



August 5, 2020

RE: GRAS Notification – Exemption Claim

Dear Sir or Madam:

Pursuant to the proposed 21C.F.R. § 170.36 (c)(1) AB Enzymes GmbH hereby claims that Maltogenic Amylase (IUBM 3.2.1.133) from a Genetically Modified *Bacillus subtilis* produced by submerged fermentation is Generally Recognized as Safe; therefore, they are exempt from statutory premarket approval requirements.

The following information is provided in accordance with the proposed regulation:

Proposed 21C.F.R. § 170.36 (c)(i) *The name and address of notifier.*

AB Enzymes Inc.¹
8211 W. Broward Blvd. Suite 375
Plantation, FL 33324 USA

Proposed 21C.F.R. § 170.36 (c)(ii) *The common or usual name of notified substance:*

Maltogenic Amylase (IUBMB 3.2.1.133) from a Genetically Modified *Bacillus subtilis*.

Proposed 21C.F.R. § 170.36 (c)(iii) *Applicable conditions of use:*

The maltogenic amylase is to be used in baking processes. The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements under current Good Manufacturing Practices. There are no maximal limits set, just suggested dosages.

Proposed 21C.F.R. § 170.36 (c)(iv) *Basis for GRAS determination:*

This GRAS determination is based upon scientific procedures.

Proposed 21C.F.R. § 170.36 (c)(v) *Availability of information:*

A notification package providing a summary of the information which supports this GRAS determination is enclosed with this letter. The package includes a safety evaluation of the production strain, the enzyme, and the manufacturing process, as well as an evaluation of dietary exposure. Complete data and information that are the basis for this GRAS determination are available to the Food and Drug Administration for review and copying at reasonable times (customary business hours) at a specific address set out in the notice or will be sent to FDA upon request (electronic format or on paper).

§170.225(c)(8) - FOIA (Freedom of Information Act):

¹ AB Enzymes Inc. is the North America Division of AB Enzymes GmbH based in Plantation, Florida USA



Parts 2 through 7 of this notification do not contain data or information that is exempt from disclosure under the FOIA (Freedom of Information Act)

§170.225(c)(9) – Information included in the GRAS notification:

To the best of our knowledge, the information contained in this GRAS notification is complete, representative and balanced. It contains both favorable and unfavorable information, known to AB Enzymes and pertinent to the evaluation of the safety and GRAS status of the use of this substance.

Sincerely,

AB Enzymes GmbH



-Aug-2020 | 1 :

i.V. Candice Cryne

Regulatory Affairs Manager



-Aug-2020 | 1 :

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Junior Regulatory Affairs Specialist

GRAS Notification of a Maltogenic Amylase from a Genetically Modified *Bacillus subtilis*

AB ENZYMES GmbH

August 5th, 2020

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1 PART 1 §170.225 – SIGNED STATEMENTS AND CERTIFICATIONS

§170.225(c)(1) – Submission of GRAS notice:

In conformity with the established regulation 21 C.F.R. Section 170, subsection E, AB Enzymes GmbH hereby claims that Maltogenic Amylase (IUBM 3.2.1.133) from a genetically modified *Bacillus subtilis* produced by submerged fermentation is Generally Recognized as Safe; therefore, they are exempt from statutory premarket approval requirements.

§170.225(c)(2) -The name and address of the notifier:

AB Enzymes Inc.¹
8211 W. Broward Blvd. Suite 375
Plantation, FL 33324 USA

§170.225(c)(3) – Appropriately descriptive term:

Maltogenic amylase (IUBM 3.2.1.133) from a Genetically Modified *Bacillus subtilis*

§170.225(b) – Trade secret or confidential:

This notification does not contain any trade secret or confidential information.

§170.225(c)(4) – Intended conditions of use:

The maltogenic amylase is to be used in baking processes. The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements under current Good Manufacturing Practices. There are no maximal limits set, just suggested dosages.

§170.225(c)(5) -Statutory basis for GRAS conclusion:

This GRAS determination is based upon scientific procedures.

§170.225(c)(6) – Premarket approval:

The notified substance is not subject to the premarket approval requirements of the FD&C Act based on our conclusion that the substance is GRAS under the conditions of the intended use.

Proposed 21C.F.R. § 170.36 (c)(v) Availability of information:

A notification package providing a summary of the information which supports this GRAS determination is enclosed with this letter. The package includes a safety evaluation of the production strain, the enzyme, and the manufacturing process, as well as an evaluation of dietary exposure. Complete data and information that are the basis for this GRAS determination are available to the Food and Drug Administration for review and copying at reasonable times

¹ AB Enzymes Inc. is the North America Division of AB Enzymes GmbH (Darmstadt, Germany) based in Plantation, Florida USA



(customary business hours) at a specific address set out in the notice or will be sent to FDA upon request (electronic format or on paper).

§170.225(c)(8) - FOIA (Freedom of Information Act):

Parts 2 through 7 of this notification does not contain data or information that is exempt from disclosure under the FOIA (Freedom of Information Act).

§170.225(c)(9) – Information included in the GRAS notification:

To the best of our knowledge, the information contained in this GRAS notification is complete, representative and balanced. It contains both favorable and unfavorable information, known to AB Enzymes and pertinent to the evaluation of the safety and GRAS status of the use of this substance.

2 PART 2 §170.230 - IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS AND PHYSICAL OR TECHNICAL EFFECT OF THE NOTIFIED SUBSTANCE

2.1 Identity of the notified substance

The dossier concerns a **maltogenic amylase from a genetically modified *Bacillus subtilis***.

2.1.1 Common name of the enzyme

Name of the enzyme protein: Maltogenic amylase

Synonyms: maltogenic alpha-amylase
1,4-alpha-D-glucan alpha-maltohydrolase, glucan-1,4-alpha-maltohydrolase.

2.1.2 Classification of the enzyme

IUBMB #	3.2.1.133
Production Strain	<i>Bacillus subtilis</i> RF13018

The classification of the enzyme according to the IUBMB is as follows:

EC 3. is for hydrolases;

EC 3.2. is for glycosylases;

EC 3.2.1. is for glycosidases, i.e. enzymes hydrolyzing *O-* and *S-*glycosyl compounds;

EC 3.2.1.133 is for glucan 1,4-alpha-maltohydrolase.

2.2 Identity of the Source

2.2.1 Recipient Strain

The recipient strain used in the genetic modification for the construction of the production strain is a genetically modified derivative of a classical *Bacillus subtilis* mutant strain. This strain has been shown to be genetically stable for industrial production.

The original *Bacillus subtilis*, which has been isolated in the year 1984 by the University of Osaka from soil, was characterized as *Bacillus subtilis* by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). Which was further developed by conventional mutagenesis for better yield and the resulting mutant has been used in AB Enzymes since 2010 for the production of maltogenic amylase for food processing.

The identity of both the mutant parental strain and the genetically modified recipient strain was confirmed by Ribotyping in the years 2009 and 2016 respectively, by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) and both strains were classified as *Bacillus subtilis*.

Ribotyping is a method that can identify and classify bacteria based upon differences in rRNA. It generates a highly reproducible and precise fingerprint that can be used to classify bacteria from the genus through and beyond the species level. DNA is extracted from a colony of bacteria and then restricted into discrete-sized fragments. The DNA is then transferred to a membrane and probed with a region of the rRNA operon to reveal the pattern of rRNA genes. The pattern is recorded, digitized and stored in a database. The variations that exist among bacteria in both the position and intensity of generated rRNA bands can be used for classification and identification of bacteria.

Standardized, automated ribotyping was performed by DSMZ using the Qualicon™ RiboPrinter system. The RiboPrinter system combines molecular processing steps of ribotyping in a stand-alone, automated instrument. Steps include cell lysis, digestion of chromosomal DNA with restriction enzymes (kits for EcoRI and PvuII), separation of fragments by electrophoresis, transfer of DNA fragments to a nylon membrane, hybridization to a probe generated from the *rrnB* operon

from *E. coli*, chemiluminescent detection of the probe to the fragments containing *rrnB* operon sequences, image detection and computerized analysis of RiboPrint patterns (Bruce et al. 1995).

For further development, genetic modifications were introduced into the mutant parental strain (see steps 1-5 described in [section 2.3](#)) to improve strain and production performance, resulting in the current recipient strain used for the construction of the maltogenic amylase production strain RF13018.

Both the mutant parental strain and the genetically modified recipient strains were identified by DSMZ by using the DuPont Identification Library with a similarity to DuPont ID DUP-12544 (*Bacillus subtilis*) of 1.00.

Therefore, the recipient can be described as followed:

Genus:	<i>Bacillus</i>
Species:	<i>Bacillus subtilis</i>
Subspecies (if appropriate):	Not applicable
Commercial name:	Not applicable. The organism is not sold as such.

2.2.2 Donor:

The maltogenic amylase gene described in this application derives from *Bacillus stearothermophilus* also referred as *Geobacillus stearothermophilus*.

The *Geobacillus stearothermophilus* maltogenic α -amylase gene with a promoter and a signal sequence from *B. amyloliquefaciens* and a transcription terminator from *Thermoactinomyces vulgaris* 94-2A (Palva et al. 1981; Hofemeister et al. 1994) has been inserted into the vector backbone by restriction and ligation and by isothermal assembly techniques.

Other DNA fragments included in the expression cassette of the maltogenic amylase

The signal sequence and the **promoter** are from *Bacillus amyloliquefaciens* (Palva et al. 1981; Hofemeister et al. 1994).

Genus: *Bacillus*
Group: *Bacillus subtilis*
Species or Subspecies: *Bacillus amyloliquefaciens*

The transcription terminator [T(*amyTV*)] is from *Thermoactinomyces vulgaris* 94-2A (Hofemeister et al. 1994).

Genus: *Thermoactinomyces*
Species: *Thermoactinomyces vulgaris*

Furthermore, a native hydrolase from a *Bacillus amyloliquefaciens* AB Enzymes strain has been inserted upstream of the maltogenic alpha-amylase expression cassette. The hydrolase gene was amplified using the genomic DNA from the *Bacillus amyloliquefaciens* AB Enzymes strain with decades of safe use in production in the food industry.

The synthetic maltogenic amylase gene was inserted into a plasmid consisting of backbone elements from well-known and defined cloning *Bacillus* vectors (Kreft et al. 1978) and pUB110 (McKenzie et al. 1986, 1987). The resulting expression plasmid pMA-A001 is devoid of any transfer function (required for plasmid mobilization) or any sequence conferring antibiotic resistance. Vector selection is achieved using episomal complementation of an auxotrophy introduced into the parental strain.

The plasmid pMA-A001 contains no genes conferring antibiotic resistance.

Synthetic DNA

Maltogenic amylase gene: The maltogenic alpha-amylase gene was synthesised based on the sequence published by (Diderichsen and Christiansen 1988) and in the patent US 6,162, 628 and has been further protein engineered. The maltogenic alpha-amylase has >99% identity to the maltogenic alpha amylase from *Geobacillus stearothermophilus* and shows the same general functionality with improved properties in application.

