known allergen. In particular, the source of maltodextrin used in the production process is corn starch.

Certificates of analyses for these 3 non-consecutive production batches of the enzyme processing aid are provided in Appendix 6: Certificates of Analysis (CONFIDENTIAL).

The internal methods for the determination of:

- The maltogenic amylase activity is provided in Appendix 7: Maltogenic Amylase Activity Determination Method (CONFIDENTIAL);
- The antimicrobial activity method is provided in Appendix 8: Antimicrobial Activity Method;

The absence of production organism is determined using an internal method following the EFSA guidelines provided in section 2.1 of the guidance on the "characterisation of microorganisms used for the production of food enzymes" (EFSA CEP Panel 2019b).

Below are the links to the internationally recognised analytical methods used:
- BAM chapter 3: https://www.fda.gov/food/laboratory-methods-food/bam-aerobic-plate-count (accessed April 1 2020)
- BAM chapter 4: https://www.fda.gov/food/laboratory-methods-food/bam-4-enumeration-escherichia-coli-and-coliform-bacteria (accessed April 1 2020)
- BAM chapter 5: https://www.fda.gov/food/laboratory-methods-food/bacteriological-analytical-manual-bam-chapter-5-salmonella (accessed April 1 2020)
- NOM-117-SSA1-1994: http://www.fao.org/faolex/results/details/fr/c/LEX-FAOC013506/ (accessed April 1 2020, available in Spanish). An English translation of the method is provided in Appendix 9: NOM-117-SSA1-1994 Method – English Translation from Original Method in Spanish.

A.6 Analytical Method for Detection

This information is not required in the case of an enzymatic processing aid.

B Information Related to the Safety of a Chemical Processing Aid

This section is not applicable as the processing aid subject to this application is an enzyme.

C Information Related to the Safety of an Enzyme Processing Aid

C.1 General information on the use of the enzyme as a food processing aid in other countries

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).

Maltogenic amylase from *Geobacillus stearothermophilus* is authorised and used in various geographic areas in the world including, but not limited to, the USA, the European Union, Canada, Australia, New Zealand, China, Brazil, South Africa, Switzerland.

C.2 Information on the potential toxicity of the enzyme processing aid

The enzyme expressed by the production strain is protein engineered, differing from the wild type maltogenic amylase sequence by three amino acids (See Appendix 16: Genetic Modification of the Source Organism (CONFIDENTIAL)). In 2009 the Enzyme Technical Association (ETA) published a summary of a survey of toxicology (genotoxicity and oral toxicity) study results for protein engineered enzymes, which had been shared by ETA members (Enzyme Technical Association, 2009).

In all, no adverse effects for protein engineered enzymes were reported for either genotoxicity or oral toxicity tests, and the ETA concluded that "the general safety profile of enzymes produced using protein engineering is no different from that already established via extensive toxicological studies for non-protein engineered enzymes as reviewed in Pariza and Johnson (2001), Olempska-Beer *et al*. (2006) and in many cases, reviewed by the FDA in the GRAS Notice Program." This is in line with Pariza and Cook (2010), in which the authors note that modifying a few amino acids likely will not turn a non-toxic enzyme into a toxin, due to the great similarity of protein engineered enzymes and their non-engineered counterparts as well as the great dissimilarity of most food and feed enzymes from known toxins.

To assess if maltogenic amylase has similarity with 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. All the retrieved records are provided in Appendix 10: Results of the TOXNET Search. 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). 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 (provided as 'Annex 1 - 20181128_uniprot-keyword%3Atoxin.fasta' to this application) 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 analysed and unreviewed TrEMBL database. The amino acid sequence of maltogenic α-amylase expressed from production strain were queried against the custom toxin database using the BLAST function in Geneious software (Search process detailed in Appendix 11: Step-by-step process for toxin search. 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.

No toxicological study has been conducted with the maltogenic α-amylase subject to this application. Toxicological studies conducted with equivalent maltogenic α-amylase preparations, including homologous enzymes from *G. stearothermophilus*, are briefly tabulated below to further substantiate the safety of our

maltogenic α-amylase food enzyme. All the tests were performed in compliance with the Organisation of Economic Co-operation and Development (OECD) Guidelines for the Testing of Chemicals.

As detailed below (Cf. P.24-25) there is a significant similarity between the amino acid sequence of the enzyme of this application with that of maltogenic α-amylase from *Bacillus stearothermophilus* (now *Geobacillus stearothermophilus*) expressed in *Bacillus subtilis* BRG-1 and Maltogenic amylase from *B. stearothermophilus* expressed in *B. subtilis* RF12029 / EL 2009083 which both have a history of safe human consumption and are considered as GRAS (U.S. FDA 2018a and 2018b). Therefore, information on the stability of the enzyme to degradation in gastric or intestinal model digestion systems has not been conducted.

Repeat-dose Toxicity

The safety of several maltogenic α-amylase has been assessed in repeat-dose toxicological studies in rats. Results are reported in Table 4 below for studies that have been reviewed and accepted by EFSA and FDA. Maltogenic α-amylase did not result in noteworthy toxicological effects and NOAELs were reported to be from 318.4 to 986 mg TOS/kg bw/day when reported on a TOS basis and up to 1,000 mg/kg bw/day for a study that did not report doses on a TOS basis.

Species, Strain, (No./Sex/Group; age/weight)	Duration	Test Item, Dose (mg/kg bw/day) and Route	NOAEL (mg/kg bw/day)	Reference
Crl:CD Sprague-Dawley rats 10 of each sex/group Age and weight NR	90 days (OECD Guideline No. 408, 2018 and GLP)	Maltogenic α-amylase (from engineered *B. subtilis* NZYM-OC) 0, 112, 371 or 1,124 mg TOS/kg bw/day (1) Oral (gavage)	371 mg TOS/kg bw/day	EFSA-Q-2014-00922 EFSA CEP Panel, 2018a
Sprague–Dawley Mol:SPRD rats 10 of each sex/group Age and weight NR	90 days (OECD Guideline No. 408, 2018 and GLP)	Maltogenic α-amylase (from engineered *B. subtilis* NZYM-SO) 0, 96.5, 318.4 or 964.8 mg TOS/kg bw/day (2) Oral (gavage)	318.4 mg TOS/kg bw/day	EFSA-Q-2015-00046 EFSA CEP Panel, 2018b
Sprague-Dawley rats 10 of each sex/group Age and weight NR	90 days (OECD Guideline No. 408, 2018 and GLP)	Maltogenic α-amylase (from engineered *B. subtilis* NZYM-SM) 0, 97, 320 or 968 mg TOS/kg bw/day (3) Oral (gavage)	320 mg TOS/kg bw/day	EFSA-Q-2015-00096 EFSA CEF Panel, 2018a
Wistar rats 10 of each sex/group Age and weight NR	90 days (OECD Guideline No. 408, 2018 and GLP)	Maltogenic α-amylase (from engineered *B. subtilis* MAM) 0, 99, 296 or 986 mg TOS/kg bw/day Oral (gavage)	986 mg TOS/kg bw/day	EFSA-Q-2013-00790 EFSA CEF Panel, 2018b
Wistar rats 10 of each sex/group Age and weight NR	90 days (OECD Guideline No. 408, 2018 and GLP)	Maltogenic α-amylase (from engineered *E. coli* BLASC)	838 mg TOS/kg bw/day	EFSA-Q-2015-00446 EFSA CEP Panel, 2019a

Species, Strain, (No./Sex/Group; age/weight)	Duration	Test Item, Dose (mg/kg bw/day) and Route	NOAEL (mg/kg bw/day)	Reference
		0, 210, 419 or 838 mg TOS/kg bw/day Oral (gavage)		
SPF Sprague-Dawley rats 5 of each sex/group Age and weight NR	14 days (dose range-finding study)	Maltogenic α-amylase (from *B. stearothermophilus* expressed in *B. subtilis* BRG-1) 0, 1.0, 3.3, or 10.0 mL/kg/day (0, 96.8, 319.5, or 968.2 mg TOS/kg bw/day) Oral (gavage)	968.2 mg TOS/kg bw/day	GRN 751 "No Questions" (U.S. FDA, 2018a)
SPF Sprague-Dawley rats 10 of each sex/group Age/weight NR	13 weeks (OECD Guideline No. 408, 2018 and GLP)	Maltogenic α-amylase (from *B. stearothermophilus* expressed in *B. subtilis* BRG-1) 0, 1.0, 3.3, or 10.0 mL/kg/day (0, 96.8, 319.5, or 968.2 mg TOS/kg bw/day) Oral (gavage)	968.2 mg TOS/kg bw/day	GRN 751 "No Questions" (U.S. FDA, 2018a)
Wistar rats 10/sex/group 7 weeks old Weight: m: 187 to 211 g f: 141 to 159 g	13 weeks (OECD Guideline No. 408, 2018)	Maltogenic amylase (from *B. stearothermophilus* expressed in B.*subtilis* RF12029 / EL 2009083) 0, 100, 300, or 1,000 mg/kg bw/day Oral (gavage)	1,000 mg/kg bw/day (TOS not reported)	GRN 746 "No Questions" (U.S. FDA, 2018b)

Table 4: Repeat-Dose Studies Conducted with Maltogenic α-Amylase Preparations (NR: Not Reported)

(1) A statistically significant decrease in counts of total leucocytes, and of lymphocytes, eosinophils, monocytes and large unstained cells was observed in high-dose females as compared to controls.

(2) Statistically significant differences to the controls included a lower mean cell haemoglobin in high-dose males and a decrease in white blood cell and lymphocyte counts in high-dose females. Microscopically, minimal hyperplasia/hyperkeratosis of the non-glandular epithelium was reported at the junction of the glandular and non-glandular stomachs (the limiting ridge) in high-dose males and females. This microscopic change was considered by the EFSA Panel as treatment related. It cannot be excluded that this represents a toxicologically relevant effect.

(3) Statistically significant changes were observed at high dose in several kidney relevant parameter, among which N-acetyl-β-D-glucosaminidase (NAG), which is an indicator or kidney damage.

As shown in Figure 1 below, the maltogenic α-amylase processing aid that is subject to this application is closely related to the one from *B. stearothermophilus* expressed in *B. subtilis* BRG-1 (U.S. FDA, 2018a) and the one from from *B. stearothermophilus* expressed in *B.subtilis* RF12029 / EL 2009083 (U.S. FDA, 2018b), that are both considered as GRAS.

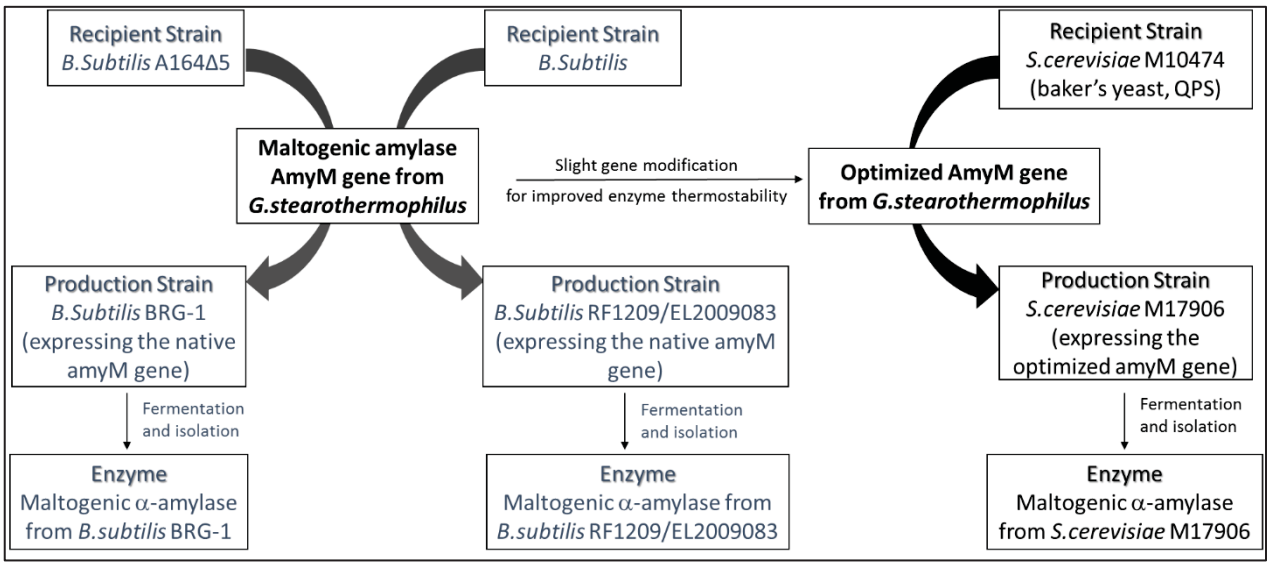


Figure 1: Production Strain Lineage

In fact both GRAS enzymes are obtained through the expression of the native maltogenic α-amylase amyM gene from *Geobacillus stearothermophilus*[1] (formerly known as *Bacillus stearothermophilus*) in 2 different *Bacillus subtilis* strains, when the maltogenic α-amylase subject to this application is obtained by the expression of the same gene, that has been slightly modified for improved thermostability of the enzyme and is near 100% homologous to the native gene (Detailed information regarding the DNA sequence of the native and optimized amyM genes are available in Appendix 16: Genetic Modification of the Source Organism (CONFIDENTIAL), in a strain of *S.cerevisiae* (traditional baker's yeast), one of the safest microorganisms used in food and feed production and that has been designated Qualified Presumption as Safe (QPS) status in Europe (EFSA BIOHAZ Panel 2020a).

It is not possible to directly relate the maltogenic α-amylase subject to this application to the other maltogenic α-amylases listed in Table 4 above.

Summary of the toxicity data for the maltogenic α-amylase from *Bacillus subtilis* BRG-1 is provided in 'Annex 3 - Toxicity data summary for Maltogenic amylase from *G. stearothermophilus* produced in Bacillus subtilis BRG-1 (GRN 751)'.

The full toxicity data set for the maltogenic α-amylase from *Bacillus subtilis* RF12029/EL 2009083 is provided in 'Annex 4 - Toxicity report for Maltogenic amylase from G. stearothermophilus produced in Bacillus subtilis RF12029-EL 2009083 (GRN 746)'.

[1] Native amino acid sequence of maltogenic α-amylase from *Geobacillus stearothermophilus* (UniProt Accession P19531 AMYM_GEOSE) is available online: https://www.uniprot.org/uniprot/P19531

The maltogenic α-amylase from *Bacillus subtilis* BRG-1 is secreted during fermentation into the fermentation media and is recovered from the culture broth by vacuum drum filtration or centrifugation. It is then concentrated by ultrafiltration and/or evaporation. A germ filtration step is then applied, for removal of residual production strain organisms and as a general precaution against microbial degradation, followed by another concentration, spray-drying and formulation.

The maltogenic α-amylase from *Bacillus subtilis* RF12029/EL 2009083 is secreted during fermentation into the fermentation media and is recovered from the culture broth by filtration or centrifugation. It is then concentrated. A germ filtration step is then applied, for removal of residual production strain organisms and as a general precaution against microbial degradation, followed by formulation.

The recovery process of the enzyme is equivalent for the maltogenic α-amylase subject to this application as compared with the 2 others mentioned above. The only difference is that during fermentation, the enzyme is produced intracellularly in the yeast. Therefore, an additional lysis step is necessary to extract the enzyme from the yeast cell. Insoluble cell debris (cell wall) formed during the lysis step are separated from the solution containing the enzyme by centrifugation or filtration, as explained in section A.4 Manufacturing Process.