2.3 Genetic modification

The *Bacillus subtilis* strain RF13018 was constructed for maltogenic amylase gene production. The genetically engineered *Bacillus subtilis* recipient strain (s.b.) was transformed with the plasmid pMA-A001 carrying the synthetic gene encoding the maltogenic amylase and a native hydrolase from *Bacillus amyloliquefaciens*. The maltogenic amylase has >99% identity to the maltogenic alpha amylase from *Geobacillus stearothermophilus*. The plasmid pMA-A001 contains no genes conferring antibiotic resistance.

At AB Enzymes, *Bacillus subtilis* strains have been used and developed for a long period of time, in the production of various enzymes, including maltogenic amylase. Also, other enzymes have been developed to be used in food industrial applications. The reason for the genetic modification of the microorganism was to produce greater quantities of the desired enzyme. The resulting production strain RF13018 secretes high amounts of maltogenic amylase into its culture supernatant, resulting in high maltogenic amylase activity in the cultivation broth.

The strain **RF13018** was constructed in six genetic modification steps:

STEP 1-5: Markerless deletions in the genome of the parental strain:

The *B. subtilis* recipient strain was constructed according to the well described methods for markerless deletions in the genome of *Bacillus* species (Vehmaanperä et al. 1991; Lordănescu 1975; Rachinger et al. 2013) to arrive at a host strain with improved production performance and an

intended auxotrophy for vector selection. In addition, the resulting strain had lost its ability to sporulate. The deletion vectors constructed for this purpose were only used for targeted and markerless deletion of native genes and are not present anymore in the final recipient strain. The deletions of the native genes from the genome of the original *Bacillus subtilis* mutant (i.e. parental strain of the recipient) were carefully monitored by PCR and sequencing. It was verified that no DNA-fragments of the deletion vectors remained in the cell.

STEP 6: Construction of production strain RF13018 - Introduction of pMA-A001 into the *Bacillus subtilis* recipient strain:

In the sixth and final step, plasmid pMA-A001 containing the expression cassette for the maltogenic amylase was introduced into the recipient strain by protoplast transformation according to the method of Chang and Cohen (1979). Transformants were plated on appropriate agar plates for selection of pMA-A001 carrying cells being able to complement the host's auxotrophy.

The production strain RF13018 is deposited in the "Centraalbureau voor Schimmelcultures" (CBS) in the Netherlands with the deposit number CBS145947.

2.3.1 Genetic stability of the production strain

When implemented, the fermentation process always starts from identical replicas of the RF13018 (production strain) seed ampoule. Production preserves from the "Working Cell Bank" are used to start the fermentation process. A Working Cell Bank is a collection of ampoules containing a pure culture. The cell line history and the production of a Cell Bank, propagation, preservation and storage is monitored and controlled. The WCB is prepared from a selected strain. A WCB ampoule 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 WCB ampoule. The accepted WCB ampoule is used as seed material for the inoculum.

The production starts from “Working Cell Bank” preserves. A Petri dish is inoculated from the culture collection preserve in such a way that single colonies can be selected. Altogether individual colonies are picked up from plates and inoculated into shake flasks. Care is taken to select only those colonies which present the familiar picture (same phenotype). Colonies are used for inoculating 2 rounds of shake flask cultivation. Subsequently these are combined for the inoculation of the first process bioreactor.

Testimony to the stability of the strain is given by monitoring the growth behavior and by comparable levels of maltogenic amylase activity in a number of fermentation batches performed for the RF13018 strain. The activity measurements from parallel fermentations showed that the productivity of the RF13018 strain remains similar. This clearly indicates that the strain is stable. The data of the analysis of enzyme activities from preparation from different fermentation batches of the recombinant RF13018 strain is presented in [Appendix # 1](#).

2.3.2 Structure and stability of vector and/or nucleic acid remaining in the GMM

The vector pMA-A001 consists of:

- Defined elements derived from plasmids pBC16-1 (Kreft et al. 1978) and pUB110 (Gryczan et al. 1978).
- pUB110 was isolated the first time by Gryczan *et al.* in 1978. Ever since it has been used worldwide for the cloning in *Bacilli*. pUB110 is known to be able to be stably maintained in *B. subtilis*, but also in *B. stearothermophilus*, *B. licheniformis*, *B. megaterium* and *B. pumilus* (Nugent 1989).
- The *Geobacillus stearothermophilus* maltogenic α -amylase gene coding for the mature protein was inserted in an expression cassette composed of promoter and signal sequence from *B. amyloliquefaciens* and transcription terminator from *Thermoactinomyces vulgaris* 94-2A (Palva et al. 1981; Hofemeister et al. 1994).
- A native hydrolase derived from *Bacillus amyloliquefaciens*.

- The gene from the parental recipient strain *B. subtilis* complementing the host's auxotrophy which was formerly introduced by deleting this gene from the recipient's strain genome (as described above).

pBC16-1 and pUB110 can be regarded as safe vectors, because of their fully known nucleotide sequence and the known biological functions of the open reading frames, which reveal no potential hazards.

No genes conferring antibiotic resistance or encoding any transfer functions are present in pMA-A001.

Plasmid instabilities (e.g., structural or segregational vector instabilities) could theoretically occur and could potentially cause changes of the production strain during propagation in the production process. Structural and segregational plasmid stability of pMA-A001 has been demonstrated over about 200 generations. Fermentations at production level (counting from the production pre-culture onwards) typically last for maximally 150 generations.

2.3.3 Demonstration of the absence of the GMM in the product

The absence of the GMM in the final enzyme preparation of RF13018 is achieved through filtering after the fermentation process. All viable cells of the production strain RF13018 are removed during the down-stream processing: the fermentation broth is filtered through a pressure filter, concentrated by ultrafiltration (nominal molecular weight cut-off 10000 Da), and finally filtered with sheet filters. The procedures are completed by trained staff based on documented standard operating procedures complying with the requirements of the quality system.

The maltogenic amylase food enzyme is free of detectable, viable production organism. The absence of the production strain is confirmed for every production batch. Three different samples were analysed for absence of the production strain as summarized in [Appendix #1](#). Absence of

the production strain in the final product is confirmed by a Roal² in-house method, which is validated in-house and company specific.

2.3.4 Inactivation of the GMM and evaluation of the presence of remaining physically intact cells

The RF13018 enzyme preparation is free from detectable, viable production organism as demonstrated in the certificate of analysis, [Appendix #1](#). As the absence of the production strain is confirmed for every production batch, no additional information regarding the inactivation of the GMM cells is required.

2.3.5 Information on the possible presence of recombinant DNA

The *Bacillus subtilis* RF13018 enzyme preparation is produced by an aerobic submerged microbial fermentation using a genetically modified *Bacillus subtilis* strain. All viable cells of the production strain, RF13018, are removed during the down-stream processing: the fermentation broth is filtered with pressure filters and subsequent sheet filters, concentrated by ultra-filtration, optionally followed by sheet filtration(s).

After this the final product does not contain any detectable bacterial colony forming units or recombinant DNA. Three separate food enzyme samples (concentrates from industrial scale production and pilot scale fermentations) were tested for the presence of recombinant DNA using highly sensitive and specific PCR techniques. No recombinant DNA (recDNA) of the production strain was shown to be present above the detection limits.

² Roal Oy is the sole manufacturer of AB Enzymes' enzyme preparation. Roal Oy is based in Finland

2.3.6 Absence of Antibiotic Genes and Toxic Compounds

As mentioned above, the transformed DNA does not contain any antibiotic resistance genes. Furthermore, the production of known toxins according to the specifications elaborated by the General Specification for Enzyme Preparations Used in Food Processing Joint FAO/WHO Expert Committee on Food Additives, Compendium of Food Additive Specifications, FAO Food and Nutrition Paper (Food and Agriculture Organization of the United Nations 2006) has been also tested from the fermentation products. Adherence to specifications of microbial counts is routinely analyzed. Three production batches produced by the production strain *Bacillus subtilis* RF13018 (concentrates) were analyzed and no antibiotic or toxic compounds were detected (Appendix #1).

2.4 ENZYME PRODUCTION PROCESS

2.4.1 Overview

The food enzyme is produced by ROAL Oy³ by submerged fermentation of *Bacillus subtilis* RF13018 in accordance with current Good Manufacturing Practices for Food (GMP) and the principles of Hazard Analysis of Critical Control Points (HACCP). As it is run in the EU, it is also subject to the Food Hygiene Regulation (852/2004).

The enzyme preparation described herein is produced by controlled fed-batch submerged fermentation. The production process involves the fermentation process, recovery (downstream processing) and formulation and packaging. Finally, measures are taken to comply with cGMPs and HACCP. The manufacturing flow-chart is presented in Appendix #2.

It should be noted that the fermentation process of microbial food enzymes is substantially equivalent across the world. This is also true for the recovery process: in a clear majority of cases, the enzyme protein in question is only partially separated from the other organic material present in the food enzyme.

³ See footnote #2

2.4.2 Fermentation

The production of food enzymes from microbial sources follows the process involving fermentation as described below. Fermentation is a well-known process that occurs in food and has been used for the production of food enzymes for decades. The main fermentation steps are:

- Inoculum
- Seed fermentation
- Main fermentation

2.4.3 Raw Materials

The raw materials used in the fermentation and recovery processes are standard ingredients that meet predefined quality standards controlled by Quality Assurance for ROAL Oy. The safety is further confirmed by toxicology studies. The raw materials conform to either specifications set out in the Food Chemical Codex, 12th edition, 2020 or The Council Regulation 93/315/EEC, setting the basic principles of EU legislation on contaminants and food, and Commission Regulation (EC) No 1881/2006 setting maximum limits for certain contaminants in food. The maximum use levels of antifoam and flocculants are $\leq 0.15\%$ and $\leq 1.5\%$ respectively.

2.4.4 Materials used in the fermentation process (inoculum, seed and main fermentation)

- Potable water
- A carbon source
- A nitrogen source
- Salts and minerals
- pH adjustment agents
- Foam control agents

2.4.5 Inoculum

A suspension of a pure culture of RF13018 is aseptically transferred to shake flasks containing fermentation medium.

When a sufficient amount of biomass is obtained the shake flasks cultures are combined to be used to inoculate the seed fermentor.

2.4.6 Seed fermentation

The inoculum is aseptically transferred to a pilot fermentor and then to the seed fermentor. The fermentations are run at a constant temperature and a fixed pH. At the end of the seed fermentation, the inoculum is aseptically transferred to the main fermentor.

2.4.7 Main Fermentation

The fermentation in the main fermenter is run as normal submerged fed-batch fermentation. The content of the seed fermenter is aseptically transferred to the main fermenter containing fermentation medium.

In order to control the growth of the production organism and the enzyme production, the feed-rate of this medium is based upon a predetermined profile or on deviation from defined set points.

The fermentation process is continued for a predetermined time or until laboratory test data show that the desired enzyme production has been obtained or that the rate of enzyme production has decreased below a predetermined production rate. When these conditions are met, the fermentation is completed.

2.4.8 Recovery

The purpose of the recovery process is:

- to separate the fermentation broth into biomass and fermentation medium containing the desired enzyme protein,
- to concentrate the desired enzyme protein and to improve the ratio enzyme activity/Total Organic Substance (TOS).

During fermentation, the enzyme protein is excreted by the producing microorganism into the fermentation medium. During recovery, the enzyme-containing fermentation medium is separated from the biomass.

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

- Pre-treatment
- Primary solid/ liquid separation
- Concentration
- Polish and germ filtration

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

2.4.9 Materials

Materials used, if necessary, during recovery of the food enzyme include:

- Flocculants
- Filter aids
- pH adjustment agents

Potable water can also be used in addition to the above-mentioned materials during recovery.

2.4.10 Pre-Treatment

Flocculants and/or filter aids are added to the fermentation broth, in order to get clear filtrates, and to facilitate the primary solid/liquid separation. Typical amount of filter aids is 3%.

2.4.11 Primary solid/liquid separation

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

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

2.4.12 Concentration

The liquid containing the enzyme protein needs to be concentrated to achieve the desired enzyme activity and/or to increase the ratio enzyme activity/TOS before formulation. Temperature and pH are controlled during the concentration step, which is performed until the desired concentration has been obtained. The filtrate containing the enzyme protein is collected for further recovery and formulation.

2.4.13 Polish and germ filtration

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

2.4.14 General Production Controls and Specifications

To comply with cGMPs and HACCP principles for food production, the following potential hazards in food enzyme production are accounted for and controlled during production as described below:

Identity and purity of the producing microorganism:

The assurance that the production microorganism efficiently produces the desired enzyme protein is of utmost importance to the food enzyme producer. Therefore, it is essential that the identity and purity of the microorganism is controlled.

Production of the required enzyme protein is based on a well-defined Working Cell Bank (WCB). The WCB is a collection of ampoules containing a pure culture prepared from an isolate of the production strain. The cell line history, propagation, preservation and the production of a Working Cell Bank is monitored and controlled. A WCB 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 WCB. The accepted WCB is used as seed material for the inoculum.

Microbiological hygiene:

For optimal enzyme production, it is important that hygienic conditions are maintained throughout the entire fermentation process. Microbial contamination can result to decreased growth of the production organism, and consequently, in a low yield of the desired enzyme protein, resulting in a rejected product.

Measures utilized by ROAL OY 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 microorganisms
- Cleaning and sterilization:
 - Validated standard cleaning and sterilization procedures of the production area and equipment: all fermentor, vessels and pipelines are washed after use with a CIP-system (Cleaning in Place), where hot caustic soda and nitric acid are used as cleaning agents. After cleaning, the vessels are inspected manually; all valves and connections not in use for the fermentation are sealed by steam at more than 120°C; critical parts of down-stream equipment are sanitized with disinfectants approved for food industry
- Sterilization of all fermentation media:
 - all the media are sterilized with steam injection in fermentors or media tanks
- Use of sterile air for aeration of the fermentors:
 - Air and ammonia water are sterilized with filtration (by passing a sterile filter).
- Hygienic processing:
 - Aseptical transfer of the content of the WCB ampoule, inoculum flask or seed fermentor
 - Maintaining a positive pressure in the fermentor
- Germ filtration

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

- Training of staff:
 - all the procedures are executed by trained staff according to documented procedures complying with the requirements of the quality system.
- Procedures for the control of personal hygiene
- Pest control
- Inspection and release by independent quality organization according to version-controlled specifications

- Procedures for cleaning of equipment including procedures for check of cleaning efficiency (inspections, flush water samples etc.) and master cleaning schedules for the areas where production take place
- Procedures for identification and implementation of applicable legal requirements
- Control of labelling
- Requirements to storage and transportation

Chemical contaminants:

It is also important that the raw materials used during fermentation are of good quality and do not contain contaminants which might affect the product safety of the food enzyme and/or the optimal growth of the production organism and thus enzyme yield.

It is ensured that all raw materials used in production of food enzymes are of food grade quality or have been assessed to be fit for their intended use and comply with agreed specifications.

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

These in-process controls comprise:

Microbial controls:

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

Monitoring of fermentation parameters may include:

- pH
- Temperature

- Aeration conditions

The measured values of these parameters are constantly monitored during the fermentation process. The values indicate whether sufficient biomass or enzyme protein has been developed and the fermentation process evolves according to plan.

Deviations from the pre-defined values lead to adjustment, ensuring an optimal and consistent process.

Enzyme activity and other relevant analyses (like dry matter, refraction index or viscosity):

This is monitored at regular intervals and at critical steps during the whole food enzyme production process.

2.4.15 Formulation and Packaging

Subsequently, the food enzyme is formulated. The resulting product is defined as a 'food enzyme preparation'. Maltogenic amylase from *Bacillus subtilis* is sold as solid preparations.

For all kinds of food enzyme preparations, the food enzyme is adjusted to the desired activity and is standardized and preserved with food-grade ingredients or additives.

2.4.16 Stability of the enzyme during storage and prior to use

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

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

2.5 Composition and specifications

2.5.1 Characteristics of the enzyme preparation

The characteristics of the enzyme preparation are:

Property		Requirement	
Activity		min.	4200 MAZ/g
Appearance		Light beige	
Particle Size Distribution		Max 1% > 250 μ m	

2.5.2 Formulation of a typical enzyme preparation

Composition	
Constituent	%
Enzyme Concentrate	32-37
Sodium Chloride	1-2
Sunflower oil	0.2
Maltodextrin	Remainder

2.5.3 Molecular mass and amino acid sequence of the enzyme

The maltogenic amylase protein subject for this dossier consists of 686 amino acid residues with a calculated molecular mass of 75 kDa (or 75,000 Da).

2.5.4 Purity and identity specifications of the enzyme preparation

It is proposed that the food enzyme maltogenic amylase should comply with the internationally accepted JECFA specifications for chemical and microbiological purity of food enzymes (Food and Agriculture Organization of the United Nations 2006):

Lead:	Not more than 5 mg/kg
<i>Salmonella</i> sp.:	Absent in 25 g of sample
Total coliforms:	Not more than 30 per gram
<i>Escherichia coli</i> :	Absent in 25 g of sample
Antimicrobial activity:	Not detected
Mycotoxins:	Not applicable for bacteria

The proof that the food enzyme complies with these specifications is shown by the analyses on 3 different batches (see [Appendix #1](#)) and summarized below:

2.5.5 Composition of the enzyme preparation

Batch Number	P190007B	P190008B	191132317	Average
Ash (%)	0.19	0.16	0.97	0.44
Water (%)	88	88	91.3	89.1
Protein (%)	2.37	2.79	2.44	2.53
TOS (%)	11.8	11.8	7.7	10.43
Activity (MAZ/g)	2130	2350	1480	1986.67
Activity/mg TOS	180.51	199.15	192.21	190.62

TOS values were calculated using the following formula: % TOS = 100 % - (% Ash + % Moisture + % Diluents) as recommended by JECFA. The 3 samples do not contain any diluents.

Other enzymatic activities: the food enzyme is standardized on maltogenic amylase activity. Apart from it, the production organism *Bacillus subtilis* produces other endogenous *Bacillus* proteins, e.g. amylase. However, they are present in a small amount and those enzyme activities are already present in the human diet and are not relevant from a safety point of view.

Therefore, there are no relevant side activities from an application and/or safety point of view.

2.6 Enzymatic Activity

The main activity of the food enzyme preparation for this application is maltogenic amylase (IUB 3.2.1.133). The function of maltogenic amylase is in catalyzing hydrolysis of α (1-4) glycosidic bonds in polysaccharides for the removal of successive α -maltose residues from the non-reducing ends of the chains.

Substrates: The substrates in which maltogenic amylase acts upon are starch and related polysaccharides and oligosaccharides.

- A molecule of starch is comprised of many glucose units joined by glycosidic bonds. All vegetables produce starch for energy storage. Starch is a carbohydrate extracted from agricultural raw materials which is widely present in multiple everyday food (and non-food) applications. Starch is considered to be the most important carbohydrate in the human diet. For this purpose, starch is used chemically and enzymatically processed into a variety of different products such as starch hydrolysates, glucose syrups, fructose, maltodextrin derivatives or cyclodextrins, used in the food industry.

Reaction products: The result of the catalytic activity of the maltogenic amylase enzymatic reaction is maltose (disaccharide formed from 2 units of glucose joined with an α (1 \rightarrow 4) bond) formed (Outtrup and Norman 1984).

- 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, triticale) as they break down their starch stores to use for food, which is why it was named after malt. 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.

Consequently, adverse effects are not to be expected. In addition, based on the substrate of maltogenic amylase being found in human food, it can be concluded that the enzyme is part of the human diet.

The method used to analyze the activity of the enzyme is company specific and is capable of quantifying maltogenic amylase activity as defined by its IUBMB classification. The enzyme activity is usually reported in MANU/g or MAZ/g.

2.6.1 Side activities of the enzyme protein which might cause adverse effects

Food enzymes are known to have side activities in the form of other proteins i.e. other enzymes. This is because food enzymes are biological concentrates containing apart from the desired enzyme protein (expressing the activity intended to perform a technological purpose in a certain food process, also called 'main enzyme activity'), other substances as well. This is the reason why JECFA developed the TOS concept for food enzymes and why it is important that the source of a food enzyme is safe.

To add on, like all living cells, microorganisms produce a variety of enzymes responsible for the hundreds of metabolic processes that sustain their life. As microorganisms do not possess a digestive system, many enzymes are excreted to digest the material on which the microorganisms grow. Most of these enzymes are hydrolases that digest carbohydrates, proteins and lipids (fats). These are the very same activities that play a role in the production of fermented food and in the digestion of food by - amongst others - the intestinal micro flora in the human body. In addition, if a food raw material contains a certain substrate (e.g. carbohydrate, protein or lipid), then, by nature, it also contains the very same enzymatic activities that break down such a substrate; e.g. to avoid its accumulation.

Furthermore, the presence in food of such enzyme activities and the potential reaction products is not new and should not be of any safety concern. During the production of food enzymes, the

main enzyme activity contains several other enzymes excreted by the microbial cells or derived from the fermentation medium. As in the case of the enzyme for this application, the side activity comes directly from the production strain. It is generally accepted that the enzyme proteins themselves do not pose any safety concern and are recognized to be generally considered as safe along with known not to cause adverse effects. Apart from maltogenic amylase, the food enzyme also contains other enzymatic side activities in small amount which are naturally and typically produced by the production organism *Bacillus subtilis*, mainly amylases and proteases. Currently, AB Enzymes is not aware or has been aware of adverse effects from the side activities present in the maltogenic amylase enzyme preparation.