During the lysis step, water soluble components, commonly named as yeast extract, are also released. Yeast extracts are mainly composed of amino acids, peptides, carbohydrates and salts (Milic *et al*. 2007). Yeast extracts are safe compounds that are widely used in food as flavoring agents or to increase the nutritional value of food (Boonraeng et *al.* 2000). Additionally, bakers' yeast extracts are considered as GRAS by the FDA[2]. Therefore, they are not considered to be of toxicological concern and no specific steps are performed during the recovery process of the enzyme to remove them from the enzyme processing aid.

Genotoxicity Studies

The genotoxicity and mutagenicity of several maltogenic α-amylase preparations were assessed in several assays including bacterial reverse mutation test, *in vitro* mammalian chromosomal aberration test, and *in vitro* mammalian cell micronucleus test, and reviewed by EFSA and FDA (see Table 5 below). Based on the conditions of these studies, it was concluded that maltogenic α-amylase does not have the potential to induce structural or numerical chromosome aberrations in cultured mammalian cells and lack mutagenic potential (an increase in the number of revertants was observed in only one study, but EFSA hypothesised that this result could be due to an issue with the used protocol).

Test System	Type	Test Item and Dose	Results	Reference
Salmonella Typhimurium (TA98, TA100, TA1535 and TA1537) and *E. coli* WP2 uvrA pKM 101	Bacterial Reverse Mutation Assay (OECD Guideline No. 471, 1997a and GLP)	Maltogenic α-amylase (from engineered *B. subtilis* NZYM-OC) Up to 3,963 µg TOS/plate (+/-S9)(1)	Negative	EFSA-Q-2014-00922 EFSA CEP Panel, 2018a

[2] https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=184.1983

Test System	Type	Test Item and Dose	Results	Reference
Salmonella Typhimurium (TA98, TA100, TA1535 and TA1537) and *E. coli* WP2 uvrA	Bacterial Reverse Mutation Assay (OECD Guideline No. 471, 1997a and GLP)	Maltogenic α-amylase (from engineered *B. subtilis* NZYM-SO) Up to 2,908 µg TOS/plate (+/-S9)	Negative	EFSA-Q-2015-00046 EFSA CEP Panel, 2018b
Salmonella Typhimurium (TA98, TA100, TA1535 and TA1537) and *E. coli* WP2 uvrA	Bacterial Reverse Mutation Assay (OECD Guideline No. 471, 1997a and GLP)	Maltogenic α-amylase (from engineered *B. subtilis* NZYM-SM) Up to 3,273 µg TOS/plate (+/-S9)	Negative	EFSA-Q-2015-00096 EFSA CEF Panel, 2018a
Salmonella Typhimurium (TA98, TA100, TA1535 and TA1537) and *E. coli* WP2 uvrA	Bacterial Reverse Mutation Assay (OECD Guideline No. 471, 1997a and GLP)	Maltogenic α-amylase (from engineered *B. subtilis* MAM) Up to 355 µg TOS/plate (+/-S9)	Dose-related increase in the number of revertants observed (-S9) at doses > 236 µg TOS/plate in *E. coli* WP2 uvrA Could be due to testing conditions	EFSA-Q-2013-00790 EFSA CEF Panel, 2018b
Salmonella typhimurium strains (TA97a, TA98, TA100, TA102, and TA1535)	Bacterial Reverse Mutation Assay (OECD Guideline No. 471, 1997a and GLP)	Maltogenic α-amylase (from engineered *E. coli* BLASC) Up to 4190 µg TOS/plate (+/-S9)	Negative	EFSA-Q-2015-00446 EFSA CEP Panel, 2019a
Salmonella typhimurium (TA98, TA100, TA1535 and TA1537), and *E. coli* WP2 uvrA	Bacterial Reverse Mutation Assay (OECD Guideline No. 471, 1997a)	Maltogenic α-amylase (from *B. stearothermophilus* expressed in *B. subtilis* BRG-1) Up to 5,000 µg/plate or 455 µg TOS/plate (+/-S9)	Negative	GRN 751 "No Questions" (U.S. FDA, 2018a)
Salmonella typhimurium strains (TA98, TA100, TA102, TA1535 and TA1537)	Bacterial Reverse Mutation Assay (OECD Guideline No. 471, 1997a)	Maltogenic α-amylase (from *B. stearothermophilus* expressed in *B. subtilis* RF12029 / EL 2009083) Up to 5,000 µg/plate (+/-S9)	Negative	GRN 746 "No Questions" (U.S. FDA, 2018b)
Human peripheral blood lymphocytes	*In vitro* mammalian cell micronucleus test (OECD Guideline No. 487, 2016 and GLP)	Maltogenic α-amylase (from engineered *B. subtilis* NZYM-OC) Up to 535 µg TOS/mL	Negative	EFSA-Q-2014-00922 EFSA CEP Panel, 2018a
Human peripheral blood lymphocytes	*In vitro* chromosomal aberration test (OECD	Maltogenic α-amylase (from engineered *B. subtilis* NZYM-SO)	Negative	EFSA-Q-2015-00046

Test System	Type	Test Item and Dose	Results	Reference
	Guideline 473, 1997b and GLP)	Up to 445 µg TOS/mL		EFSA CEP Panel, 2018b
Human peripheral blood lymphocytes	*In vitro* chromosomal aberration test (OECD Guideline 473, 1997b and GLP)	Maltogenic α-amylase (from engineered *B. subtilis* NZYM-SM) Up to 455 µg TOS/mL	Negative	EFSA-Q-2015-00096 EFSA CEF Panel, 2018a
Whole blood cultures	*In vitro* chromosomal aberration test (OECD Guideline 473, 1997b and GLP)	Maltogenic α-amylase (from engineered *B. subtilis* MAM) Up to 355 µg TOS/mL	Negative	EFSA-Q-2013-00790 EFSA CEF Panel, 2018b
Whole blood cultures	*In vitro* chromosomal aberration test (OECD Guideline 473, 1997b and GLP)	Maltogenic α-amylase (from engineered *E. coli* BLASC) Up to 4,190 µg TOS/mL	Negative	EFSA-Q-2015-00446 EFSA CEP Panel, 2019a
Human peripheral blood lymphocytes	*In vitro* chromosomal aberration test (OECD Guideline 473, 1997b)	Maltogenic α-amylase (from *B. stearothermophilus* expressed in *B. subtilis* BRG-1) Up to 5,000 µg/mL/plate or 455 µg TOS/mL/plate (+/-S9)	Negative	GRN 751 "No Questions" (U.S. FDA, 2018a)
Chinese hamster V79 cells	*In vitro* chromosomal aberration test (OECD Guideline 473, 1997b)	Maltogenic α-amylase (from *B. stearothermophilus* expressed in *B. subtilis* RF12029 / EL 2009083) Up to 5,000 µg /plate (+/-S9)	Negative	GRN 746 "No Questions" (U.S. FDA, 2018b)

Table 5: Genotoxicity Studies Conducted with Maltogenic α-Amylase Preparations

(1) +S9 = with metabolic activation; -S9 = without metabolic activation.

C.3 Information on the potential allergenicity of the enzyme processing aid

Enzymes are proteinaceous molecules, and like other proteins, they possess the potential to elicit allergenic responses. As reported by Pariza and Foster (1983), "*Allergies and primary irritations from enzymes used in food processing should be considered a low priority item of concern except in very unusual circumstances*".
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 (AMFEP, 1998). 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 inactivated during food processing and denatured proteins have 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. 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).

To confirm that the maltogenic α-amylase enzyme does not contain amino acid sequences similar to known allergens that might produce an allergenic response, a sequence homology search was conducted according to the approach outlined by Codex Alimentarius (2009) and EFSA (EFSA GMO Panel 2010) in order to confirm the lack of potential for allergenic cross-reactivity. This search was conducted using the AllergenOnline[3] database version 19 and FASTA36. The database contains a comprehensive list of putative allergenic proteins developed *via* a peer-reviewed process for the purpose of evaluating food safety. The maltogenic α-amylase amino acid protein sequence expressed in *S. cerevisae* production strain is available in Appendix 16: Genetic Modification of the Source Organism (CONFIDENTIAL).

In accordance with the guidelines endorsed by Codex Alimentarius Commission (2009) and EFSA (EFSA GMO Panel, 2010) for the safety evaluation of newly expressed proteins from genetically modified plants and microorganisms, the database was searched using a sliding window of 80-amino acids sequences derived from the full-length amino acid sequence. According to the approach adopted by the Codex Alimentarius Commission and EFSA, 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.

Using this sequence homology search strategy, the maltogenic α-amylase amino acid sequence shared >35% identity for at least one 80 amino acids segment with allergens from *Aspergillus oryzae*, *Schizophyllum commune*, *Aedes aegypti*, and *Aspergillus fumigatus*. (See Table 6 below).

Sequence G.I. No	description	Organism	Best % Identity
94706935	Alpha-amylase A type-1/2 precusor	*Aspergillus oryzae*	42.54
166531	Taka-amylase A (Taa-G1) precursor	*Aspergillus oryzae*	42.54
302681819	Glycoside hydrolase family 15	*Schizophyllum commune* H4-8	36.24
126713	Probable maltase	*Aedes aegypti*	36.20
3549630	Alkaline protease, partial	*Aspergillus fumigatus*	35.30
2295	Uncleaved alkaline protease	*Aspergillus fumigatus*	35.30

Table 6: Homology Search Results on AllergenOnline Database for Maltogenic α-Amylase Amino Acid Sequence (80 amino acids sequences, >35% identity)

[3] AllergenOnline is an allergen protein database containing 2,129 peer-reviewed allergenic protein sequences (Version 19; released on February 10, 2019) that is curated by the Food Allergy Research and Resource Program (FARRP) of the University of Nebraska. The database is available at: http://www.allergenonline.org/

Low level homology to these fungal allergens suggests potential cross-reactivity to these proteins; Moreover, these allergens are characterized as inhalation sensitizers rather than food allergens and therefore no allergenic risk from food uses of the enzyme were identified. Sensitization to enzymes have been reported in workers involved in enzyme manufacturing; however, these are allergenic reactions that occur after inhalation exposure to enzymes. Nevertheless, the risk of worker sensitization to enzymes are mitigated by improvements in safe handling and use of personal protective equipment (Bindslev-Jensen *et al.,* 2006).

A sequence homology search was also conducted using the exact 8-mer approach, which is considered to be highly conservative, and did not identify any matches.

Additionally, 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 analyses, full length FASTA or BLASTP searches may be the most predictive approach for allergenic reactions (Aalberse, 2000; Goodman and 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. A second homology search was therefore conducted using the full length FASTA36 alignment of the amino acid protein sequence with known allergens using the AllergenOnline database (using default settings, i.e. *E* value cutoff = 1 and maximum alignments of 20). All the results obtained from this search strategy are presented in Table 7 below. No alignment with allergenic proteins at or above the 35% threshold of concern for allergenicity was found, indicating the unlikely potential for cross-reactivity to these allergens.

Sequence G.I. No	Description	Organism	Amino Acid Length	E-Value	% Identity
166531	Taka-amylase A (Taa-G1) precursor	*Aspergillus oryzae*	499	3.2e-13	28.4
94706935	Alpha-amylase A type-1/2	*Aspergillus oryzae*	499	4.4e-13	28.2
126713	Probable maltase	*Aedes aegypti*	579	2.9e-10	24.6
33667932	Blo t 4 allergen	*Blomia tropicalis*	506	2.9e-8	23.0
302681819	Glycoside hydrolase family 15 and carbohydrate-binding module family 20	*Schizophyllum commune*	576	6.3e-8	22.6
118638278	Allergen Aca s 4	*Acarus siro*	517	2.3e-7	23.3
821092692	Alpha-amylase	*Periplaneta Americana*	494	2.7e-6	25.3
685848328	Der f 4 allergen	*Dermatophagoides farina*	525	5.5e-6	23.7
85002763	Alpha-amylase	*Blattella germanica*	515	0.015	22.7

Table 7: Homology Search Results of AllergenOnline Database for maltogenic α-Amylase Amino Acid Full Sequence

The allergenicity of maltogenic α-amylase was also considered through a search of the available scientific literature; however, no relevant information was identified in PubMed when searching for the terms "maltogenic alpha-amylase" or "maltogenic amylase" and "allergen*" (advanced search in all fields, Dec.24 2019). A review of 3 major allergen databases, AllergenOnline, COMPARE[4], and WHO/IUIS allergen database[5], did not reveal any entries for putative allergens originating from *G. stearothermophilus* or *S. cerevisiae*.

Based on the information provided above, no evidence exists that might indicate that maltogenic α-amylase from the production strain would produce an allergenic response following consumption of foods to which the enzyme is added. A search of the available scientific literature did not reveal any evidence indicating allergenicity to maltogenic α-amylase in consumers of foods to which the enzyme is added. Furthermore, any residual enzyme potentially carried over into the final ingredient would likely be inactivated and denatured under the conditions of food processing during production of the final food products. Therefore, the use of the maltogenic α-amylase enzyme is not anticipated to pose any allergenicity concerns for consumers. Finally, no major food allergen is used during the manufacturing process of the enzyme processing aid.

C.4 Safety assessment reports prepared by international agencies or other national government agencies, if available

The safety of the enzyme processing aid subject of this application has been independently and collectively, critically evaluated by a panel of scientific experts, and was determined to be Generally Recognized as Safe (GRAS) for use as a food enzyme in the production of baked goods in the U.S. The GRAS notice has been filed with the U.S. Food and Drug Administration (GRN 842) and recognised as GRAS by the FDA ("FDA has no questions"). The No Question Letter received from the FDA is provided in Appendix 12: FDA No Question Letter.

Moreover, the safety of maltogenic α-amylase food enzymes similar to the enzyme processing aid subject of this application has already been reviewed by regulatory agencies and authoritative bodies, including FSANZ (See Table 18 below).

It is worth noting that, similarly to the genetic modification described in this application to obtain the production strain, several of the microbial sources utilize the maltogenic α-amylase gene of *Geobacillus Stearothermophilus* (formally classified as *Bacillus Stearothermophilus*) in the genetic modification.

[4] The COMprehensive Protein Allergen REsource (COMPARE) database is a manually-curated allergenic protein database maintained by the Health and Environmental Sciences Institute (HESI). The COMPARE database contains about 2,081 allergenic proteins in total. The database is available at: http://db.comparedatabase.org/

[5] The WHO/IUIS allergen database contains 948 allergenic proteins and is maintained by the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-Committee. The database is available at: http://www.allergen.org/index.php

Jurisdiction	Source	Food Category	Maximum Level of Use	Reference
EU	Genetically modified *Bacillus subtilis* (strain NZYM-OC)	Baking processes	54.5 mg TOS/kg flour for cakes and 13.6 for bread	EFSA-Q-2014-00922 EFSA CEP Panel, 2018a
	Genetically modified *Bacillus subtilis* (strain NZYM-SO)	Baking processes	47.6 mg TOS/kg flour for cakes and 11.9 for bread	EFSA-Q-2015-00046 EFSA CEP Panel, 2018b
	Genetically modified *Bacillus subtilis* (strain NZYM-SM)	Baking processes	15 mg TOS/kg flour	EFSA-Q-2015-00096 EFSA CEF Panel, 2018a
		Starch processing	49.5 mg TOS/kg starch	
	Genetically modified strain of *Escherichia coli* (strain BLASC)	Baking processes	8.47mg TOS/kg flour	EFSA-Q-2015-00446 EFSA CEP Panel, 2019a
		Starch processing	33.89 mg TOS/kg starch	
		Brewing processes	16.95 mg TOS/kg malted barley	
	Genetically modified strain of *Bacillus subtilis* (strain MAM)	Baking processes	15.6 mg TOS/kg flour	EFSA-Q-2013-00790 EFSA CEF Panel, 2018b
Canada	Genetically modified strain of *Bacillus licheniformis* (strain MDT06-221)	-Bread, flour, whole wheat flour -Pasta -Unstandardized bakery products	cGMP	List of Permitted Food Enzymes (Health Canada, 2020)
	Genetically modified strains *Bacillus subtilis* BRG-1 (pBRG1); *Bacillus subtilis* DN1413 (pDN1413); *Bacillus subtilis* LFA 63 (pLFA63); *Bacillus subtilis* RB-147 (pRB147)	-Starch used in production of dextrins, dextrose, glucose syrup, glucose solids (dried glucose syrup) or maltose -Bread, flour; whole wheat flour -Unstandardized bakery products		
	Genetically modified *Bacillus subtilis* BS154; *Bacillus subtilis* RF12029	-Bread; Flour; Whole wheat flour -Unstandardized baking products		
U.S.	*Bacillus stearothermophilus* produced in *Bacillus subtilis* BRG-1	-Processing starch in food	cGMP	GRN 751 "No Questions" (U.S. FDA, 2018a)
	Geobacillus stearothermophilus produced in *Bacillus subtilis* RF12029 / EL 2009083	-Baking processes	cGMP	GRN 746 "No Questions" (U.S. FDA, 2018b)
FSANZ	*Bacillus subtilis* containing the gene for maltogenic α-amylase isolated from *Geobacillus stearothermophilus*	Not detailed		Australia New Zealand Food Standards Code – Schedule 18 – Processing aids (FSANZ)
JECFA	Maltogenic amylase from *Bacillus Stearothermophilus* expressed in *Bacillus Subtilis*	-Retardation of staling in baked goods -Preparation of high maltose glucose syrup	ADI not specified	JECFA (1998)

Table 8 Non-Exhaustive List of Existing Authorizations for Maltogenic α-Amylase as a Food Enzyme

D Additional Information Related to the Safety of an Enzyme Processing Aid Derived from a Microorganism

D.1 Information on the source microorganism

The source organism (production strain) is obtained by genetic engineering of a *Saccharomyces cerevisiae* strain (traditional baker's yeast) to express an optimized variant of the maltogenic α-amylase gene from *Geobacillus stearothermophilus*. The production strain has already been used industrially since July 2019, to produce commercial batches of the maltogenic α-amylase.

The production strain has been confirmed to be a *Saccharomyces cerevisiae* strain and does not appear to be of a hybrid nature, as demonstrated by Whole Genome Sequencing (WGS) analysis. (See Appendix 13: Whole Genome Sequencing Analysis Report (CONFIDENTIAL))

It is customary to use the Pariza-Johnson decision tree (Pariza and Johnson, 2001) to evaluate the safety of modified strains for enzyme production. The analysis includes, but is not limited to, the identity of the host strain, characteristics of the introduced DNA (the sources and functions of the introduced genetic material), an outline of the genetic construction of the production strain, a characterization of the production strain, and potential for the production strain to have pathogenic, toxigenic or antibiotic resistance characteristics. If the production microorganism meets the criteria described by Pariza and Johnson, it can be determined safe as used for food production.

Pariza and Johnson base the decision tree concept on their 1983 publication (Pariza and Foster, 1983) 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 recombinant DNA 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 recombinant DNA strain improvement technologies." (Pariza and 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 and Johnson, 2001).

The safety of the production strain and consequently the one of the maltogenic α-amylase food enzyme was assessed using the Pariza and Johnson decision tree (see Figure 2 below). The production strain is genetically modified using standard recombinant DNA techniques, and the gene is integrated into a designated loci of the *Saccharomyces cerevisiae* recipient strain. The production strains 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. Based on this approach, the maltogenic α-amylase food enzyme derived from genetically modified *S. cerevisiae* is accepted and suitable for food production.

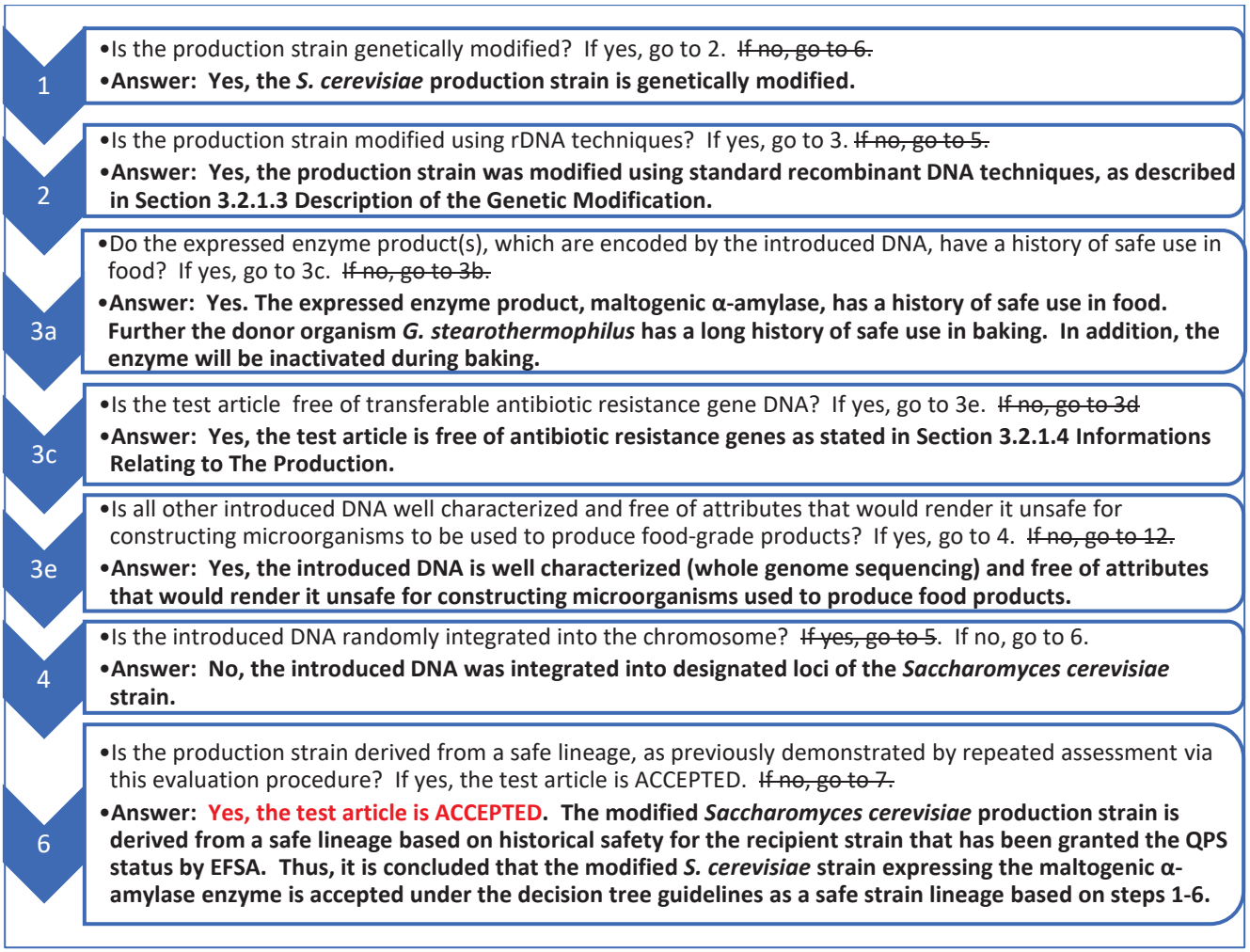


Figure 2: Pariza-Johnson Decision Tree Analysis of Maltogenic α-Amylase Enzyme Production Strain *S. cerevisiae*

D.2 Information on the pathogenicity and toxicity of the source microorganism

S. cerevisiae is a non-pathogenic and non-toxigenic species that has been demonstrated to be suitable for food production through an extensive history of safe use in several countries in food applications such as bakery, winemaking, brewery, and distillery and is not anticipated to produce any toxin secondary metabolites or have antibiotic activity.

According to EFSA, yeasts used in food production, particularly bakers/brewer's yeast, are considered among the safest of microorganisms (EFSA Scientific Committee, 2007). 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 (EFSA BIOHAZ Panel 2020a).

In its guidance on the characterisation of microorganisms used for the production of food enzymes (EFSA CEP Panel, 2019b), EFSA CEP Panel states that food enzymes produced by adequately characterised genetically modified organisms from QPS recipient strains can be considered as QPS if the absence of DNA of the production strain is demonstrated, which is the case for M17906 (See Appendix 14: Absence of GMO DNA

(CONFIDENTIAL). This approach has also been emphasized by the EFSA BIOHAZ Panel (EFSA BIOHAZ Panel 2020a and 2020b).

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.

In addition, to determine whether the production strain contained any antibiotic resistance or virulence genes, whole-genome Illumina sequence data of the production slant was analysed with multiple databases. Details regarding the analytical methods are provided in Appendix 13: Whole Genome Sequencing Analysis Report (CONFIDENTIAL).

ResFinder. Analysis of the whole-genome Illumina sequence data with ResFinder to detect acquired antimicrobial resistance genes detected no genes for any of the available antimicrobials.

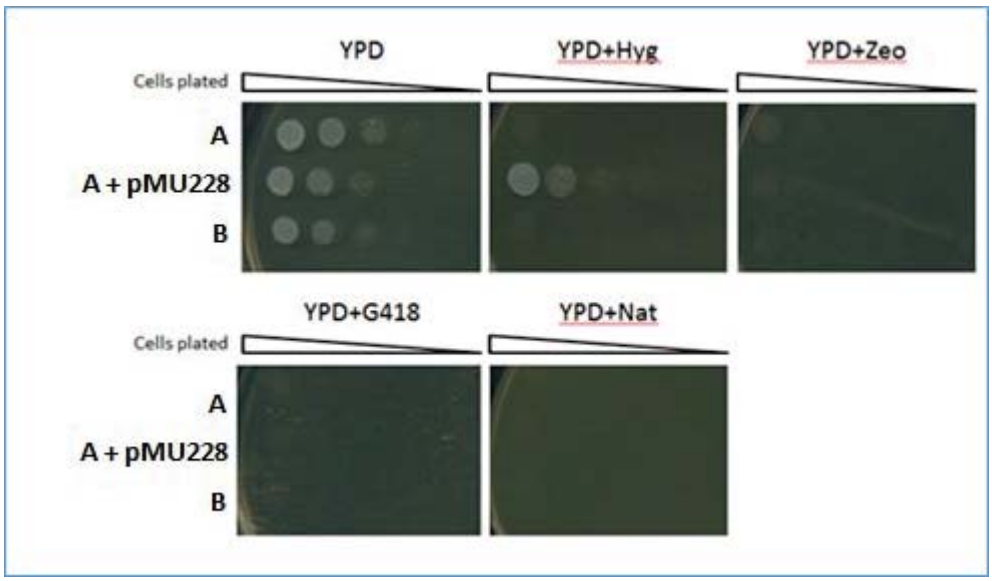
VirulenceFinder. Analysis of the whole-genome Illumina sequence data with VirulenceFinder to detect virulence factors showed no hits for any of the screened categories (virulence genes for *Listeria*, *Enterococcus*, and *Escherichia coli*, toxin, hostimm, and exoenzyme genes for *S. aureus*, and Shiga-toxin genes).

PlasmidFinder. Analysis of the whole-genome Illumina sequence data with PlasmidFinder to detect plasmids showed no hits.

Ariba – ARG-ANNOT/NCBI. Analysis of the whole-genome Illumina sequence data with Ariba to detect antimicrobial resistance genes in the ARG-ANNOT and NCBI databases showed no hits.

Ariba – In-house antibiotic resistance and gene editing genes. Analysis of the whole-genome Illumina sequence data with Ariba to detect antibiotic resistance and gene editing genes used in-house (See Table 2 in Appendix 13: Whole Genome Sequencing Analysis Report (CONFIDENTIAL)) showed no hits. In addition, *GAL2* from *S. cerevisiae* S288C was used as a positive control, and resulted in a hit, confirming proper function of the Ariba workflow.

Furthermore, the lack of growth on media containing various antibiotics (refer to Figure 3 below) supports the lack of antibiotic resistant genes.



Strain	Description
A	Non-modified host strain
A+ pMU228	Control strain into which the co-transformation plasmid pMU228 was transformed and retained
B	Modified strain that expresses maltogenic amylase variant
Media	**Description**
YPD	Yeast extract media with 40 g/L glucose
YPD + Hyg	YPD + 300µg/mL hygromycin (Hyg)
YPD + Zeo	YPD + 600µg/mL zeocin (zeo)
YPD + G418	YPD + 200µg/mL geneticin (g418)
YPD + NAT	YPD + 100µg/mL norseothricin (NAT)

Figure 3: Growth of *S. cerevisiae* Production Strain on Select Media

D.3 Information on the genetic stability of the source microorganism

Information regarding genetic stability of the source organism is provided in Appendix 15: Genetic Stability of the Source Organism (CONFIDENTIAL)

E Additional Information Related to the Safety of an Enzyme Processing Aid Derived from a Genetically-Modified Microorganism

E.1 Information on the methods used in the genetic modification of the source organism

All the requested information regarding the genetic modification of the source organism is provided in Appendix 16: Genetic Modification of the Source Organism (CONFIDENTIAL).

F Information Related to the Dietary Exposure of the Processing Aid

F.1 A list of food or food groups likely to contain the processing aid or its metabolites

Based on the food group descriptions in the Food Additives Schedule 15 (table S15-5), maltogenic α-amylase enzyme from a modified strain of Saccharomyces cerevisiae would be used on the following food groups:

- 7 Bread and bakery products

F.2 The levels of residues of the processing aid or its metabolites for each food or food group

The proposed maximum level of use for maltogenic α-amylase in its intended application is listed below.

Application	Raw Material (RM)	Maximal recommended use levels (mg TOS[(1)]/kg RM)
Baking	Flour	28.6

[(1)] TOS (Total organic solids) = 100% - (A+W+D)
where A = % ash, W = % water, and D = % diluents and/or other formulation ingredients

The enzyme processing aid will perform its technological function during the baking process. It is then expected to be inactivated by heat at higher temperature (95°C for 10 minutes) as shown in Figure 4 below, and has no further technological effect after baking.

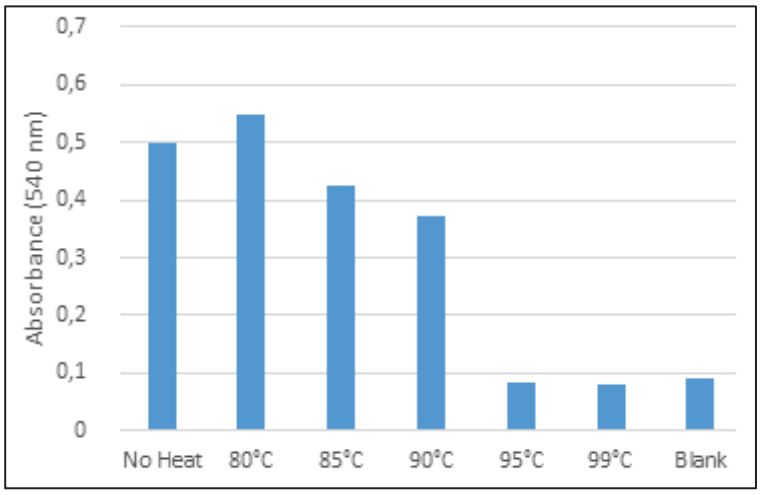


Figure 4: Thermostability of the Maltogenic α-Amylase

In this test, 150 µL of 100 µg/mL enzyme solutions at pH 5.0 were pre-incubated for 10 minutes at the following temperatures: room temperature, 80, 85, 90, 95, and 99°C. Then, 50 µL of each solution were added to 100 µL of a 1% wheat starch substrate solution, at pH 5.0. The enzyme/substrate solutions were incubated for 10 minutes at 60°C. Following this incubation the enzyme/substrate solutions were incubated at 99°C for 5 minutes in DNS reagent (1% w/v 3,5-dinitrosalicylic acid, 1% w/v sodium hydroxide, 0.05% w/v sodium sulfide in deionized water). The absorbance of the solution was then determined spectrophotometrically at

540 nm as a measure of the enzyme activity, the intensity of the colour being proportional to the concentration of maltose present in the sample and consequently to the enzymatic activity.