2.7 Allergenicity

There have been reports of enzymes manufactured for use in food to cause inhalation allergy in workers exposed to the enzyme dust in manufacturing facilities. In the case of maltogenic amylase, there is a possibility of causing such occupation allergy in sensitive individuals. However, the possibility of an allergic reaction to the maltogenic amylase residues in food seems remove. To address allergenicity by ingestion of the enzyme, the following may be considered:

- The allergenic potential of enzymes was studied by (Bindslev-Jensen et al. 2006) and reported in the publication: "*Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry*". The investigation conducted involved enzymes produced by wild-type and genetically modified strains as well as wild-type enzymes and protein engineered variants. To add on, the investigation comprised 400 patients with a diagnosed allergy to inhalation allergens, food allergens, bee or wasp. The conclusion from the study was that ingestion of food enzymes in general is not likely to be a concern regarding food allergy.
- In the past, the AMFEP Working Group on Consumer Allergy Risk from Enzyme Residues in Food performed an in-depth analysis of the allergenicity of enzyme food products (Daurvin et al. 1998). The overall conclusion is that exposure to enzyme proteins by

ingestion, as opposed to exposure by inhalation, are not potent allergens and that sensitization to ingested enzymes is rare.

Based on this information, there are no scientific indications that small amounts of enzymes in food can sensitize or induce allergic reactions in consumers.

There are additional considerations that support the assumptions that the ingestion of enzyme protein is not a concern for food allergy, which are the following:

- The vast amounts of known proteins are not considered as food allergens and based on previous experience, the enzyme industry is not aware of any enzyme proteins used in food that are homologous to known food allergens
- Only a small amount of the food enzyme is used during food processing, which leads to very small amount of enzyme protein present in the final food. A high concentration generally equals a higher risk of sensitization, whereas a low level in final food equals a lower risk (Goodman et al. 2008).
- For cases where the proteins are denatured which is the case for this maltogenic amylase due to the food process conditions (i.e. baking), the tertiary conformation of the enzyme molecule is destroyed. These types of alterations to the conformation in general, are associated with a decrease in the antigenic reactivity in humans. In the clear majority of investigated human cases, denatured proteins are much less immunogenic than the corresponding native proteins (Valenta and Kraft 2002; Valenta 2002; Takai et al. 2000; Nakazawa et al. 2005; Kikuchi et al. 2006).
- To add on, residual enzyme still present in the final food will be subjected to digestion in the gastro-intestinal system, that reduces further the risk of enzyme allergenicity. While stability to digestion is considered as a potential risk factor of allergenicity, it is believed that small protein fragments resulting from digestion are less likely to be allergenic (Food and Agriculture Organization of the United Nations January/2001; Goodman et al. 2008).

- Recently, AMFEP published version #1 of Guidelines on the Safe Handling of Enzymes in the Bakery Supply Chain which discusses the allergenic potential of baking enzymes in consumer exposure. One of the studies conducted on fungal alpha amylases demonstrated that food allergenic reactions to fungal alpha amylases is rare and respiratory allergy to enzymes does not directly follow a food allergy due to the enzyme (AMFEP and Fedima 2018).
- Lastly, enzymes have a long history of safe use in food processing, with no indication of adverse effects or reactions. Moreover, a wide variety of enzyme classes (and structures) are naturally present in food. This is in contrast with most known food allergens, which are naturally present in a narrow range of foods.

2.7.1 Allergenicity Search

To specifically evaluate the risk of the maltogenic amylase enzyme cross reacting with known allergens and induce a reaction, the sequence homology testing to known allergens was performed. The testing involved using an 80-amino acid (aa) sliding window search, 8-amino acid search and conventional FASTA alignment of the full-length protein sequence (overall homology), with the threshold of 35% identity as recommended by the FAO/WHO in 2001 (Food and Agriculture Organization of the United Nations January/2001) and the Codex Alimentarius in 2003 (Codex Alimentarius Commission 2003) for the 80mer sliding window search.

The database used in the sequence homology comparison test was the "AllergenOnline" database (<http://www.allergenonline.org>) from the University of Nebraska-Lincoln (Version 19, February 19, 2019). The amino acid sequence of the maltogenic amylase enzyme found in this dossier was scanned using three search methods. The first method was a FASTA alignment for the full-length maltogenic amylase sequence to any allergenic proteins in the Allergen online database. All resulting alignments showed identities to allergenic proteins below the above mentioned 35% identity threshold and far below 50% identity. The best hit of the FASTA alignment of the mature maltogenic amylase protein to the database proteins showed an identity of 28.6% with Taka-amylase A precursor (Taa-G1) produced by the fungal species *Aspergillus oryzae* and similarities

of less than 57.6% to eukaryotic amylases and other glycoside hydrolases. Because the maltogenic amylase from the production strain belongs to the same class of enzymes (amylase) it is not surprising that a certain degree of similarity and identity occurs. However, Aalberse suggested “cross-reactivity is rare below 50% amino acid identity and in most situations requires more than 70% identity” (Aalberse 2000, Goodman et al. 2008) making it unlikely that the maltogenic amylase in question can be presumed to be allergenic based on full-length sequence relatedness to known allergens.

In addition to the full-length FASTA search the 80-mer sliding window analysis was used. Each possible 80 amino acid segment of the maltogenic amylase was scanned against the Allergenonline database. The search revealed only a few matches with hit 1 and 2 being the above mentioned Taka-amylase from *Aspergillus oryzae*. The highest identity percentage for 80-mers was found for Taka-amylase 42.54%. The observed identities for the other hits are slightly above 35% i.e. 36.2% [glycoside hydrolase from *Schizophyllum commune H-8* & maltase from *Aedes aegypti*] and 35.3%, [alkaline protease and uncleaved protease from *Aspergillus*]. The interpretation of the FASTA search results requires evaluation of both the Expectation (*E*) values and the percent identity (<http://www.allergenonline.org>). As the percent identities for the matches with the protease from *Aspergillus* are only minimally higher than the 35% threshold, combined with a high *E* score (a high *E* score indicates a low similarity between the query and the matched sequence) these matches can be disregarded.

The FAO/WHO would recommend considering a possible cross-reactivity when there is more than 35% identity in the amino acid sequence of the expressed protein with known allergenic proteins using an 80 amino acids sliding window and a suitable gap penalty (Food and Agriculture Organization of the United Nations January/2001). This recommendation was challenged however recently, according to Ladics et al. (2007) by comparing the predictive value of a full-length (conventional) FASTA search to the 80-mer analysis “a conventional FASTA search provides more

relevant identity to the query protein and better reflects the functional similarities between proteins. It is recommended that the conventional FASTA analysis be conducted to compare identities of proteins to allergens". This judgement on the predictive inferiority of the 80-mer approach was supported recently by Goodman et al. (2008) and Goodman, Tetteh (2011). The authors suggested to consider to raise the identity threshold for the 80-mer alignment to 50%: "because the purpose of the bioinformatics search is to identify matches that may require further evaluation by IgE binding, full-length sequence evaluation or an increase in the threshold from 35% identity toward 50% for the 80 amino acid alignment should be considered" (Goodman and Tetteh 2011). Using the latter recommendation, the maltogenic amylase in question would be below threshold (showing low percent identity) even using the 80-mer sliding window approach.

In addition to the previously described analyses, the maltogenic amylase protein sequence was subject to a very precautionary 8-mer identity match analysis as recommended by some guidances. These short peptide matches have not been validated as predictive tools to identify potentially cross-reactive proteins as found in Goodman's studies in 2008 and in 2005 (Goodman et al. 2008; Goodman et al. 2005). Originally, the eight-amino-acid match was selected based on the idea that it would represent potential IgE binding sites. The search for 8 amino acid exact matches was performed by using the amino acid sequence of maltogenic amylase with a number of 679 8-mers as a query. No match to any known allergen was found confirming that the maltogenic amylase is of no concern.

To summarize, the bioinformatics approach to estimate potential allergenicity and cross-reactivity based on relatedness to known allergens and taking into account the most recent scientific recommendations on the interpretation of such data leads us to conclude that the maltogenic amylase produced by *Bacillus subtilis* RF13018 is of no concern.

2.8 Technological purpose and mechanism of action of the enzyme in food

As an enzyme, maltogenic amylase's main function is to act as a biocatalyst. Through the assistance of an enzyme, biochemical reactions occur to convert a certain substrate into a certain reaction product. The technical effect on the food or food ingredient is caused by the conversion of the substrate to the reaction product caused by the enzymatic reaction involving maltogenic amylase. Once the conversion occurs, the enzyme can no longer perform a technological function.

As mentioned in [section 2.6](#) of this notice, the **substrates** for maltogenic amylase are starch and related polysaccharides and oligosaccharides which occur naturally in nature and are part of the human diet.

The **function** of maltogenic amylase is to catalyze the hydrolysis of α (1-4) glycosidic bonds in polysaccharides for the removal of successive α -maltose residues from the non-reducing ends of the chains.

Like most enzymes, the maltogenic amylase performs its technological function during food processing. The maltogenic amylase from *Bacillus subtilis* RF13018 object of this dossier is specifically intended to be used in **baking** (e.g. bread, bread buns, tortillas, crackers, sweet baked potatoes). In these processes, the maltogenic amylase is used as a processing aid in food manufacturing and is not added directly to final foodstuffs.

The baking industry is a large consumer of starch and starch-modifying enzymes. Amylases have been used in baking cereal-based processes for decades (especially alpha-amylases) and their use in the bakery industry is continuously increasing. In the late eighties, maltogenic amylases, as well as other enzymes active on starch, have been suggested to act on bread staling.

Since alpha-amylases cause stickiness of baked goods, especially when overdosed, it was suggested that these problems could be solved using an exoamylase, since they do not produce

the branched maltooligosaccharides of DP20-100. Such enzymes produce linear oligosaccharides of 2–6 glucose residues. In particular, maltogenic amylases produces maltose and modifies starch at a temperature when most of the starch starts to gelatinize, therefore delaying retrogradation of the starch compound (Diderichsen and Christiansen 1988).

These applications have been specifically approved for a number of years in USA, which together with the extensive use for decades globally justifies the technological need of maltogenic amylase in these food processes.

Below, the benefits of the use of industrial maltogenic amylase in baking are described.

The beneficial effects are of value to the food chain because they lead to better and/or more consistent product characteristics by reducing the rate of staling during storage. Moreover, the application leads to more effective production processes, resulting in better production economy. The reduced staling rate results in less waste bread which results in environmental benefits such as more efficient use of agricultural raw materials, and the reduction of green-house gas emissions by savings in energy consumption in milling and baking and by reduced transportation (Ulber and Sell 2007).

Baking Process:

Maltogenic amylase can be used in the manufacturing of bakery products such as, but not limited to bread, steamed bread, bread buns, tortillas, cakes, pancakes and waffles. Bread baking starts with dough preparation by mixing flour, water, yeast and salt and possibly additives. Flour consists mainly of gluten, starch, non-starch polysaccharides and lipids.

Immediately after dough preparation, the yeast starts to ferment the available sugars into alcohols and carbon dioxide, which causes rising of the dough. Amylases can be added to the dough to

degrade the damaged starch in the flour into smaller dextrins, which are subsequently fermented by the yeast.