Dietary Exposure

The Budget Method was used to obtain an estimate of the potential dietary exposure to the maltogenic α-amylase processing aid in foods intended for consumption for the general population on the basis that the enzyme processing aid is used in bread and other baking products.

The Budget Method is used as a screening tool and provides an overestimate of dietary exposure by using conservative assumptions in terms of use level and food consumption (FAO/WHO, 2009). This approach assumes that there is a maximum physiological amount of foods which can be consumed daily and that the portion of solid foods that contain the food enzyme preparation may be set at 25% for food additives used in a wide range of foods (FAO/WHO, 2009)[6]. Beverages were not included in the Budget Method calculation since the proposed uses of the maltogenic α-amylase food enzyme preparation is specific to food. The result is an estimate of the dietary exposure to the food enzyme preparation in the form of a Theoretical Maximum Daily Intake (TMDI). The assumptions of the Budget Method are outlined below.

Level of Consumption of Solid Foods

The FAO/WHO report on the *Principles and Methods for the Risk Assessment of Chemicals in Food* (FAO/WHO, 2009) specifies the standard values for food intakes at 0.05 kg/kg body weight/day (based on an estimated energy density of 2 kcal/g) for solid foods. Using the default body weight for adults of 70 kg, this is equivalent to an intake of 3.5 kg.

Level of Presence of Food Enzyme in Solid Foods

The amount of the maltogenic α-amylase food enzyme preparation assumed to be present in solid foods is based on the maximum level of the food enzyme in flour (i28.6 mg TOS/kg flour). This conservative approach is made assuming that bread and other baking products prepared with the flour containing the food enzyme are only composed of flour.

Proportion of Solid Foods That May Contain the Food Enzyme

According to the budget method, a standard proportion of all solid foods of 12.5% are assumed to contain the food enzyme (FAO/WHO, 2009). As a conservative approach, 25% of solid foods may be made with the food enzyme (assumption for additives used in a wide range of foods (FAO/WHO, 2009)). This assumes that a typical adult weighing 70 kg consumes 0.88 kg of solid food which are produced using the food enzyme preparation.

Theoretical Maximum Daily Intake of Enzyme

Based on conservative estimates of exposure calculated using the budget method, the TMDI of the maltogenic α-amylase enzyme processing aid was calculated to be 0.358 mg TOS/kg body weight/day. The calculations for the derivation of the TMDI of the food enzyme preparation from all solid foods and the resulting total estimated intakes are presented in Table 9 below.

[6] Based on the assumptions of the FAO/WHO report on the Principles and Methods for the Risk Assessment of Chemicals in Food (FAO/WHO, 2009), 12.5% of solid foods are assumed to contain the ingredient produced using the food enzyme preparation, however this should be increased to 25% in the case of ingredients (produced using the food enzyme) used in a wide range of food categories.

Products	Level of Consumption of Solid Foods (kg/kg bw/day)	Proportion of Solid Foods Containing Food Enzyme (%)	Maximum Level of Food Enzyme in Solid Foods (mg TOS/kg)	Total Exposure to Food Enzyme Preparation[a] (mg TOS/kg bw/day)
Solid Foods	0.05	25	28.6	**0.358**

bw = body weight; TMDI = Theoretical Maximum Daily Intake; TOS = total organic solids

[a] Calculation: (Level of Consumption of Solid Foods) * (Proportion of Solid Foods Containing Food Enzyme/100) * (Maximum Level of Food Enzyme in Solid Foods)

Table 9: TMDI of Maltogenic α-Amylase Based on the Maximum Use Levels in Solid Foods Using the Budget Method

Dietary Exposure to Any Other Substance Formed in or on Food

The maltogenic α-amylase enzyme processing aid acts on the linkages in amylose, amylopectin and related glucose polymers, catalyzing hydrolysis and breaking them down to form maltose. These products are regular components of food and not expected to have any adverse effects on humans.

Dietary Exposure to Contaminants or By-products

Fermentation parameters including pH, aeration, temperature, and off-gas production are monitored during the fermentation process and deviations from the pre-defined values lead to adjustment to ensure an optimal and consistent process. Therefore, no harmful contaminants or by-products are expected. Furthermore, routine batch analysis is conducted to ensure the product complies with established specifications and is free of contaminants.

Conclusion on Dietary Exposure Assessment

The estimated human exposure to the maltogenic α-amylase enzyme processing aid was calculated using the Budget Method, reflecting the proposed uses of the enzyme as a processing aid to be used in baking processes to reduce crumb firmness and prevent staling. The assumptions have been conservative to ensure there is no under-estimation of intakes of the food enzyme preparation. The Budget Method uses standard values to calculate the TDMI based on conservative assumptions regarding dietary intake of solid foods. In the assessment, the enzyme was assumed to be present at the maximum usage level in all applications of food and is assumed to be present at these levels in the final food as consumed.

The TMDI calculated for maltogenic α-amylase food enzyme preparation using the Budget Method was 0.358 mg TOS/kg body weight per day based on the maximum intended use levels of the food enzyme in the intended food uses. Furthermore, the consumer exposure to other substance formed in food is not anticipated to be of toxicological concern and contaminants/by-products are routinely monitored in the manufacturing product to ensure food-grade specifications are met.

When compared with NOAEL provided in applications on equivalent maltogenic a-amylase food enzyme preparations that have been recently evaluated by EFSA or FDA, it appears that the lowest NOAEL of 318.4 mg TOS/kg bw per day found among these opinions (EFSA CEP Panel, 2018b; see data in Table 4) corresponds to 889 times the TMDI for the food enzyme preparation.

F.3 For foods or food groups not currently listed in the most recent Australian or New Zealand National Nutrition Surveys (NNSs), information on the likely level of consumption

Not applicable

F.4 The percentage of the food group in which the processing aid is likely to be found or the percentage of the market likely to use the processing aid

We estimate that the enzyme would be used as a processing aid in about 20% to 25% of the tonnage of bread and bakery products sold in Australia and New Zealand.

F.5 Information relating to the levels of residues in foods in other countries

Applications and levels of use of the maltogenic α-amylase processing aid in other countries is the same as presented in section F.2.

F.6 For foods where consumption has changed in recent years, information on likely current food consumption

Not applicable

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Appendices

Non-Confidential Appendices

The following non-confidential appendices are provided on the following pages.

Appendix 1:	Statutory Declaration
Appendix 2:	Checklists
Appendix 8:	Antimicrobial Activity Method
Appendix 9:	NOM-117-SSA1-1994 Method – English Translation from Original Method in Spanish
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Confidential Appendices

The following confidential appendices are provided in a separate document.

Appendix 3:	Technological effect of the Enzyme Processing Aid (CONFIDENTIAL)
Appendix 4:	Manufacturing Process Flow Chart (CONFIDENTIAL)
Appendix 5:	Manufacturing Process – List of Raw Materials and Processing Aids (CONFIDENTIAL)
Appendix 6:	Certificates of Analysis (CONFIDENTIAL)
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Appendix 13:	Whole Genome Sequencing Analysis Report (CONFIDENTIAL)
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Appendix 1: Statutory Declaration

STATUTORY DECLARATION

Statutory Declarations Act 1959

I, Jim Moshovelis, Director, Scientific & Regulatory Solutions of Killarney Heights NSW Australia,

make the following declaration under the *Statutory Declarations Act 1959*:

1. the information provided in this application fully sets out the matters required
2. the information provided in this application is true to the best of my knowledge and belief
3. no information has been withheld that might prejudice this application, to the best of my knowledge and belief

I understand that a person who intentionally makes a false statement in a statutory declaration is guilty of an offence under section 11 of the *Statutory Declarations Act 1959*, and I believe that the statements in this declaration are true in every particular.

[redacted]

Declared at 17th on August 2020.

Before me,

[redacted]

Signature:

Full Name:

Qualification:

Address:

Appendix 2: Checklists

Checklist for General requirements

This Checklist will assist you in determining if you have met the mandatory format and information requirements as detailed in Guideline 3.1.1 – General requirements. All applications **must** include this Checklist.

General requirements (3.1.1)		
Check	**Page No.**	**Mandatory requirements**
☑		A Form of application ☑ *Application in English* ☑ *Executive Summary (separated from main application electronically)* ☑ *Relevant sections of Part 3 clearly identified* ☑ *Pages sequentially numbered* ☑ *Electronic copy (searchable)* ☑ *All references provided*
☑	7	B Applicant details
☑	7	C Purpose of the application
☑	8	D Justification for the application ☑ *Regulatory impact information* ☑ *Impact on international trade*
☑	9	E Information to support the application ☑ *Data requirements*
☑	9	F Assessment procedure ☑ *General* ☐ *Major* ☐ *Minor* ☐ *High level health claim variation*
☑	9	G Confidential commercial information ☑ *CCI material separated from other application material* ☑ *Formal request including reasons* ☑ *Non-confidential summary provided*
☑	10	H Other confidential information ☐ *Confidential material separated from other application material* ☐ *Formal request including reasons*
☑	10	I Exclusive Capturable Commercial Benefit ☐ *Justification provided*
☑	11	J International and other national standards ☑ *International standards* ☑ *Other national standards*
☑	11	K Statutory Declaration
☑	11	L Checklist/s provided with application ☑ *3.1.1 Checklist* ☑ *All page number references from application included* ☑ *Any other relevant checklists for Chapters 3.2–3.7*

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Checklist for applications for substances added to food

This Checklist is in addition to the Checklist for Guideline 3.1.1 and will assist you in determining if you have met the information requirements as specified in Guidelines 3.3.1–3.3.3.

Processing aids (3.3.2)		
Check	**Page No.**	**Mandatory requirements**
☑	12	A.1 Type of processing aid
☑	12	A.2 Identification information
☑	13	A.3 Chemical and physical properties
☑	15	A.4 Manufacturing process
☑	19	A.5 Specification information
N/A		A.6 Analytical method for detection
N/A		B.1 Industrial use information (chemical only)
N/A		B.2 Information on use in other countries (chemical only)
N/A		B.3 Toxicokinetics and metabolism information (chemical only)
N/A		B.4 Toxicity information (chemical only)
N/A		B.5 Safety assessments from international agencies (chemical only)
☑	20	C.1 Information on enzyme use on other countries (enzyme only)
☑	21	C.2 Toxicity information of enzyme (enzyme only)
☑	27	C.3. Allergenicity information of enzyme (enzyme only)
☑	30	C.4. Overseas safety Assessment Reports
☑	32	D.1 Information on source organism (enzyme from microorganism only)
☑	33	D.2 Pathogenicity and toxicity of source microorganism (enzyme from microorganism only)
☑	35	D.3 Genetic stability of source organism (enzyme from microorganism only)
☑	35	E.1 Nature of genetic modification of source organism (enzyme from GM source microorganism)
☑	36	F.1 List of foods likely to contain the processing aid
☑	36	F.2 Anticipated residue levels in foods
☑	39	F.3 Information on likely level of consumption
☑	39	F.4 Percentage of food group to use processing aid
☑	39	F.5 Information on residues in foods in other countries (if available)
☑	39	F.6 Where consumption has changed, information on likely consumption

N/A = not applicable for enzymatic processing aids

Appendix 3: Technological effect of the Enzyme Processing Aid (CONFIDENTIAL)

Appendix 3 is provided in a separate document.

Appendix 4: Manufacturing Process Flow Chart (CONFIDENTIAL)

Appendix 4 is provided in a separate document.

Appendix 5: Manufacturing Process – List of Raw Materials and Processing Aids (CONFIDENTIAL)

Appendix 5 is provided in a separate document.

Appendix 6: Certificates of Analysis (CONFIDENTIAL)

Appendix 6 is provided in a separate document.

Appendix 7: Maltogenic Amylase Activity Determination Method (CONFIDENTIAL)

Appendix 7 is provided in a separate document.

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Appendix 8: Antimicrobial Activity Method

Antimicrobial activity

Objective

This procedure is to determine antibacterial activity in enzyme preparations derived from microbial sources, following JECFA guidelines[1] for the 3 commercial batches submitted for the application.

Material and Methods

1. **Enzyme sample preparation**

   Enzyme samples were prepared by adding 1 g of the food enzyme preparation powder to 9 mL of sterile water (10% enzyme preparation). 0.1 mL of the enzyme preparation was applied to a filter disk (Whatman® Antibiotic Assay Discs, 9 mm) to saturate the disk with the enzyme preparation. Six disks of were prepared for each sample to test on six bacterial strains.

2. **Bacterial culture preparation**

   Six strains were ordered as lyophilized preparations from ATCC (https://www.atcc.org/en.aspx): *Staphylococcus aureus* (ATCC 6538); *Escherichia coli* (ATCC 11229); *Bacillus cereus* (ATCC 11778); *Bacillus circulans* (ATCC 4516); *Streptococcus pyrogenes* (ATCC 12344); and *Serratia marcescens* (ATCC 14041), and revived following manufacturer's instructions. The strains were then grown overnight and stocked in TSA with 20% glycerol at 80 °C. Cultures for this experiment set were inoculated directly from the glycerol stocks of these strains in Trypton Soya Broth. The cells were let grown for 24 hours with shaking at 250 rpm, 35±2 °C. Trypton Soya Agar (TSA) plates were prepared by pouring 15 mL TSA to a Petri dish and allowed to harden. Seeded TSA was prepared by diluting the 24 Hr bacterial cultures by 10 times into TSA (20 times for *Streptococcus pyrogenes*), and overlaying the 15 mL-TSA plates with 10 mL of the seeded TSA.

3. **Antibacterial activity testing**

   The prepared disks were place on the surface of the hardened culture plates. Plates were incubated at 4 °C for overnight to allow proper diffusion. Following the 4 °C incubation, plates were incubated at 37±2 °C for 24 hours. After incubation, plates were visually examined for the presence of a clear zone around the disk. If a clear zone of a total diameter of 12 mm is observed, the enzyme preparation is considered inhibitory for this strain.

Results

As shown in Table 1, neither of the 3 food enzyme preparations showed inhibitory effects for any of the six strains tested in this experiment set. Hence we conclude that these 3 enzyme samples exhibit no antimicrobial activity for these six strains.

Table 1 Antibacterial activity results showing the presence (+) and absence of (-) of a clear zone of 12 mm or above.

Strains	*Staphylococcus aureus*	*Escherichia coli*	*Bacillus cereus*	*Bacillus circulans*	*Streptococcus pyrogenes*	*Serratia marcescens*
Lot LLM1903-01	-	-		-	-	-
Lot LLM1904-01	-	-		-	-	-
Lot LLM1905-01	-	-		-	-	-

References

1. Joint FAO/WHO Expert Committee on Food Additive. COMBINED COMPENDIUM OF FOOD ADDITIVE SPECIFICATIONS. Available at: http://www.fao.org/3/A0691E07.htm#7.1.3. (Accessed 13th Nov. 2019)

Appendix 9: NOM-117-SSA1-1994 Method – English Translation from Original Method in Spanish

08-16-95 Mexican Official STANDARD NOM-117-SSA1-1994, Goods and services. Test method for the determination of cadmium, arsenic, lead, tin, copper, iron, zinc and mercury in food, drinking water and water purified by atomic absorption spectrometry.

In the margin a stamp with the National Shield, which says: United Mexican States - Health Secretary.

OFFICIAL MEXICAN STANDARD NOM-117-SSA1-1994, GOODS AND SERVICES. TEST METHOD FOR THE DETERMINATION OF CADMIUM, ARSENIC, LEAD, TIN, COPPER, IRON, ZINC AND MERCURY IN FOOD, DRINKING WATER AND PURIFIED WATER BY ATOMIC ABSORPTION SPECTROMETRY.

JOSE MELJEM MOCTEZUMA, General Director of Sanitary Control of Goods and Services, by agreement of the National Advisory Committee for Standardization of Regulation and Sanitary Promotion, based on articles 39 of the Organic Law of the Federal Public Administration; 3rd. section XXII and XXIV, 13 section I, 194 section I, of the General Health Law; 3rd. section XI, 38 section II, 40 section I, VI, VIII, XI and XIII, 41, 43, and 47 section IV of the Federal Law on Metrology and Standardization; 8o. section IV and 13 section I of the Internal Regulations of the Ministry of Health; and those applicable to the Regulations of the General Health Law in the Area of Sanitary Control of Activities, Establishments, Products and Services, and

CONSIDERING

That on April 28, 1994, in compliance with the provisions of Article 46 section I of the Federal Law on Metrology and Standardization the General Directorate of Sanitary Control of Goods and Services presented to the Committee National Advisory for Standardization of Regulation and Health Promotion, the Preliminary draft of this Official Mexican Standard.

That on August 15, 1994, pursuant to the Committee's agreement in article 47 fraction I of the Federal Law on Metrology and Standardization, the Draft of this Official Mexican Standard was provided to the effect that within the following ninety calendar days after said publication, the interested parties submitted their comments to the National Advisory Committee for Standardization of Regulation and Health Promotion.

That in a previous date, the responses to the comments received by the aforementioned Committee were published in the Official Gazette of the Federation, in terms of article 47 fraction III of the Federal Law on Metrology and Standardization.

That in consideration of the above considerations, counting on the approval of the National Advisory Committee for the Standardization of Regulation and Promotion Sanitary, the following is issued:

OFFICIAL MEXICAN STANDARD NOM-117-SSA1-1994, GOODS AND SERVICES. TESTING METHOD FOR THE DETERMINATION OF CADMIUM, ARSENIC, LEAD, TIN, COPPER, IRON, ZINC AND MERCURY IN FOOD, DRINKING WATER AND PURIFIED WATER BY SPECTROMETRY OF ATOMIC ABSORPTION.

PREFACE

The following organizations and institutions participated in the preparation of this Standard:

HEALTH SECRETARY
General Directorate of Sanitary Control of Goods and Services
National Laboratory of Public Health
SECRETARY OF AGRICULTURE, LIVESTOCK AND RURAL DEVELOPMENT
National Center for Animal Parasitology
National Water Commission
FEDERAL CONSUMER ATTORNEY
NATIONAL AUTONOMOUS UNIVERSITY OF MEXICO
Faculty of Chemistry
AUTONOMOUS METROPOLITAN UNIVERSITY
Iztapalapa Unit
LABORATORY FERMI, SA
LABORATORY ICCABI, SA DE CV
INDUSTRIAS VINICOLAS PEDRO DOMECQ, SA DE CV
MEXICAN SOCIETY OFR NORMALISATION AND CERTIFICATION, SC
NORMEX

INDEX

0. Introduction

The presence of certain chemical elements in food, beverages, drinking water and purified water is a serious problem for human health due to their toxicity.

1. Objective and field of application

1.1. This Official Mexican Standard establishes the test methods for atomic absorption spectrometry determination of cadmium, arsenic, lead, tin, copper, iron, zinc and mercury present in food, beverages, purified water and drinking water.

1.2. This Official Mexican Standard is of obligatory observance in the territory national law for individuals or companies that require this method in national or imported products, for official purposes.

2. Basis

The atomic absorption method is based on passing a monochromatic light beam of a frequency such as it can be absorbed by the analyte that is present in the form of atomic vapor. Measurement of the light intensity before and after passing through the atomic steam allows to determine the absorption percentage.

The amount of absorption increases with the concentration of the atoms in the medium, that is, the measure of absorption increases with concentration of the item in the sample, whether it is in its original condition or subject to pre-treatment.

3. Definitions

For the purposes of this Standard, the following definitions apply:

3.1. Instrument calibration blank, is the acid solution used as diluent.

3.2. Reagent blank, is the solution that contains all the reagents used at the same volumes and concentrations in sample processing. This blank should follow the digestion and sample preparation steps.

3.3. Fortified reagent blank, is the solution that is prepared from an aliquot of the reagent blank, adding an aliquot of the solution standard concentrated "stock solution", to give a final concentration that produces an acceptable absorbance (approximately 0.1) for the analyte. The fortified reagent blank must follow the same digestion schedule as sample preparation.

3.4. Spectrometry, is a branch of spectroscopy related to spectra measurement.

3.5. Atomic absorption spectrometry, is a branch of instrumental analysis in which an element is atomized in a way that allows observation, selection and measurement of its absorption spectrum.

3.5.1. Flame atomic absorption spectrometry is the method by which the element is determined using an atomic absorption spectrometer, used in conjunction with a nebulization system and an atomization source.

The atomization source is a burner that uses different mixtures of gases, the most frequent are air-acetylene and nitrous-acetylene oxide.

3.5.2. Graphite furnace atomic absorption spectrometry, is the method whereby the element is determined by an atomic absorption spectrometer, used in conjunction with a graphite furnace. The principle is essentially the same as in direct flame aspiration atomic absorption, except that an oven is used instead of the flame to atomize the sample.

3.5.3. Atomic absorption spectrometry for hydride generation, is a method similar to that of cold steam. Samples react in an external device with a reducing agent, generally borohydride. The products reaction gases are taken to a sampling cell located in the optical step of the atomic absorption spectrometer, in this case the products reaction compounds are volatile hydrides. These molecular compounds are not capable of giving an atomic absorption signal, therefore the cell is heated to dissociate the gaseous hydride into free atoms. When the gaseous hydride is dissociated in the heated cell into free atoms, the atomic absorption grows and it falls as the atoms are created and they escape from the absorption cell. The maximum absorption or peak height is measured as an analytical signal. The elements that can be determined with this technique are: As, Bi, Ge, Pb, Sb, Se, Te and Sn.

3.5.4. Cold vapor atomic absorption spectrometry; this method is another approach to improve the sensitivity of atomic absorption, optimizing the sampling efficiency in the premix burner, where the mercury is chemically reduced to free atomic state by reacting the sample with a strong reducer (stannous chloride or sodium borohydride) in a closed reaction container. Free volatile mercury is entrained from the reaction by bubbling air or nitrogen through the solution. Atoms of trailing mercury are transported to an absorption cell that is placed in the light path of the atomic absorption spectrometer. As mercury atoms go through the sampling cell, the measured absorbance increases, indicating the increase in concentration in the light path.

3.6. Spectroscopy, is an area of physics and chemistry dedicated to the study of the generation, measurement and interpretation of energy spectra (electromagnetic or particle) that results either from the emission or absorption of radiant energy or particles of a substance when bombarded with electromagnetic radiation, electrons, neutrons, protons, ions or by heating, excitation with a magnetic electric field, used to investigate nuclear and atomic structure.

3.7. Standard addition method, is the one that involves the preparation of standards in the sample matrix, adding known quantities of a standard to one or more aliquots of the sample and that compensates the effects of exaltation or depression of the analyte signal, but does not correct interference additives that cause a deviation from the baseline and in which the results obtained are valid if:
The analytical curve is linear.
The chemical form of the analyte is the same as in the sample.
The interference effect is constant in the working interval.
The signal is corrected for additive interference.

3.8. Quality control sample, is a sample external to the laboratory, which contains an aliquot of known concentration of the analyte, whose absorbance values must be within the linear range of the method.

3.9. Fortified sample, is a sample to which an aliquot of known concentration of the analyte, diluted in the appropriate acid in such a way that the resulting solution has an absorbance of about 0.1.

4. Symbols and abbreviations

When in this Standard reference is made to the following symbols and abbreviations is understood by:

As	arsenic
Bi	bismuth
Cd	cadmium
Cu	copper
Fe	iron
Ge	germanium
Hg	mercury
Pb	lead
Sb	antimony
Se	Selenium

Sn	tin
Te	tellurium
Zn	zinc
µmho	micromho
°C	degrees Celsius
%	percent
Lb	pound
G	gram
Cm	centimeter
Kg	kilogram
Mg	milligram
L	liter
ml	milliliter
µg	microgram
sec	second
rpm	revolutions per minute
nm	nanometer
AR	analytical reagent
N	normal
M	molar
w	weight
v	volume
/	per
DLI	detection limit of the instrument
DLM	detection limit of the method
QCS	quality control sample
No	number

5. Reagents and materials

5.1. Reagents

Certified standard reference solutions for each of the metals.

Water, must be distilled deionized, with a maximum conductivity of 1 µmho / cm at 25°C.

Nitric acid (specific density 1.41), extra pure grade.

Nitric acid (specific density 1.41), very low mercury content.

Perchloric acid (specific density 1.67), extra pure grade.

Hydrochloric acid (specific density 1.19), extra pure grade.

Sulfuric acid (specific density 1.84), extra pure grade.

1 N sulfuric acid from the extra pure grade solution.

Nitric acid 65% v / v grade AR.

Hydrogen peroxide (specific density 1.12).

Sodium hydroxide grade AR.

Clean and dry compressed air.

Gases: acetylene, nitrous oxide, argon and nitrogen, degree of atomic absorption.

7% w / v Magnesium Nitrate Hexahydrate Solution. Dissolve 70 g of $Mg(NO_3)_2.6H_2O$ in 1000 ml of 1N HCl.

1N hydrochloric acid. Dilute 8.3 ml of HCl and make up to 100 ml of water.

50% v / v nitric acid. Dilute 50 ml of 65% v / v HNO_3 extra pure grade in 50 ml of water.

8 M hydrochloric acid. Dilute 66.0 ml of HCl and make up to 100 ml with water.

Hydrochloric acid 0.5 N. Dilute 4.15 ml of HCl and make up to 100 ml with water.

Potassium Iodide Solution at 15% w / v. Dissolve 15 g of KI in 100 ml of water (This solution must be prepared at the time of use).

Potassium Iodide Solution at 20% w / v. Dissolve 20 g of KI in 100 ml of water (This solution must be prepared at the time of use).

Potassium Chloride Solution (10 mg / ml K). Dissolve 1.91 g of KCl in water and dilute to 100 ml with water.

50% w / v Magnesium Nitrate Solution. Dissolve 50 g of $Mg(NO_3)_2.6H_2O$ in 100 ml of water.

1.5% w / v hydrochloric acid solution. Dilute 1.5 ml of HCl in 100 ml of deionized distilled water.

1% w / v sodium hydroxide solution. Weigh 1 g of sodium hydroxide and dilute to 100 ml with deionized distilled water.

4% w / v sodium borohydride solution in sodium hydroxide solution at 1% w / v. Weigh 4 g of sodium borohydride into 100 ml of a solution of 1% w / v sodium hydroxide. Filter under vacuum.

Reducing solution for mercury. Mix 50 ml of concentrated sulfuric acid with approximately 300 ml of water. Cool to room temperature and dissolve 15 g of sodium chloride, 15 g of hydroxylamine sulfate or chloride and 25 g of stannous sulfate or chloride solution. Dilute to 500 ml.

Dilution solution for mercury. In a 1 l flask, containing 300 to 500 ml of deionized distilled water, add 58 ml of concentrated nitric acid (with very low mercury content) and 67 ml of concentrated sulfuric acid. Dilute to volume with water.

1 µg / ml As working solution. Dilute 1 ml of the 1000 standard solution µg / ml to 1 l with 1N sulfuric acid prepared from the extra pure grade solution. Prepare fresh every day.

5.2. Materials
500 ml and 800 ml Kjeldahl flasks.
Reflux system with refrigerant.
Vycor crucibles of 40 to 50 ml capacity.
Platinum crucibles of 40 to 50 ml capacity.
Erlenmeyer flasks of different capacities.
Volumetric flasks of different capacities.
50 ml flat bottom round flasks.
Parr pumps.
Eppendorf micropipettes or pipettes of different capacities.

Plastic tips for micropipettes.
Whatman No. 2 filter paper.
Boiling pearls.
Plastic rods.
15 ml graduated propylene or propylene test tubes.
Propylene or propylene containers.
Filtering funnels of different capacities.
Common laboratory equipment.

All the used material must be washed according to the following instructions.
The soap used should preferably be neutral.
Rinse perfectly under running water.
Immerse glass or plastic material in a container (preferably plastic) containing a 30% grade AR nitric acid solution.
Leave it covered and resting for a period of 24 hours.
Remove nitric acid excess several rinses (5 or 6 times) with deionized water.
Let drain and dry.
Store as soon as dry to avoid particle contamination in the air.

6. Apparatus and instruments

6.1. Appliances

Hollow cathode or discharge lamps without electrodes for determining arsenic, cadmium, copper, tin, iron, mercury, lead and zinc.
Radio frequency source in case of using discharge lamps.
Autosampler and water recirculator.
Heating plate with regulator that reaches a temperature of 400 to 450°C.
Microwave.
Autoclave that reaches 121 ± 5°C or 15 lb of pressure.
Laboratory centrifuge capable of maintaining 1600 rpm.

6.2. Instruments

The instruments listed below must be calibrated and adjusted before operation.

Atomic absorption spectrometer equipped with accessories for flame, graphite furnace, hydride or cold steam generator, depending on the method followed.
Analytical balance with sensitivity of 0.1 mg.
Muffle capable of maintaining a temperature of 550 ± 10°C.
Heating oven (stove) with temperature range of 120 ± 5°C.

7. Sample preparation

7.1. Digestion for the determination of Cd, Cu, Fe, Pb and Zn.

7.1.1. Wet digestion.

7.1.1.1. Weigh to the nearest ± 0.1 mg, an appropriate amount of sample.

For the determination by the flame absorption method, weigh a maximum of 40 g of juice or drink, 20 g of food containing 50 to 75% water and 10 g of solid or semi-solid food. Limit the fat or oil content to a maximum of 4 g and the total organic matter at 5 g.

7.1.1.2. Add 10 ml of concentrated nitric acid and let sit overnight or directly start digestion.

7.1.1.3. Use Kjeldhal flask or flask connected to the coolant system.

7.1.1.4. Heat gently.

7.1.1.5. Digest the sample 3 hours or longer if necessary (some samples require the addition of more nitric acid) until the appearance of translucent color, if amber remains, add hydrogen peroxide dropwise with continuous stirring (exothermic reaction).