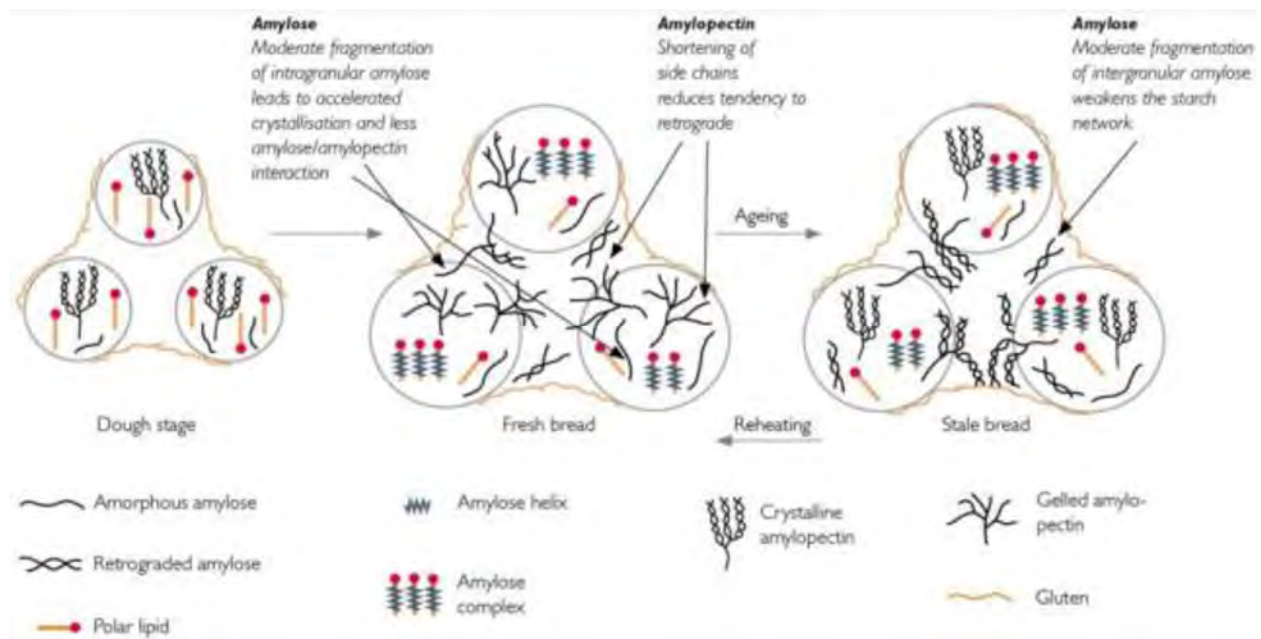
After rising, the dough is baked. When the bread is removed from the oven, a series of changes start. These changes include increase of crumb firmness, loss of crispness of the crust, decrease in moisture content of the crumb and loss of bread flavor. All undesirable changes that do occur upon storage together are called staling. Staling is of considerable economic importance for the baking industry since it limits the shelf life of baked products. Staling is a highly complex phenomenon with firming being the most well-known and important symptom (Gray and Bemiller 2003).

During the dough stages of baking, most of the starch in the flour is in semi-crystalline granules. As higher temperatures are reached in the oven the granular starch begins to gelatinize – to absorb water, swell and lose crystallinity. As the granules begin to rupture, much of the highly soluble amylose is leached out of the granule into the open matrix of the bread.

After baking, as the bread cools, the solubilized amylose retrogrades or recrystallizes within few hours. This is an intermolecular association in which the long, linear amylose chain hydrogen-bond to form an ordered, very stable array. At the same time, the amylose will complex with polar lipids (either naturally occurring or adjunct added). Together, these restructurings are responsible for the oven set of the bread.

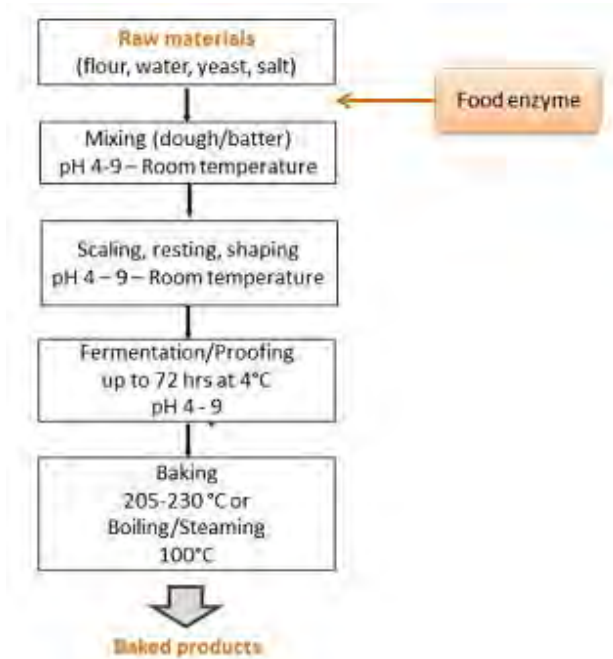
After this initial rapid retrogradation of the amylose, a much slower rate of retrogradation of the amylopectin occurs. During storage, an extensive, partially crystalline, permanent amylopectin network is formed, with junction zones formed by intermolecular recrystallization of amylopectin branches. 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. 2009a).

Thus, retrogradation (recrystallization) of the starch fraction in bread is considered very important in staling. Especially the extent of amylopectin retrogradation correlates strongly with the firming rate of bread.



By degrading the outer amylopectin branches to a large extent and releasing malto-oligosaccharides (maltose) during baking, maltogenic amylase forms a high level of very short amylopectin chains. Short amylopectin chains are correlated with reduced amylopectin retrogradation. Due to the action of maltogenic amylase the outer chains of amylopectin become too short to crystallize, and crystalline junction zone formation is inhibited. Consequently, the formation of a permanent amylopectin network during storage is largely prevented, and the networks of soft, freshly bread is retained, and the bread staling is reduced (Goesaert et al. 2009b).

The process flows of the baking process is presented in the next page:



Therefore, **the benefits of the conversion of starch with the help of maltogenic amylase in baking** can be summarized as follows:

- Reduce capability of amylopectin retrogradation, by shortening the amylopectin chain (down to the branch points) during the baking process.

Beside the main intention to modify the starch structure of the dough (shortening the amylopectin chain structure), some beneficial effects may be associated with effects on the final food, which are however not exclusively obtainable by means of enzyme treatment: they can be achieved without the use of enzymes through e.g. modified, maybe more expensive, production processes, the use of chemicals or recipe changes:

- Ensure an improved / uniformed/ softer / more elastic and less gummy-sticky crumb structure of the bakery product, which might otherwise be impaired by fluctuating processing of the bakery products;

- Possible effects are less product variation, ensuring uniform/standardized quality products;
- All this leading to improved eating quality that would ensure a better consumer acceptability of the final products.

Use of maltogenic amylase in baking ensures a maximum compatibility with modern industrial processes (also leading to less product variations, hereby ensuring standardized quality products). The enzyme is technologically justified and has been demonstrated to be effective in achieving its stated purpose. Adequate assurance is also provided that the enzyme in the form and amounts prescribed are consistent with achieving its technological function.

Maltogenic amylase performs its technological function during the first steps of the baking process (when temperatures rise in the oven). The maltogenic amylase is denatured by heat during baking (when higher temperatures are above 80°C are raised) and has no further technological effect after baking.

2.9 Use Levels

Commercial food enzyme preparations are generally used following the *Quantum Satis* (QS) principle, i.e. at a level not higher than the necessary dosage to achieve the desired enzymatic reaction, according to Good Manufacturing Practice. The amount of enzyme activity added to the raw material by the individual food manufacturer must be determined case by case, based on the desired effect and process conditions.

Therefore, the enzyme manufacturer can only issue a recommended enzyme dosage range. Such a dosage range is the starting point for the individual food producer to fine-tune this process and determine the amount of enzyme that will provide the desired effect and nothing more. Consequently, from a technological point of view, there are no 'normal or maximal use levels' and

maltogenic amylase is used according to the QS principle. A food producer who would add much higher doses than the needed ones would experience untenable costs as well as negative technological consequences.

The dosage of a food enzyme depends on the activity of the enzyme protein present in the final food enzyme preparation (i.e. the formulated food enzyme). However, the activity Units as such do not give an indication of the amount of food enzyme added.

Microbial food enzymes contain, apart from the enzyme protein in question, also some substances derived from the producing microorganism and the fermentation medium. The presence of all organic materials is expressed as Total Organic Solids (TOS). From a safety point of view, the dosage on basis of TOS is relevant. It must also be noted that the methods of analysis and the expression of the Units are company specific. Consequently, in contrast to when the amount is expressed in TOS activity Units of a certain enzyme cannot be compared when coming from different companies. Because of these reasons, the use levels are expressed in TOS in the table below.

The table below shows the range of recommended use levels for each application where the maltogenic amylase from *Bacillus subtilis* RF13018 may be used:

Food Application	Raw material (RM)	Suggested recommended use levels (mg TOS/kg RM)
Baking and other cereal based processes	Flour	22

2.10 Fate in food

It is not the food enzyme itself, but the result of the enzymatic conversion that determines the effect in the food or food ingredient (including raw materials). This effect remains, irrespective of whether the food enzyme is still present or removed from the final food.

Maltogenic amylase performs its technological function during baking processes. In some cases, the enzyme may no longer be present in the final food. In other cases, where the enzyme protein is still present in the final food, it does not perform any technological function in the final food, just like the maltogenic amylase present in food.

To be able to perform a technological function in the final food, many conditions must be fulfilled at the same time:

- The enzyme protein must be in its 'native' (non-denatured) form, AND
- The substrate must still be present, AND
- The enzyme must be free to move (able to reach the substrate), AND
- Conditions like pH, temperature and water content must be favorable

In baking, the maltogenic amylase, is used in the treatment of flour and in the production of baking improvers and mixes. The maltogenic amylase is denatured by heat during baking (when higher temperatures above 80°C are raised) and has no further technological effect after baking.

Based on the conditions of use and the activity of maltogenic amylase under such conditions, it can be concluded the presence of (residues of) enzyme maltogenic amylase in the final food does not lead to an effect in or on the final foods.

3 Part 3 § 170.325- Dietary Exposure

The best method to determine an estimate of human consumption for food enzymes is using the so-called Budget Method (Hansen 1966; Douglass et al. 1997). Through this method, the Theoretical Maximum Daily Intake (TMDI) can be calculated, based on conservative assumptions. These conservative assumptions regard physiological requirements for energy from food and the energy density of food rather than on food consumption survey data.

The original role of the Budget Method was for determining food additive use and is known to result in conservative estimations of the daily intake.

The Budget Method is based on the following assumed consumption of important foodstuffs and beverages (for less important foodstuffs, e.g. snacks, lower consumption levels are assumed):

Average consumption over the course of a lifetime/kg body weight/day	Total solid food (kg)	Total non-milk beverages (l)	Processed food (50% of total solid food) (kg)	Soft drinks (25% of total beverages) (l)
	0.025	0.1	0.0125	0.025

To determine the TMDI of maltogenic amylase enzyme preparation, the calculation used the maximum use levels. In addition, the calculation accounts for how much food or beverage is obtained per kg raw materials (as shown in the table below). All of the TOS is assumed to be in the final product.