7.1.1.6. Cool.

7.1.1.7. Retrieve, filter and bring to a known volume in a volumetric flask.

7.1.1.8. Run a reagent blank and fortified sample for each set of digestion.

7.1.1.9. Read on the apparatus of choice (atomic absorption spectrometer by flame or graphite furnace).

7.1.2. Dry digestion.

7.1.2.1. Weigh to the nearest ± 0.1 mg, an appropriate amount of sample.

For the determination by the flame absorption method, weigh a maximum of 40 g of juice or drink, 20 g of food containing 50 to 75% water and 10 g of solid and semi-solid foods. Limit the fat or oil content to a maximum of 4 g and the total organic matter at 5 g.

7.1.2.2. Add 10 ml of concentrated nitric acid and let sit overnight or directly start digestion. In products with a high concentration of protein add a 7.0% w/v magnesium nitrate solution and mix completely, dry for approximately 6 hours on the stove at a temperature of 90 to 95°C.

7.1.2.3. Place the sample in a flask and raise the temperature slowly from 2 to 4°C per minute up to 350°C. Maintain the temperature until the fumes cease.

7.1.2.4. Gradually raise the temperature from 500 to 550°C to prevent the sample is incinerated and maintain that temperature for 16 hours or all the night.

7.1.2.5. Turn off the flask and allow to cool.

7.1.2.6. A second calcination step may be required to remove some coal waste, using the following procedure:

Wash the walls of the crucible with 2 ml of 50% nitric acid. Place the sample on a heating plate set at 120°C to remove excess acid. Place the sample in a cold flask and raise the temperature gradually from 500 to 550°C, keeping it for the necessary time. Repeat this procedure as many times as necessary until free of remaining carbon.

7.1.2.7. Dissolve the ashes completely in 5 ml of 1N hydrochloric acid, transfer the dissolved sample to a propylene tube or to a flask with a known volume, rinse the crucible with twice 5 ml aliquots of 1N hydrochloric acid and transfer to the same tube or flask to obtain a volume of 15 ml in the first and take the capacity in the second, cover and mix, if there is presence of particles or insoluble matter, filter on Whatman No. 2 paper, before determination.

7.1.2.8. Run a reagent blank and fortified sample for each set of digestion.

7.1.2.9. Read on the apparatus of choice (atomic absorption spectrometer: flame or graphite furnace).

7.2. Wet digestion for the determination of Sn.

7.2.1. Proceed as in point 7.1.1.1.

7.2.2. Do not add nitric acid if total digestion is not carried out in the same day.

7.2.3. Add 30 ml of concentrated nitric acid to the flask and heat gently for 15 minutes under the hood to start digestion, avoiding an excessive foam production.

7.2.4. Gently boil until you have a remaining 3 to 6 ml or until the sample begins to dry at the bottom. Do not allow the sample to calcine.

7.2.5. Remove sample from heat.

7.2.6. At the same time run two reagent blanks.

7.2.7. Add 25 ml of concentrated hydrochloric acid, heat gently for about 15 minutes, until all the chlorine is released. Increase the temperature gradually until boiling.

7.2.8. Evaporate to obtain 10 to 15 ml, using a similar flask with 15 ml of water as a volume standard.

7.2.9. Add approximately 40 ml of water.

7.2.10. Shake and transfer to a 100 ml flask and rinse with 10 ml of water.

7.2.11. When hydrochloric acid is present in digestion, samples they can stay overnight or longer.

7.2.12. Add 1 ml of potassium chloride solution to each flask.

7.2.13. Cool down to room temperature.

7.2.14. Dilute with water and add more water to compensate for the volume of fat in the flask.

7.2.15. Mix perfectly and filter 30 to 50 ml through Whatman No. 2 paper and collect the filtrate in a propylene container, polypropylene or polyethylene.

7.2.16. Do not filter the blanks. Cap the bottles during analysis. The solutions are stable for several months.

7.2.17. Run a reagent blank and fortified sample for each set of digestion.

7.2.18. Read on the apparatus of choice (atomic absorption spectrometer: flame or graphite furnace).

7.3. Wet digestion for the determination of Hg.

7.3.1. Reflux system.

7.3.1.1. Weigh to the nearest ± 0.1 mg, the appropriate amount of sample, depending on the type of it, in a digestion flask and add pearls of boiling.

7.3.1.2. Connect the flask to the reflux system and gradually add the necessary amount of concentrated nitric acid and heat for half an hour or until changes in digestion are observed.

7.3.1.3. Let cool and add a mixture of nitric acid and sulfuric acid concentrates (1 + 1).

7.3.1.4. Heat and add more nitric acid drop by drop on the walls of the container, until the dark color of the solution disappears.

7.3.1.5. Cool.

7.3.1.6. If there is grease or wax, filter the solution.

7.3.1.7. Run a reagent blank and fortified sample for each set of digestion.

7.3.1.8. Read on the apparatus of choice (atomic absorption spectrometer of cold steam).

7.3.2. System closed.

7.3.2.1. Weigh to the nearest ± 0.1 mg, the appropriate amount of sample, depending on the type of it, in the digestion container.

7.3.2.2. Add the necessary amount of concentrated nitric acid.

7.3.2.3. Cover and perfectly seal the digestion container.

7.3.2.4. If the digestion container is an Erlenmeyer flask, place it in autoclave at 15 lbs for 30 minutes. If Parr pump is used, heat in grill controlling the temperature to a maximum of 300°C for 30 minutes.

7.3.2.5. Cool down to room temperature.

7.3.2.6. In case the digestion is not complete add peroxide of hydrogen and repeat digestion.

7.3.2.7. Filter in case of grease or wax and analyze the content of Hg.

7.3.2.8. Run a reagent blank and fortified sample for each set of digestion.

7.3.2.9. Read on the apparatus of choice (atomic absorption spectrometer of cold steam).

7.4. Digestion for the determination of As.

7.4.1. Wet-dry digestion.

7.4.1.1. Proceed as in point 7.3.2 until digestion is complete and then continue with the following steps.

7.4.1.2. Pipette an aliquot of the digested sample solution and place it in a Vycor crucible or beaker.

7.4.1.3. Add 1 ml of 7% w / v magnesium nitrate solution and heat in grill on low heat until dry.

7.4.1.4. Increase the plate heat to a maximum of 375°C.

7.4.1.5. Place the flask in the flask at 450°C to oxidize any residue of carbon and break down excess magnesium nitrate for at least 30 minutes.

7.4.1.6. Cool and dissolve the residue in 2.0 ml of 8M hydrochloric acid.

7.4.1.7. Add 0.1 ml of 20% w / v potassium iodide to reduce As(V) to As(III).

7.4.1.8. Let stand for longer than 2 minutes and transfer to a flask and take to the capacity with water.

7.4.1.9. Run a reagent blank and fortified sample for each set of digestion.

7.4.1.10. Read on the apparatus of choice (atomic absorption spectrometer with adaptation for graphite furnace or hydride generator).

7.4.2. Dry digestion.

7.4.2.1. Weigh accurately to ± 0.1 mg, the required amount of sample in a Vycor or platinum crucible.

7.4.2.2. Add the necessary volume of 50% w / v magnesium nitrate.

7.4.2.3. Homogenize with a clean plastic rod spreading the mixture in the crucible.

7.4.2.4. Place the sample in a muffle gradually raising the temperature up to 300°C for 2 hours. Then gradually raise the temperature to 500°C for 16 hours or overnight.

7.4.2.5. Cool to room temperature and moisten the ashes with 50% v / v nitric acid.

7.4.2.6. Heat on the grill until the acid is removed.

7.4.2.7. Bring the crucibles to a flask by gradually raising the temperature from 23 to 500°C, keeping it 30 min until total evaporation.

7.4.2.8. Transfer the ashes from the crucible to a volumetric flask using a 10 ml portion of 0.5 N hydrochloric acid.

7.4.2.9. Rinse the crucibles with 5 ml of distilled water and transfer to a flask, add 1 ml of 15% potassium iodide solution and mix.

7.4.2.10. Let it rest for 15 minutes and take to the capacity.

7.4.2.11. Run a reagent blank and fortified sample for each set of digestion.

7.4.2.12. Read on the apparatus of choice (atomic absorption spectrometer with adaptation for graphite furnace or hydride generator).

7.5. Digestion for the determination of Cd, As, Pb, Sn, Cu, Fe, Zn and Hg by microwave.

Weigh to the nearest ± 0.1 mg, maximum 0.500 g of sample, add 6 ml of concentrated nitric acid and 2 ml of 30% hydrogen peroxide, close perfectly the reaction container and proceed according to the manufacturer's manual.

7.6. Determination of metals in drinking water and purified water.

Colorless, transparent, odorless, single-phase samples can be analyzed directly by atomic absorption spectrometry, without digestion.

Prior to said analysis, add to 100 ml of sample, 1 ml of nitric acid. If a precipitate is observed, carry out a digestion adding 1 more ml of concentrated nitric acid, heat to 85°C until the volume is reduced to 20 ml taking care that it does not boil. Heat at reflux 30 minutes and transfer to a 50 ml volumetric flask. Spin at 1600 rpm for 30 minutes or leave rest overnight and analyze the supernatant.

8. Process

8.1. Atomic absorption spectrometry by flame.

8.1.1. Calibration. It is necessary to check that you have an initial calibration and periodically acceptable.

8.1.1.1. The operational configuration of the instrument and the system begins data acquisition. Allow a period of not less than 30 minutes for the heating of discharge lamps without electrodes.

8.1.1.2. Instrument stability should be verified by analysis of a standard solution 20 times more concentrated than the detection limit of the instrument (DLI) for the analyte, read a minimum of five times and calculate the resulting standard deviation, which must be less than 5%.

8.1.1.3. The instrument must be calibrated for the analyte to be determined using the calibration blank and calibration standards prepared at 3 or 4 concentration levels within the dynamic concentration range of the analyte.

8.1.1.4. Set the instrument to 0 with the calibration blank. Enter the analyte calibration standards from lowest to highest concentration and record at least three replicates of the absorbance of each.

8.1.1.5. Create a calibration curve by plotting absorbance as a function of concentration.

This can be carried out with computers that are programmed directly, in which only need to enter the standards and mark their theoretical concentration.

8.1.2. Instrument operation.

The performance of the instrument is verified by using targets of calibration, calibration standards and a quality control sample (QCS).

8.1.2.1. After the calibration has been performed, it should be verified that the instrument works properly for the analyte. For this purpose, a QCS is analysed. If measurements vary by ± 10% or more, as compared with established value for the QCS, the analysis should be interrupted and the possible cause of error should be investigated; the instrument should be recalibrated and the new calibration verified.

8.1.2.2. To verify that the instrument does not present drift, for every 10 analysis the calibration blank should be analyzed. If the true value of analyte differs ± 10% or more, the instrument must be recalibrated. If the mistake persists the problem should be identified and corrected.

If the sample matrix is responsible for the drift or affects the response of the analyte it may be necessary to work for standard additions.

8.1.2.3. The demonstration of the initial operation of the instrument is made setting the detection limits of the method (DLM) for the analyte and the linear calibration interval. A target of fortified reagents with an equivalent analyte concentration of 2 to 5 times the estimated detection limit. At least 4 read replicates are made of the absorbance of the fortified reagent blank processed through all the analytical method. DLMs are calculated according to:

DLM = t * s

t = Student's "t" value at a 99% confidence interval and an estimated standard deviation for n-1 degrees of freedom. t = 3.14 for 7 replicates.
s = standard deviation of the replicates of the analysis.

The linear calibration interval is established from at least 4 standards of different concentration, one of which must be close to the upper limit of the linear interval.

8.1.3. Determination

8.1.3.1. Adjust the atomic absorption instrument under the conditions suitable for the determination of the analyte according to the indications of the instrument manual.

8.1.3.2. Enter the reagent blank and the sample to analyze and record absorbance values. At least one reagent blank must be analyzed with each group of samples. The obtained values show the quality of the reagents used and the degree of contamination of the laboratory.

8.1.3.3. With computers that can be programmed, the obtained reading gives directly the concentration of the element in the concentration units used.

8.1.3.4. At least one fortified reagent blank should be tested for each sample group. Accuracy is calculated as percent recovery (according to section 8.1.3.6).

8.1.3.5. 10% per group with at least 1 sample, must be fortified. The added concentration should be approximately 0.1 absorbance units.

8.1.3.6. The percentage of recovery for the analyte should be calculated according to:

$$R = \frac{\mathrm{CM} - \mathrm{C}}{CA} * 100$$

R = % recovery
CM = Concentration of the fortified sample
C = Sample concentration
CA = Equivalent concentration of analyte added to the sample.

If the recovery of the analyte in the fortified sample is outside the previously set interval and the fortified reagent blank is correct, there may be a problem with the sample matrix.
Data should be verified by the standard addition method.

8.2. Graphite furnace atomic absorption spectrometry.

8.2.1. Calibration.

8.2.1.1. Proceed according to points 8.1.1.1 to 8.1.1.4.

8.2.1.2. Make a calibration curve by plotting peak area or maximum height against analyte concentration.

Calibration using a computer or calculator based on adjustment on response concentration data is accepted.

This can be carried out on computers that are programmed directly, in which only need to enter the standards and mark their theoretical concentration.

8.2.2. Instrument operation.

8.2.2.1. Proceed according to 8.1.2.1 to 8.1.2.3.

8.2.3. Determination.

8.2.3.1. Adjust the atomic absorption instrument under the conditions suitable for analyte determination, according to recommendations of the instrument manual.

The temperature program for the graphite furnace may vary depending on the sample matrix. In the case of non-specific interference (molecular absorption or light scattering), it is recommended to consult the existing bibliography regarding the methods available to eliminate them, as well as in the case of matrix interferences.

8.3. Atomic absorption spectrometry by hydride generator.

8.3.1. Calibration.

8.3.1.1. Proceed according to points 8.1.1.1 to 8.1.1.4.

8.3.1.2. Starting from the 1000 mg / l As standard solution, prepare a 1 mg/l As solution in hydrochloric acid of appropriate concentration for the method. Plot an absorbance calibration curve (maximum of the height of peak) as a function of analyte concentration for a range of concentration of 0 to 10 µg/l of As under the same conditions of the matrix of the sample.

8.3.2. Instrument operation.

8.3.2.1. Proceed according to points 8.1.2.1 to 8.1.2.3.

8.3.3. Determination.

8.3.3.1. Adjust the atomic absorption instrument under the conditions suitable for the determination of As: 193.7 nm wavelength and lamp discharge without electrodes. Position and adjust the absorption cell according to the manufacturer's manual. Adjust the gas flow (nitrogen or argon).

8.3.3.2. Adjust to 0 absorbance with 1.5% hydrochloric acid calibration blank following the instructions in the manufacturer's manual.

8.3.3.3. Optimize the instrument response with a calibration standard to the analyte (usually 10 ml of a 5 µg/l As solution gives an absorbance of 0.2), adjusting the purge time I, the reaction time and the purge time II.

8.3.3.4. Take a known volume of the directed sample and follow the same procedure than with calibration standards.

8.4. Cold vapor atomic absorption spectrometry.

8.4.1. Calibration.

8.4.1.1. Proceed as in points 8.1.1.1 to 8.1.1.4.

8.4.1.2. From the working solution of 1 µg/ml prepare standards of Calibration containing 0, 0.2, 0.4, 0.6, 0.8 and 1.0 µg of Hg to vials of reaction. To each bottle add 100 ml of the dilution solution and 20 ml of the reduction solution. Plot the absorbance calibration curve (height peak temperature) as a function of analyte concentration.

8.4.2. Instrument operation.

8.4.2.1. Proceed according to points 8.1.2.1 to 8.1.2.3.

8.4.3. Determination.

8.4.3.1. Adjust the atomic absorption instrument under the conditions suitable for the determination of Hg: wavelength of 253.6 nm, slit 0.7 nm and hollow cathode lamp. Fit and adjust the absorption cell according to the manufacturer's manual. Adjust the gas flow (nitrogen or argon).

8.4.3.2. Adjust to 0 absorbance with the calibration blank (dilution and reduction) following the instructions in the manufacturer's manual.

8.4.3.3. Optimize the instrument response with a calibration standard to the analyte.

8.4.3.4. Take 25 ml of the digested sample and follow the same procedure as with calibration standards.

9. Expression of results

Calculation method.
Interpolate the absorbance or peak height values of the analyzed sample on the calibration curve and obtain the mg / kg of the element in the sample and perform the calculations using the following formula:

$$mg/kg = \frac{\mathrm{A} * \mathrm{B}}{C}$$

Where:

A = Concentration in mg / kg of the sample to be interpolated on the calibration.
B = Final volume to which the sample was taken (ml).
C = Sample weight (g) or sample volume (ml) in the case of water.

With computers that can be programmed, the reading obtained gives directly the element concentration in mg/kg or µg/kg.

10. Test report

The results will be reported in mg / kg or µg / kg of the element to be determined.

11. Conformity with international standards

This Official Mexican Standard is not equivalent to any international standard.

12. Bibliography

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14. Compliance with the Standard

The supervision of compliance with this Standard corresponds to the Secretariat of health.

15. Validity

This Official Mexican Standard will enter into force as mandatory 30 days after its publication in the Journal Federation Officer.

Effective suffrage. No Re-election.

Mexico City, June 29, 1995 - The General Director, José Meljem Moctezuma - Rubric.

Appendix 10: Results of the TOXNET Search

The following information was generated from the Toxicology Bibliographic Information (TOXLINE), a database of the National Library of Medicine's TOXNET system (http://toxnet.nlm.nih.gov) on November 28, 2018.

Query: The word maltogenic (All Fields).

Singular and plural forms were searched.

The chemical name amylase was identified.

The following terms were added from ChemIDplus:

mylase 100

amylases

diastase jan

diastase

CAS Registry Number: 9000-92-4

Molecular and enzymatic characterization of a maltogenic amylase that hydrolyzes and transglycosylates acarbose.

Cha HJ; Yoon HG; Kim YW; Lee HS; Kim JW; Kweon KS; Oh BH; Park KH

Eur J Biochem. 1998, Apr 01; 253(1):251-62. [European journal of biochemistry] [PubMed]PubMed Citation

Enhanced maltose production through mutagenesis of acceptor binding subsite +2 in Bacillus stearothermophilus maltogenic amylase.

Sun Y; Duan X; Wang L; Wu J

J Biotechnol. 2016, Jan 10; 217:53-61. [Journal of biotechnology] [PubMed]PubMed Citation

Structural elements of thermostability in the maltogenic amylase of Geobacillus thermoleovorans.

Mehta D; Satyanarayana T

Int J Biol Macromol. 2015, Aug; 79:570-6. [International journal of biological macromolecules] [PubMed]PubMed Citation

Thermostability improvement of maltogenic amylase MAUS149 by error prone PCR.

Ben Mabrouk S; Ayadi DZ; Ben Hlima H; Bejar S

J Biotechnol. 2013, Dec; 168(4):601-6. [Journal of biotechnology] [PubMed]PubMed Citation

Dimerization mediates thermo-adaptation, substrate affinity and transglycosylation in a highly thermostable maltogenic amylase of Geobacillus thermoleovorans.

Mehta D; Satyanarayana T

PLoS One. 2013; 8(9):e73612. [PloS one] [PubMed]PubMed Citation

Directed evolution of a maltogenic alpha-amylase from Bacillus sp. TS-25.

Jones A; Lamsa M; Frandsen TP; Spendler T; Harris P; Sloma A; Xu F; Nielsen JB; Cherry JR

J Biotechnol. 2008, Apr 30; 134(3-4):325-33. [Journal [PubMed]PubMed Citation

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J Microbiol Biotechnol. 2008, Aug; 18(8):1401-7. [Journal of microbiology and biotechnology] [PubMed]PubMed Citation

Maltogenic amylase

Anonymous

WHO Food Additives SeriesVol:40 (1998) pp 91-100 [RISKLINE]

Antioxidative effects of glycosyl-ascorbic acids synthesized by maltogenic amylase to reduce lipid oxidation and volatiles production in cooked chicken meat.

Lee SB; Nam KC; Lee SJ; Lee JH; Inouye K; Park KH

Biosci Biotechnol Biochem. 2004, Jan; 68(1):36-43. [Bioscience, biotechnology, and biochemistry] [PubMed]PubMed Citation

L-glutamate enhances the expression of Thermus maltogenic amylase in Escherichia coli.

Jung HM; Park KH; Kim SY; Lee JK

Biotechnol Prog. 2004 Jan-Feb; 20(1):26-31. [Biote [PubMed]PubMed Citation

Changes in the catalytic properties and substrate specificity of Bacillus sp. US149 maltogenic amylase by mutagenesis of residue 46.

Ben Mabrouk S; Ayadi-Zouari D; Ben Hlima H; Bejar S

J Ind Microbiol Biotechnol. 2013, Sep; 40(9):947-53. [Journal of industrial microbiology & biotechnology] [PubMed]PubMed Citation

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Kim YW; Choi JH; Kim JW; Park C; Kim JW; Cha H; Lee SB; Oh BH; Moon TW; Park KH

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World Health Organ Tech Rep Ser. 1999; 884:i-viii, 1-96. [World Health Organization technical report series] [PubMed]PubMed Citation

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DETERMINING THE SAFETY OF MALTOGENIC AMYLASE PRODUCED BY RECOMBINANT DNA TECHNOLOGY

ANDERSEN JR; DIDERICHSEN BK; HJORTKJAER RK; DE BOER AS; BOOTMAN J; WEST H; ASHBY R

J FOOD PROT; 50 (6). 1987. 521-526. [BIOSIS]

Branched oligosaccharides concentrated by yeast fermentation and effectiveness as a low sweetness humectant.

YOO S-H; KWEON M-R; KIM M-J; AUH J-H; JUNG D-S; KIM J-R; YOOK C; KIM J-W; PARK K-H

JOURNAL OF FOOD SCIENCE; 60 (3). 1995. 516-519. [BIOSIS]

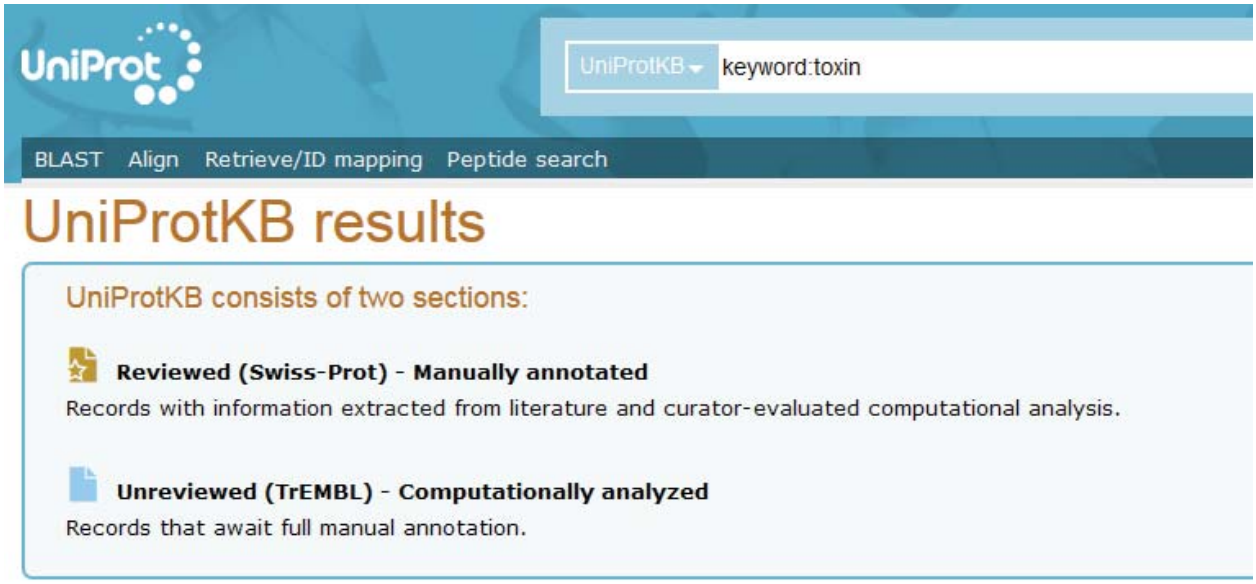
Safety evaluation of certain food additives and contamiants. Maltogenic amylase

Anonymous

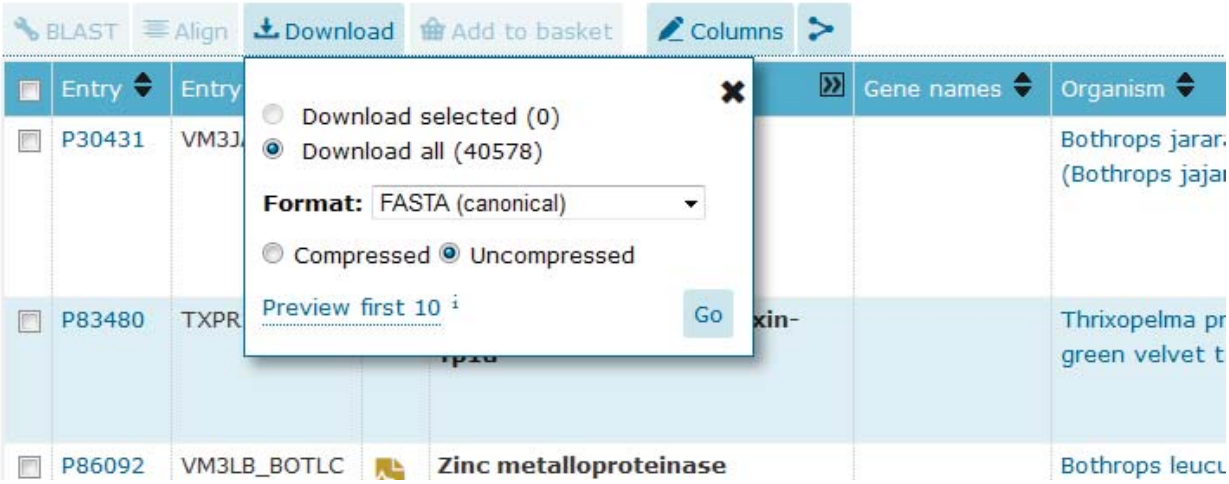
WHO Food Additives SeriesVol:40 (1998) pp 91-100 [RISKLINE]

Appendix 11: Step-by-step process for toxin search

1. In Uniprot (https://www.uniprot.org/) search UniProtKB with the terms "keyword:toxin". You will get results from Swiss-Prot and TrEMBL databases.

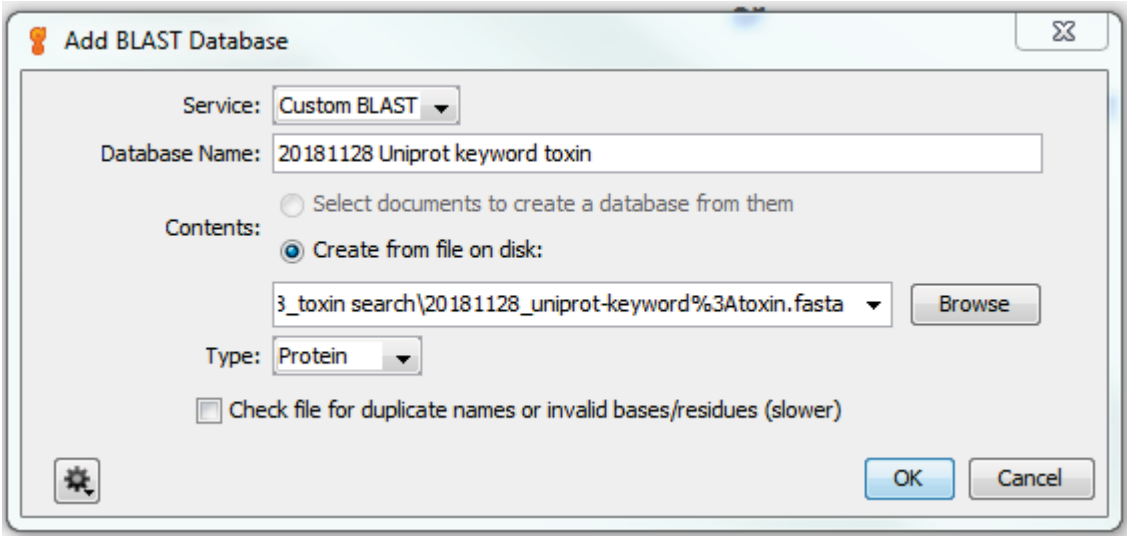


2. Download the resulting list as an uncompressed file. Save the file in a location where you can easily find it. It might help to note the data of download in the file name so that it's easy to remember when the list was downloaded (this is important because the Uniprot toxin database might change over time). In any case you will want to note the date of download for your search writeup.