Applications		Raw material (RM)	Suggested recommended use level (mg TOS/kg RM)	Final food (FF)	Ratio RM/FF*	Suggested level in final food (mg TOS/kg food)
SOLID FOODS	Baking and other cereal based processes	Flour	22	Baking Flours, bread improvers and pre-mixes	0.71	15.62

*Assumptions behind ratios of raw material to final food

Baking

- Bakery products fall in the category of solid foods.
- Flour is the raw material for bakery product and the yield will vary depending on the type of final food produced. From 1 kg of flour you would have 4 kg of cakes, 1.4 kg of bread or 1.1 kg of cracker. Cracker may represent the most conservative input from the bakery processes. However, consumption of bread is higher than that of crackers, therefore this is why bread is used as the assumption for the calculation of dietary exposure from bakery processes.
- The yield of 1.4 kg of bread per 1 kg of flour correspond to a RM/FF ratio of 0.71 kg of flour per kg bakery product is used.

The Total TMDI can be calculated on basis of the maximal values found in food (solid) and beverage multiplied by the average consumption of food and beverage/kg body weight/day.

The Total TMDI will consequently be calculated as follows:

TMDI in food (mg TOS/kg body weight/day)	TMDI in beverage (mg TOS/kg body weight/day)	Total TMDI (mg TOS/kg body weight/day)
0.0125 x 15.62 = 0.19525	0	0.19525

It should be stressed that this Total TMDI is based on conservative assumptions and represents a highly exaggerated value because of the following reasons:

- It is assumed that ALL producers of the above-mentioned foodstuffs use the specific maltogenic amylase enzyme from *Bacillus subtilis* RF13018;
- It is assumed that ALL producers apply the HIGHEST use level per application;
- For the calculation of the TMDI's in food and in beverages, only THOSE foodstuffs and beverages were selected containing the highest theoretical amount of TOS.

- Thus, foodstuffs and beverages containing lower theoretical amounts were not taken into account;
- It is assumed that the amount of TOS does not decrease because of the food production process;
- It is assumed that the final food containing the calculated theoretical amount of TOS is consumed DAILY over the course of a lifetime;
- Assumptions regarding food and beverage intake of the general population are overestimates of the actual average levels (Douglass et al. 1997).

The Margin of Safety (MoS) for human consumption can be calculated by dividing the NOAEL as listed in [section 6.2.1](#) of this application, by the Total Theoretical Maximal Daily Intake (TMDI). Total TMDI of the food enzyme is 1000 mg TOS/kg body weight/day. Consequently, the MoS is: $MoS = 1,000/0.19525 = 5,122$.

Total TMDI is highly exaggerated. Moreover, the NOAEL was based on the highest dose administered, and is therefore to be considered as a minimum value. Therefore, the actual Margin of Safety in practice will be some magnitudes higher. Consequently, there are no safety reasons for laying down maximum levels of use.

Conclusion:

The overall conclusion is that the use of the food enzyme maltogenic amylase RF13018 in the production of food is safe. Considering the high safety factor - even when calculated by means of an overestimation of the intake via the Budget method - there is no need to restrict the use of the enzyme in food processing. The suggested dosage for food manufacturers is not a restrictive value and could be higher or lower depending on usage within cGMPs.



4 Part 4 §170.240- Self-Limiting Levels of Use

This part is not applicable to this notified substance, see **Section 2.9** for further details regarding use levels.



5 Part 5 § 170.245- Experience Based on Common Use in Food Before 1958

This part is not applicable to this notified substance.

6 Part 6 § 170.250- GRAS Notice- Narrative

The data and information contained in this GRAS notice provides a basis that the notified substance is safe under the conditions of its intended use described herein. In the following subsections, the safety of the enzyme, the genetic modification and toxicological studies are presented. The information is generally available and PART 6 § 170.250 does not contain any confidential information. This section provides the basis that the notified substance is generally recognized, among qualified experts, and study data, to be safe under the conditions of its intended use.

All available known information has been reviewed and AB Enzymes GmbH is not aware of any data or information that is, or may appear to be, consistent with our conclusion of the notified substance GRAS status.

6.1 Safety of the Production Strain

The safety of *Bacillus subtilis* as an enzyme producer has been reviewed by de Boer Sietske, A. and Diderichsen, B. (1991) Schallmeyer et al. (2004) and Olempska-Beer et al. (2006).

Bacillus subtilis is among the most widely used bacteria for the production of enzymes and specialty chemicals. Industrial applications include (but are not restricted to) production of amylase, protease, glucanase, xylanase, etc.

In addition to *Bacillus licheniformis*, *B. subtilis* has become one of the most well-established cell factories in biotechnology especially for the production of exo-proteins like proteases and alpha-amylases (Westers et al. 2004) (Pohl and Harwood 2010) (van Dijn and Hecker 2013).

One of the oldest recorded uses of *Bacillus* is the fermentation of soybeans into Natto, a Tempe-like fermentation that uses a strain of *Bacillus* now recognized as *Bacillus subtilis* (natto). The production of Natto dates back more than a thousand years and was first practiced in Japan. Some 6×10^6 kg of Natto are consumed annually in Japan.

While *B. subtilis* produces many enzymes, including amylases and cellulases, the most important enzymes in the production of Natto are proteases. The proteases are responsible for creating its main flavor, through hydrolysis of soybean protein. Natto or the underlying microbial culture of *B. subtilis* (natto), is reported to have a number of beneficial health effects.

Furthermore *Bacillus subtilis* has been used in the food industry and biotechnology since many years for e.g., the production of amylases and glucanases for the baking and beverages markets, as well as for desizing of textiles and for starch modification for sizing of paper (Ferrari et al. 1993), the production of proteases for protein modification of e.g. milk or soybean protein or in the brewing industry (Schallmeyer et al. 2004), for use in detergent products and for de-hairing and batting in the leather industry, and for the production of xylanases as bread improver (Harbak and Thygesen 2002).

Food use safety:

B. subtilis-like organisms are ubiquitous in the environment (soil, water, plants and animals) and as a result can be also found in food (de Boer Sietske, A. and Diderichsen, B. 1991). *B. subtilis* has already been used for decades for the production of food enzymes with no known reports of adverse effects to human health or the environment (de Boer Sietske, A. and Diderichsen, B. 1991). Alpha-amylase enzyme preparation from *B. subtilis* has been used commercially since 1929, when it was used in the manufacture of chocolate syrup to reduce viscosity.

Recently the US Food and Drug Administration reviewed the safe use of food-processing enzymes from recombinant microorganisms, including *B. subtilis* (Olempska-Beer et al. 2006). An extensive risk assessment of *B. subtilis*, including its history of commercial use has been published by the US EPA (US EPA, 1997⁴). It was concluded that *B. subtilis* is not a human pathogen nor is it toxigenic.

Food enzymes derived from *B. subtilis* strains (including recombinant strains) have been evaluated by JECFA and many countries which regulate the use of food enzymes, such as the USA, France,

⁴ <https://www.epa.gov/sites/production/files/2015-09/documents/fra009.pdf>

Denmark, Australia/New Zealand and Canada, resulting in the approval of the use of food enzymes from *B. subtilis* in the production of various foods, such as baking, brewing, juice production, wine production, distillation, starch industry, protein processing, etc.

Please refer to table #1 for an extensive overview of countries that accepted *B. subtilis* as safe production organisms for a broad range of food enzymes.

Table #1 -Non-exhaustive list of authorized food enzymes (other than maltogenic amylase) used *Bacillus subtilis*:

Authority	Food enzyme	Reference
JECFA	Alpha amylase	WHO Food Additives Series 28 Report 710 , WHO Food Additives Series 28 Report 712 , TRS 806-JECFA 37/10 TRS 891-JECFA 51/17 NMRS 50/TRS 488-JECFA 15/12 TRS 928-JECFA 63/42 , TRS 928-JECFA 63/42
	Alpha-Acetolactate decarboxylase	
	Carbohydrase and Protease	
	Xylanase	
Australia/NZ	Alpha-Acetolactate decarboxylase Alpha amylase Beta amylase Asparaginase Endo-1,4-β-xylanase Beta glucanase Hemicellulase multicomponent enzyme Metalloproteinase Pullulanase Serine proteinase	Schedule 18 Processing Aids
Canada	Alpha-Acetolactate decarboxylase Amylase Asparaginase Glucanase Hemicellulase Lactase Pentosanase	5. List of Permitted Food Enzymes

	Protease Pullulanase Xylanase	
France	Alpha-Acetolactate decarboxylase Alpha amylase Beta glucanase Asparaginase Beta galactosidase Endo-beta-glucanase Glucosyltransferase Hemicellulase Protease Pullulanase Xylanase	Arrêté du 19 octobre 2006
USA⁵	Pullulanase Pectate lyase Branching glycosyltransferase 1,4-alpha branching enzyme Asparaginase Lactase Subtilisin	GRAS Notice Inventory, GRN 20 , GRAS Notice Inventory, GRN 205 GRAS Notice Inventory, GRN 114 GRAS Notice Inventory, GRN 274 GRAS Notice Inventory, GRN 406 GRAS Notice Inventory, GRN 476 GRAS Notice Inventory, GRN 579 GRAS Notice Inventory, GRN 714

⁵ GRAS affirmations and GRAS notifications

At Roal Oy and AB Enzymes GmbH, *Bacillus subtilis* has been used as enzyme producer for many years without any safety problems. *Bacillus subtilis* strains have been cultivated in the production plant of Alko Oy/Roal Oy starting from year 1993 and the parental strain from which the production strain described here is derived has been used since 2010.

6.1.1 Pathogenicity and Toxigenicity

Bacillus subtilis strains are non-pathogenic for healthy humans and animals (Boer and Diderichsen 1991). Apart from the well-established pathogenicity of *B. anthracis*, a pathogen of humans and some animals, *B. cereus*, which causes gastroenteritis, and the group of insect pathogens related to *B. thuringiensis*, most other species of *Bacillus* are regarded as nonpathogenic or cause only opportunistic infections, often in compromised patients. The lack of pathogenicity among strains of *B. subtilis* or any of its close relatives has resulted in the Food and Drug Administration granting the organism GRAS (generally regarded as safe) status.

Pathogenic *B. subtilis* strains are not described in the Bergey's Manual or in the ATCC and other catalogues. The species *B. subtilis* does not appear on the list of pathogens in Annex III of Directive 2000/54/EC on the protection of workers from risks related to exposure to biological agent at work.