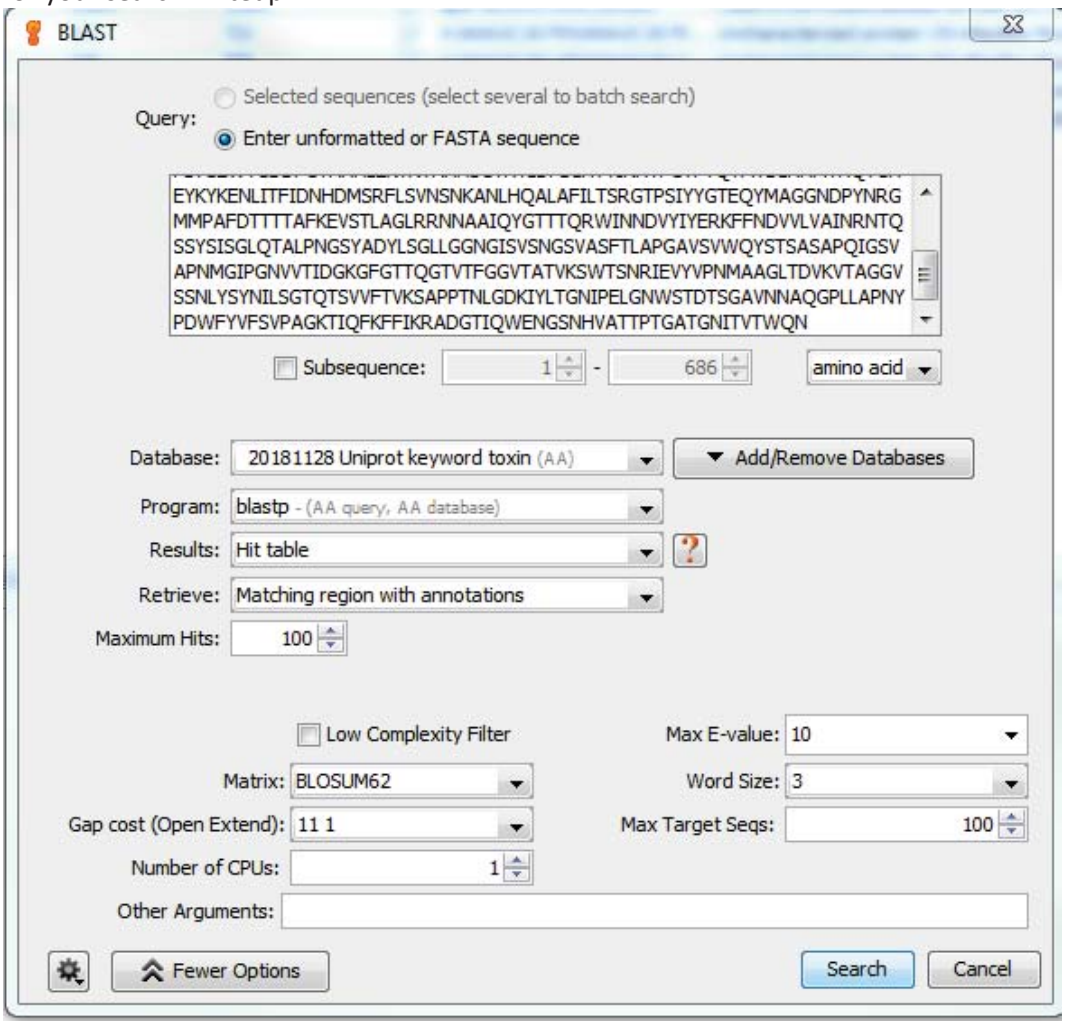


3. Open Geneious and make sure you have the Custom BLAST service: Tools→Add/Remove Databases→Set up BLAST Services.

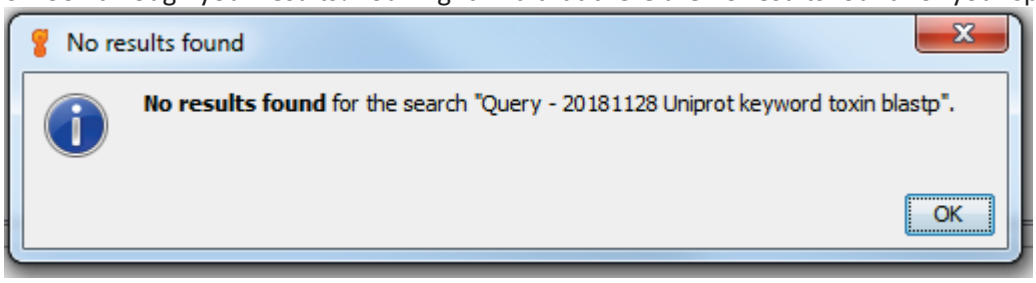
4. Make a custom database in Geneious with Tools→Add/Remove Databases→Add Sequence Database. Select service Custom BLAST, give the database a name, and create it from your saved .fasta file from step 2 above. Select type Protein.



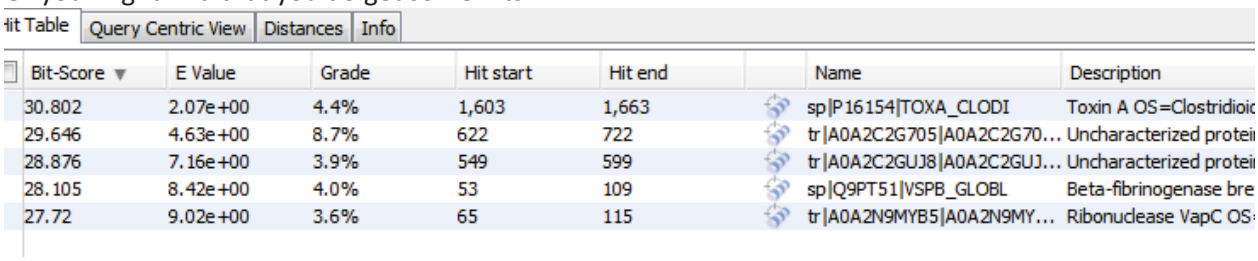
5. Perform the BLAST search in Geneious by clicking BLAST in the top bar. In the window that pops up, enter the protein sequence of interest, the custom database to search against, blastp for program, and make any adjustments or filters desired. For example, you could set the max E-value for a "hit" to a certain level (if you do this, you might want to run the BLAST with a few different E-value levels). Note the search settings for your search writeup.



6. Look through your results! You might find that there are no results found for your specific query:

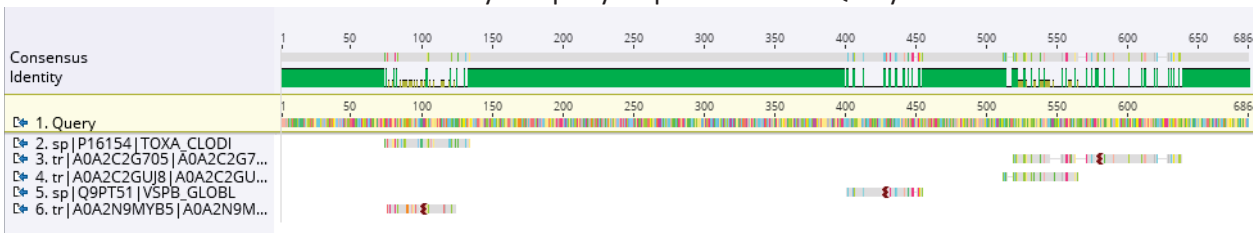


Or you might find that you do get some hits:



Bit-Score	E Value	Grade	Hit start	Hit end	Name	Description
30.802	2.07e+00	4.4%	1,603	1,663	sp|P16154|TOXA_CLODI	Toxin A OS=Clostridioic
29.646	4.63e+00	8.7%	622	722	tr|A0A2C2G705|A0A2C2G70...	Uncharacterized protei
28.876	7.16e+00	3.9%	549	599	tr|A0A2C2GUJ8|A0A2C2GUJ...	Uncharacterized protei
28.105	8.42e+00	4.0%	53	109	sp|Q9PT51|VSPB_GLOBL	Beta-fibrinogenase bre
27.72	9.02e+00	3.6%	65	115	tr|A0A2N9MYB5|A0A2N9MY...	Ribonuclease VapC OS

You can look at the how the hits match your query sequence in the "Query Centric View" tab:



7. Write up your results! Include the date you searched the Uniprot database, the BLAST settings you used, etc.

For example:
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.

Appendix 12: FDA No Question Letter

[redacted]

Dear [redacted]

The Food and Drug Administration (FDA, we) has completed its evaluation of GRN 000842. We received Mascoma LLC's (Mascoma)'s GRAS notice, dated January 15, 2019, on February 6, 2019, and filed it on March 29, 2019.

The subject of the notice is "genetically engineered maltogenic alpha-amylase enzyme produced by *Saccharomyces cerevisiae* carrying a maltogenic alpha-amylase gene from *Geobacillus stearothermophilus*" (maltogenic alpha-amylase enzyme preparation) as an enzyme used at a maximum level of 164 mg Total Organic Solids (TOS)/kg in flour for baked goods. The notice informs us of Mascoma's view that this use of maltogenic alpha-amylase enzyme preparation is GRAS through scientific procedures.

Commercial enzyme preparations that are used in food processing contain the enzyme component that catalyzes the chemical reaction as well as substances used as stabilizers, preservatives, or diluents. Enzyme preparations may also contain components derived from the production organism and from the manufacturing process, e.g., constituents of the fermentation media or the residues of processing aids. Mascoma's notice provides information about the components in the maltogenic alpha-amylase enzyme preparation.

According to the classification system of enzymes established by the International Union of Biochemistry and Molecular Biology, maltogenic alpha-amylase is identified by the Chemical Abstracts Service number 160611-47-2 and Enzyme Commission Number 3.2.1.133.[1] Mascoma provides the amino acid sequence of its alpha-amylase in its notice.

Mascoma states that the *S. cerevisiae* production organism is non-pathogenic and non-toxigenic. Mascoma describes the construction of the production strain, which includes the insertion of an expression cassette containing a genetically engineered maltogenic

[redacted]

alpha-amylase gene[2] and the native *S. cerevisiae* promoters and terminators into the genome using targeted homologous recombination. Mascoma confirmed the integration of the gene using PCR analyses and phenotypic characterizations.[3] Mascoma used quantitative PCR measurement of gene copy number, qualitative PCR genotyping, and measurement of enzyme activity to confirm the stability of the integrated DNA. Mascoma states that the final production strain does not contain any antibiotic resistance genes, virulence factors, protein toxins, or enzymes involved in the synthesis of mycotoxins.

Mascoma states that maltogenic alpha-amylase enzyme preparation is manufactured starting with the fermentation of a pure culture of the *S. cerevisiae* production strain. Mascoma states that fermentation is carried out under controlled conditions and that the enzyme is produced intracellularly. After fermentation, the yeast cells are concentrated and washed *via* centrifugation. Mascoma states that the yeast cells are disrupted either by mechanical (bead mill, high-pressure homogenization, ultrasonication) or non-mechanical (physical, chemical and/or enzymatic) techniques. The broken cell suspension is either centrifuged or filtered to remove debris from the supernatant. The enzyme-containing supernatant is then concentrated and/or diafiltered to reach the desired enzyme activity. The final enzyme solution is polished, and germ filtered to remove any residual cell material and sterilize the final preparation. Finally, the enzyme is formulated with carriers such as salt, starch, or dextrin, and dried. Mascoma states that the entire process is performed using food-grade raw materials and in accordance with current good manufacturing practices. Mascoma also states that the final maltogenic alpha-amylase enzyme preparation does not contain any major food allergens from the fermentation media.

Mascoma has established food grade specifications and states that the maltogenic alpha-amylase enzyme preparation conforms to specifications established for enzyme preparations in the Food Chemicals Codex (FCC, 10th edition, 2016), and to the General Specifications and Considerations for Enzyme Preparations Used in Food Processing established by the FAO/WHO Joint Expert Committee on Food Additives (JECFA, 2006). Mascoma provides analytical data from three batches of maltogenic alpha-amylase enzyme preparation to demonstrate that the manufacturing acceptance criteria can be met, including the absence of the production strain.

Mascoma intends to use maltogenic alpha-amylase enzyme preparation at a maximum level of 164 mg TOS/kg in flour for baked goods. Mascoma notes that the maltogenic alpha-amylase enzyme preparation will be deactivated during the process of baking. In estimating dietary exposure, Mascoma assumes that all the maltogenic alpha-amylase enzyme preparation will remain in the final food. Mascoma estimated dietary exposure

[2] The synthetic maltogenic alpha-amylase gene is based on the publicly available Genbank sequence of the *Geobacillus stearothermophilus* and codon-optimized for *S. cerevisiae*, PCR amplified, and used in the construction of the production organism.
[3] Mascoma states that multiple copies for the maltogenic amylase gene are present in the production strain; the inserted DNA contains two copies of the maltogenic amylase gene and that the host strain has three copies of the chromosome into which the insert was integrated.

to maltogenic alpha-amylase enzyme preparation to be 0.614 mg TOS/kg body weight per day (kg bw/d) from the intended use.[4,5]

Mascoma discusses published information on the safety of the *S. cerevisiae* production organism, the safety of the donor organism *G. stearothermophilus*, the safety of microbial enzyme preparations used in food processing in general, and the safety of similar alpha-amylase enzyme preparations from other donor/production organisms.

Mascoma addresses the potential for oral allergenicity to maltogenic alpha-amylase enzyme using publicly available literature and the conclusions of several organizations and expert working groups. Further, based on bioinformatic analyses, Mascoma reports that the maltogenic alpha-amylase enzyme does not share any biologically meaningful sequence homology or sequence identity to potential oral allergens. Mascoma also states that bioinformatics searches to assess potential toxicity of maltogenic alpha-amylase enzyme did not produce any information that would indicate safety concerns. Based on the totality of the information available, Mascoma concludes that it is unlikely that oral consumption of maltogenic alpha-amylase enzyme preparations from the intended use will result in allergenic or toxic responses.

Based on the data and information summarized above, Mascoma concludes that maltogenic alpha-amylase enzyme preparation is GRAS for its intended use.

Standards of Identity

In the notice, Mascoma states its intention to use maltogenic alpha-amylase enzyme preparation in several food categories, including foods for which standards of identity exist, located in Title 21 of the Code of Federal Regulations. We note that an ingredient that is lawfully added to food products may be used in a standardized food only if it is permitted by the applicable standard of identity.

Section 301(ll) of the Federal Food, Drug, and Cosmetic Act (FD&C Act)

Section 301(ll) of the FD&C Act prohibits the introduction or delivery for introduction into interstate commerce of any food that contains a drug approved under section 505 of the FD&C Act, a biological product licensed under section 351 of the Public Health Service Act, or a drug or a biological product for which substantial clinical investigations have been instituted and their existence made public, unless one of the exemptions in section 301(ll)(1)-(4) applies. In our evaluation of Mascoma's notice concluding that maltogenic alpha-amylase enzyme preparation is GRAS under its intended conditions of use, we did not consider whether section 301(ll) or any of its exemptions apply to foods

[4] Mascoma uses a budget method to calculate estimated dietary exposure to maltogenic alpha-amylase preparation based on an average consumption of 25 g of solid foods/kg bw/day, over a lifetime. Mascoma assumes that 50% of all solid foods (i.e., 12.5 g/kg bw/d) will be baked goods and will contain maltogenic alpha-amylase at the maximum intended use level.

[5] Mascoma estimates the dietary exposure to maltogenic amylase enzyme preparation to be 0.61 mg TOS/kg bw/d based on the maximum use level. FDA estimates the dietary exposure to maltogenic amylase enzyme preparation to be 0.69 mg TOS/kg bw/d based on its consumption level in the final food.

containing maltogenic alpha-amylase enzyme preparation. Accordingly, our response should not be construed to be a statement that foods containing maltogenic alpha-amylase enzyme preparation, if introduced or delivered for introduction into interstate commerce, would not violate section 301(ll).

Conclusions

Based on the information that Mascoma provided, as well as other information available to FDA, we have no questions at this time regarding Mascoma's conclusion that maltogenic alpha-amylase enzyme preparation produced by maltogenic alpha-amylase enzyme preparation produced by *Saccharomyces cerevisiae* carrying a maltogenic alpha-amylase gene from *Geobacillus stearothermophilus* is GRAS under its intended conditions of use. This letter is not an affirmation that maltogenic alpha-amylase enzyme preparation produced by maltogenic alpha-amylase enzyme preparation produced by *Saccharomyces cerevisiae* carrying a maltogenic alpha-amylase gene from *Geobacillus stearothermophilus* is GRAS under 21 CFR 170.35. Unless noted above, our review did not address other provisions of the FD&C Act. Food ingredient manufacturers and food producers are responsible for ensuring that marketed products are safe and compliant with all applicable legal and regulatory requirements.

In accordance with 21 CFR 170.275(b)(2), the text of this letter responding to GRN 000842 is accessible to the public at www.fda.gov/grasnoticeinventory.

[redacted]

Appendix 13: Whole Genome Sequencing Analysis Report (CONFIDENTIAL)

Appendix 13 is provided in a separate document.

Appendix 14: Absence of GMO DNA (CONFIDENTIAL)

Appendix 14 is provided in a separate document.

Appendix 15: Genetic Stability of the Source Organism (CONFIDENTIAL)

Appendix 15 is provided in a separate document.

Appendix 16: Genetic Modification of the Source Organism (CONFIDENTIAL)

Appendix 16 is provided in a separate document.

Annexes

The following annexes are provided separately.

Annex 1: 20181128_uniprot-keyword%3Atoxin.fasta

Annex 2: M17906 WGS phylogenetic and AMR analysis - v2020-03-09 (CONFIDENTIAL)

Annex 3: Toxicity data summary for Maltogenic amylase from G. stearothermophilus produced in Bacillus subtilis BRG-1 (GRN 751)

Annex 4: Toxicity report for Maltogenic amylase from G. stearothermophilus produced in Bacillus subtilis RF12029-EL 2009083 (GRN 746)