Bacillus subtilis is a microorganism regarded as safe globally:

- In Canada, *B. subtilis* as per CEPA (Canadian Environmental Protection Act), does not meet the criteria of section 64 of the act – dangerous substances and no further regulatory action is required for its use⁶

⁶ <http://www.ec.gc.ca/lcpe-cepa/default.asp?lang=En&n=5AE12597-1&offset=2&toc=show>

- In the USA, *B. subtilis* is exempted as a host of certified host-vector systems under the NIH Guidelines in the USA since 1994 (NIH, 1996)⁷. The US EPA has added *B. subtilis* to the list of exempted organisms in 1997 (USA EPA, 1997)⁸.
- In Europe, *B. subtilis* is classified as a low-risk-class microorganism, as exemplified by being listed as Risk Group 1 in the microorganism classification lists of the German Federal Institute for Occupational Safety and Health (BAuA, 2002) and the Federal Office of Consumer Protection and Food Safety (BVL, 2013), and not appearing on the list of pathogens from Belgium (Belgian Biosafety Server, 2010)⁹.

QPS status

The European Food Safety Agency (EFSA) maintains a list of the biological agents to which the Qualified Presumption of Safety (QPS) assessment can be applied. In 2007, the Scientific Committee set out the overall approach to be followed and established the first list of the biological agents. The QPS list is reviewed and updated annually by the Panel on Biological Hazards (BIOHAZ). If a defined taxonomic unit does not raise safety concerns or if any possible concerns can be excluded, the QPS approach can be applied and the taxonomic unit can be recommended to be included in the QPS list. The safety of *B. subtilis* as production organisms has been assessed by EFSA and it has been accorded QPS status provided that the qualification requirements are met (EFSA 2007). *B. subtilis* is therefore generally accepted as a non-pathogenic organism. In 2018 EFSA mentioned in their update to QPS, if the production organism for the recipient strain has the QPS status and the genetic modification for construction of the production strain does not pose a safety risk, then the QPS status can extend to the production strain (EFSA 2018). The production organism fulfils the specific qualifications for the QPS status, the genetic modifications do not give rise to safety concerns and the manufacturing does not give any risks, therefore the production strain *Bacillus subtilis* RF13018 qualifies for QPS status.

⁷ https://osp.od.nih.gov/wp-content/uploads/2019_NIH_Guidelines.htm

⁸ <https://www.epa.gov/sites/production/files/2015-09/documents/fra009.pdf>

⁹ <https://www.biosafety.be/content/tools-belgian-classification-micro-organisms-based-their-biological-risks>

Secondary Metabolites:

A review of the literature by the US EPA (1997) failed to reveal the production of metabolites of toxicological concern by *B. subtilis*. Although *B. subtilis* has been associated with outbreaks of food poisoning (Gilbert *et al.*, 1981 and Kramer *et al.*, 1982 as cited by Logan 1988), the exact nature of its involvement has not been established. Unlike the case in these outbreaks of food poisoning, where apparently *B. subtilis* was isolated from a food source, the strains used for food enzyme production are not present in the processed food. Only the enzyme preparation is used in the food process. *B. subtilis*, like other closely related species in the genus as *B. licheniformis*, *B. pumilus*, and *B. megaterium*, has been shown to be capable of producing lecithinase, an enzyme which disrupts membranes of mammalian cells. However, there has not been any correlation between lecithinase production and human disease for *B. subtilis*.

Concern about possible involvement of *B. cereus*-like enterotoxins in the rare cases where some *Bacillus* strains have been associated with food poisoning caused the Scientific Committee on Animal Nutrition to require specific testing of industrially used *Bacillus* strains. Subsequent testing showed the absence of *B. cereus*-like enterotoxins (Pedersen *et al.* 2002) and the current view is that the very few reports of *B. cereus*-like enterotoxins occurring in other species of *Bacillus* are likely to have resulted from misidentification of the strain involved (From *et al.* 2005).

Metabolites of human toxicological concern are usually produced by microorganisms for their own protection. Microbes in natural environments are affected by several and highly variable abiotic (e.g., availability of nutrients, temperature and moisture) and biotic factors (e.g., competitors and predators). Their ever-changing environments put a constant pressure on microbes as they are prompted by various environmental signals of different amplitude over time. In nature, this results in continuous adaptation of the microbes through inducing different biochemical systems; e.g., adjusting metabolic activity to current availability of nutrients and carbon source(s), or activation of stress or defense mechanisms to produce secondary metabolites as 'counter stimuli' to external signals (Klein and Paschke 2004; Earl *et al.* 2008). Finally, most industrial *B. subtilis* strains are from safe strain lineages that have been repeatedly tested

according to the criteria laid out in the Pariza and Johnson publication (Pariza and Johnson, 2001). See [Appendix #3](#) for Decision Tree.

Conclusion:

B. subtilis has a long history of safe use in industrial-scale enzyme production. The long industrial use and wide distribution of *B. subtilis*-like organisms in nature has never led to any symptoms of pathogenicity. Moreover, no case demonstrating invasive properties of the species has been found in the literature.

During recent years, genetic engineering techniques have been used to improve the industrial production strains of *B. subtilis* and considerable experience on the safe use of recombinant *B. subtilis* strains at industrial scale has accumulated.

Secondary metabolites are not a safety concern in fermentation products derived from industrial *B. subtilis* strains. In addition, food enzymes produced by *B. subtilis* have been subjected to a significant number of toxicological tests (including 90-day toxicological tests), as part of their safety assessment for use in food product manufacturing processes. These studies demonstrate that there are no concerns for fermentation products as produced using *B. subtilis*.

Therefore, *B. subtilis* can be considered generally safe not only as production organisms of its natural enzymes, but also as safe hosts for other safe gene products.

6.1.2 Safety of the genetic modification

The genetic modification, i.e. the transformation of the recipient strain *Bacillus subtilis* with the vector pMA-A001 results in recombinant strain RF13018. As mentioned before, the recipient belongs to a non-pathogenic species. The strain line has been used since 2010 for safe food enzyme production.

The production strain (RF13018) differs from its original parental strain in expressing maltogenic amylase and featuring a set of defined genomic deletions. Besides this, AB Enzymes has noticed no differences in the production strain RF13018 as compared to the parental strain.

Maltogenic amylase:

Maltogenic amylase (EC 3.2.1.133) catalyses the hydrolysis of a (1-4) glycosidic bonds in polysaccharides so as to remove successively α -maltose residues from the non-reducing ends of the chains. Amylases in general have been used in the food industry, particularly in baking processes, for decades (especially alpha-amylases) and their use in the bakery industry is continuously increasing. Maltogenic amylases, as well as other enzymes active on starch, have been suggested to prevent bread staling, by modifying starch at a temperature when most of the starch starts to gelatinize, therefore delaying retrogradation of the starch components which is the main reason for bread staling.

Commercial maltogenic amylase enzyme preparations from various microorganisms (including genetically modified ones) are widely accepted and *Bacillus subtilis* – whether or not genetically modified¹⁰ - is widely accepted as a safe production organism for a broad range of enzymes that have been used e.g., as processing aids in food industry for several decades.

¹⁰ Overproduction of chosen enzymes and/or modification of enzyme- (e.g. cellulase) profiles has not been observed to convey harmful properties to the host organism or its products (-animal tests- Huuskonen 1990).

Table 2 – Non-exhaustive list of authorized maltogenic amylases from similar production organisms

Authority	Food enzyme
JECFA	Maltogenic amylase from <i>Bacillus stearothermophilus</i> expressed in <i>Bacillus subtilis</i> <u>TRS 891- JECFA 51/18</u>
Australia/NZ	<i>Bacillus subtilis</i> containing the gene for maltogenic α -amylase isolated from <i>Geobacillus stearothermophilus</i> ¹¹ <u>Schedule 18 Processing Aids</u>
Canada	Maltogenic amylase from <i>Bacillus subtilis</i> BRG-1 (pBRG1); <i>Bacillus subtilis</i> DN1413 (pDN1413); <i>Bacillus subtilis</i> LFA 63 (pLFA63); <i>Bacillus subtilis</i> RB-147 (pRB147); <i>Bacillus licheniformis</i> MDT06-221; <i>Bacillus subtilis</i> RF12029 <u>5. List of Permitted Food Enzymes</u>
France	Maltogenic amylase from <i>B. stearothermophilus</i> expressed in <i>B. subtilis</i> <u>Arrêté du 19 octobre 2006</u> Maltogenic amylase from <i>B. stearothermophilus</i> expressed in <i>B. subtilis</i> strain SM, SO, OC, DS67348

The maltogenic amylase protein overproduced by RF13018 originates from *Geobacillus stearothermophilus* and is > 99% identical in its sequence and functionality to maltogenic amylase produced by the wild-type *Geobacillus stearothermophilus*.

As the maltogenic amylase protein is not toxic, our evaluation of the genetically modified *Bacillus subtilis* strain is comparable to that of the recipient strain. Based on the available information, it would be reasonable to conclude that the use of *G. stearothermophilus* maltogenic amylase gene

¹¹ *Geobacillus stearothermophilus* – former name *Bacillus stearothermophilus*

for the production of maltogenic amylase in *Bacillus subtilis* RF13018 does not lead to any particular safety concern.

Plasmid pMA-A001

Plasmid pMA-A001 contains no genes conferring antibiotic resistance and there is no transfer function present. The vector itself is fully characterized and free from potential hazards. It has been shown to be genetically stable.

Genetic stability of the strain RF13018

The transformation does not increase the natural mutation frequency. If there were any mutations happening to the genes affecting the relevant characters of the bacterium, this would be noticed in the growth characteristics in the fermentation and / or in the product obtained. This has not happened. In addition, the possibility of mutations is decreased to its minimum by inoculating the seed culture for the fermentation with controlled stocks in "Working Cell Bank".

No additional mutagenesis cycles have been performed after the RF13018 strain deposition to the culture collection.

The safety of the maltogenic amylase produced by the genetically modified *Bacillus subtilis* is supported by a standard package of genotoxicity testing as described in detailed in [section 6.2.1](#).

Because the host organism is safe and because the genetic modifications are well characterized and specific utilizing well-known plasmids for vector constructs, and the introduced genetic material does not encode and express any toxic substances, it is concluded that the use of the maltogenic amylase from genetically modified *Bacillus subtilis* RF13018 is generally considered as safe.

We consider that the colonization capacity of RF13018 in the environment must be considered rather low because of its adaptation to artificial fermentation conditions, deletion of nutrient mobilizing secreted hydrolases and inability to form spores to withstand unfavourable conditions.

The recipient has been adapted by conventional mutagenesis and has targeted gene deletions in the genome to meet production conditions in the fermenter. Such conditions, e.g., no competitive microorganisms, optimal provision of nutrients and aeration are not present in the environment.

In addition, the fitness of the strain to survive is very likely to be reduced by its high secretion performance characteristic. Most of its energy is needed for the maintenance of the plasmid and the production of maltogenic amylase and this will be of no advantage in a natural environment.

The inability of *B. subtilis* RF13018 to form spores and the deletion of relevant secreted hydrolases further greatly reduces its fitness to survive in nature, because there is no protection against common environmental stresses like extremes of pH or temperature, lack of oxygen or poor nutrient supply. In the presence of a well-adapted competing wild-type flora as found ubiquitously in soil or water, the fitness and therefore the colonization capacity of *B. subtilis* RF13018 must be considered rather low or zero.

As demonstrated above, the maltogenic amylase food enzyme from *Bacillus subtilis* RF13018 does not contain viable GMMs or their recombinant DNA. Consequently, environmental exposure of the GMM is negligible.

6.2 Data for Risk Assessment

6.2.1 Toxicological testing

The safety of the maltogenic amylase produced by the genetically modified *Bacillus subtilis* RF13018 is based on the historical safety of the strain lineage. *Bacillus subtilis* is among the most widely used bacteria for the production of enzymes and specialty chemicals. Industrial applications include (but are not restricted to) production of amylase, protease, glucanase, xylanase, etc. The RF13018 production strain and recipient are derived from a classical *Bacillus* mutant parental strain which has been proven to be safe.

In the toxicological tests performed, including a 90-days repeated dose study, no toxicity was detected. There was no indicator for toxicity in any of the dose levels tested. Therefore, a NOAEL of 1000 mg/kg/day was established. Additionally, the RF13018 strain was tested for its potential to be cytotoxic. A cytotoxicity study using Vero cells was conducted and demonstrated the strain to not be cytotoxic.

Please refer below to the summary of the cytotoxicity study below:

Cytotoxicity Study

Bacillus subtilis RF13018 underwent an analysis of the cytotoxicity of culture supernatant of the strain to Vero cells with LDH release assay. The study was conducted by BioSafe – Biological Safety Solutions Ltd at Microkatu 1M FIN-70210 KUOPIO, Finland and was completed on March 12, 2020. The study complies with Good Laboratory Practices and under the current standards of the EU.

The cells release LDH into the bloodstream after tissue damage or red blood cell hemolysis. Since LDH is a fairly stable enzyme, it has been widely used to evaluate the presence of damage and toxicity of tissue and cells. The cytotoxicity test was conducted at Biosafe by measuring the release of LDH from Vero cells exposed to 100 µL of cell-free culture supernatants (harvested by centrifugation from cultures incubated for 6 h). *Bacillus licheniformis* ATCC 14580 and *Bacillus cereus* DSM 31 (ATCC 14579) were used as a bacterial negative and positive control strains, respectively. Triton-X 100 was used as a positive control for LDH release (detergent treated cells,

considered to release 100% LDH from the cells) and background LDH release from Vero cells exposed to cell culture medium without serum as negative control.

Results:

Bacillus subtilis RF13018 (2.3%) and negative control *Bacillus licheniformis* ATCC 14580 (5.4%) cell free supernatants did not exceed 20% cytotoxicity threshold and hence were not cytotoxic to Vero cells. Bacterial positive control, *Bacillus cereus* DSM 31 (ATCC 14579), cell free culture supernatants (84.8%) were extremely cytotoxic to Vero cells.

Conclusion:

Bacillus subtilis RF13018 culture supernatant was not cytotoxic to Vero cells.

The following studies were performed as summarized in GRAS Notice 746:

- *In vitro* Bacterial reverse mutation test
- *In vitro* Chromosomal aberration test
- 13-week oral toxicity study in rats

Additionally, the strain has shown not to produce any cytotoxicity, when tested as recommended by the updated EFSA Guidance on *Bacillus* safety¹².

The original maltogenic alpha-amylase preparation produced with *Bacillus subtilis* has been subjected to several tests as part of its safety assessment for the production of food products. In toxicological tests that have been performed, including a 90-days repeated dose rat feeding study, no toxicity was detected.

For further development of the original *B. subtilis* host, genetically well-defined modifications were introduced to improve strain and product performance. Internally, our Corporate Biosafety Committee decided that the genetic changes used in the creation of the production strain are

¹² <https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2020.5965>

minor and pose no safety concerns. The genetic modifications implemented are within the range of natural variability, meaning the function of the enzyme is unchanged. Minor changes occurred in the amino acid sequence of the maltogenic amylase produced from production strain RF13018. The amino acid interchanges change the pH and temperature stability of the molecule but not to a degree that could not be achieved in natural variation. The hydrolase gene added to the production strain is also minor and does not impact the function of the maltogenic amylase as described in [section 2.2.2](#). Additionally, the production strain was shown not to produce any cytotoxic effects when tested as recommended by the updated EFSA Guidance on *Bacillus* safety.

Because:

- the original host organism is safe,
- all the genetic modifications carried out (for original host improvement) are well characterized and specifically utilizing well-known plasmids for vector construction,
- the introduced genetic material does not encode and express any toxic substances,

It is concluded that the use of the maltogenic amylase produced with the current genetically modified *Bacillus subtilis* RF13018 as a processing aid in food processes does not pose any significant risk to human health.

Because the host organism is safe and because the genetic modifications are well characterized and specific utilizing well-known plasmids for vector constructs, and the introduced genetic material does not encode and express any toxic substances, it is concluded that the use of the maltogenic amylase from genetically modified *Bacillus subtilis* RF13018 as a processing aid in food processes would pose no significant risk to human health.



7 Part 7 §170.255- List of Supporting Data and Information

This section contains a list of all the data and literature discussed in this dossier to provide a basis that the notified substance is safe under the conditions of its intended use as described in accordance with §170.250 (a)(1). All information presented in this section are publicly available.

Appendices

1. [RF13018 Composition Report](#)
2. [Flow Chart of the manufacturing process with control steps](#)
3. [Pariza and Johnson Decision Tree](#)

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FDA USE ONLY

GRN NUMBER 000974	DATE OF RECEIPT Sep 4, 2020
ESTIMATED DAILY INTAKE	INTENDED USE FOR INTERNET
NAME FOR INTERNET	
KEYWORDS	

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration
**GENERALLY RECOGNIZED AS SAFE
(GRAS) NOTICE** (Subpart E of Part 170)

Transmit completed form and attachments electronically via the Electronic Submission Gateway (*see Instructions*); OR Transmit completed form and attachments in paper format or on physical media to: Office of Food Additive Safety (*HFS-200*), Center for Food Safety and Applied Nutrition, Food and Drug Administration, 5001 Campus Drive, College Park, MD 20740-3835.

SECTION A – INTRODUCTORY INFORMATION ABOUT THE SUBMISSION

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

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

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

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

	Name of Contact Person Joab Trujillo	Position or Title Junior Regulatory Affairs Specialist
	Organization (<i>if applicable</i>) AB Enzymes Inc.	
	Mailing Address (<i>number and street</i>) 8211 W. Broward Blvd. Suite 375	

City Plantation	State or Province Florida	Zip Code/Postal Code 33324	Country United States of America
Telephone Number +1 954 800 8606	Fax Number	E-Mail Address joab.trujillo@abenzymes.com	

	Name of Contact Person	Position or Title
	Organization (<i>if applicable</i>)	
	Mailing Address (<i>number and street</i>)	

City	State or Province	Zip Code/Postal Code	Country
Telephone Number	Fax Number	E-Mail Address	

SECTION C – GENERAL ADMINISTRATIVE INFORMATION

1. Name of notified substance, using an appropriately descriptive term
Maltogenic Amylase (IUBMB 3.2.1.133) from a Genetically Modified Bacillus subtilis

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

- Electronic Submission Gateway Electronic files on physical media
 Paper
If applicable give number and type of physical media

3. For paper submissions only:

Number of volumes _____

Total number of pages _____

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

- Yes (Proceed to Item 5) No (Proceed to Item 6)

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

- a) GRAS Notice No. GRN 746 _____
 b) GRAS Affirmation Petition No. GRP _____
 c) Food Additive Petition No. FAP _____
 d) Food Master File No. FMF _____
 e) Other or Additional (describe or enter information as above) _____

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

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

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

- Yes (Proceed to Item 8)
 No (Proceed to Section D)

8. Have you designated information in your submission that you view as trade secret or as confidential commercial or financial information (Check all that apply)

- Yes, information is designated at the place where it occurs in the submission
 No

9. Have you attached a redacted copy of some or all of the submission? (Check one)

- Yes, a redacted copy of the complete submission
 Yes, a redacted copy of part(s) of the submission
 No

SECTION D – INTENDED USE

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

The Maltogenic Amylase enzyme is to be used in baking processes. The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements under current Good Manufacturing Practices. There are no maximal limits set, just suggested dosages.

Recommended Use Levels = 22 mg TOS/kg RM

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

(Check one)

- Yes No

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

(Check one)

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

SECTION E – PARTS 2 -7 OF YOUR GRAS NOTICE

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

- PART 2 of a GRAS notice: Identity, method of manufacture, specifications, and physical or technical effect (170.230).
- PART 3 of a GRAS notice: Dietary exposure (170.235).
- PART 4 of a GRAS notice: Self-limiting levels of use (170.240).
- PART 5 of a GRAS notice: Experience based on common use in foods before 1958 (170.245).
- PART 6 of a GRAS notice: Narrative (170.250).
- PART 7 of a GRAS notice: List of supporting data and information in your GRAS notice (170.255)

Other Information

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

Yes No

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

Yes No

SECTION F – SIGNATURE AND CERTIFICATION STATEMENTS

1. The undersigned is informing FDA that AB Enzymes Inc.

(name of notifier)

has concluded that the intended use(s) of Maltogenic Amylase (IUBMB 3.2.1.133) from a Genetically Modified Bacillus subtilis

(name of notified substance)

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

2. AB Enzymes Inc. *(name of notifier)* agrees to make the data and information that are the basis for the conclusion of GRAS status available to FDA if FDA asks to see them; agrees to allow FDA to review and copy these data and information during customary business hours at the following location if FDA asks to do so; agrees to send these data and information to FDA if FDA asks to do so.

8211 W. Broward Blvd. Suite 375 Plantation, Florida 33324 USA

(address of notifier or other location)

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

3. Signature of Responsible Official,
Agent, or Attorney

Joab Trujillo

Digitally signed by Joab Trujillo
Date: 2020.09.01 15:51:02 -04'00'

Printed Name and Title

Joab Trujillo Junior Regulatory Affairs Specialist

Date (mm/dd/yyyy)

09/01/2020

SECTION G – LIST OF ATTACHMENTS

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

Attachment Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)
	Form3667 AB Enzymes Maltogenic Amylase.pdf	Administrative
	Cover Letter for Maltogenic Amylase GRAS Notice.pdf	Administrative
	GRAS Notice Maltogenic Amylase.pdf	Submission
	1_RF13018 Composition Report.pdf	Submission
	2_Flow Chart of the manufacturing process with control steps. pdf	Submission
	3_Pariza and Johnson Decision Tree.pdf	Submission
	References for Maltogenic Amylase GRAS Notice.zip	Submission

OMB Statement: Public reporting burden for this collection of information is estimated to average 170 hours per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to: Department of Health and Human Services, Food and Drug Administration, Office of Chief Information Officer, PRASStaff@fda.hhs.gov. (Please do NOT return the form to this address). An agency may not conduct or sponsor, and a person is not required to respond to, a collection of information unless it displays a currently valid OMB control number.