May 5, 2021

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 Alpha amylase (IUBMB 3.2.1.1) 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:*
Alpha amylase (IUBMB 3.2.1.1) from a Genetically modified *Bacillus subtilis*

Proposed 21C.F.R. § 170.36 (c)(iii) *Applicable conditions of use:*
The alpha amylase enzyme is to be used as a processing aid 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 (Germany) based in Plantation, Florida USA

AB Enzymes, Inc.
8211 W. Broward Blvd. Suite# 375
Plantation, Florida 33324
+888-512-2176

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

DocuSigned by:
[redacted]
452B3329EDDB45F...

i.V. Candice Cryne

Senior Global Regulatory Affairs Manager

05-May-2021 | 16:30 BST

DocuSigned by:
[redacted]
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Joab Trujillo

Junior Regulatory Affairs Specialist

05-May-2021 | 16:36 BST

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AB Enzymes GmbH – Feldbergstrasse 78 , D-6412 Darmstadt

GRAS Notification of an Alpha-amylase from a Genetically Modified *Bacillus subtilis*

AB ENZYMES GmbH

May 5, 2021

Contents

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 Alpha-amylase (IUBMB 3.2.1.1) 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:

Alpha-amylase (IUBMB 3.2.1.1) 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 alpha-amylase enzyme is to be used as a processing aid 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.

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

§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 (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 an **Alpha-amylase from a genetically modified *Bacillus subtilis*.**

2.1.1 Common name of the enzyme

Name of the enzyme protein: Alpha-amylase

Synonyms: Glycogenase, glycoside hydrolase, Endo-amylase, 1,4-α-D-glucan glucanohydrolase

2.1.2 Classification of the enzyme

IUBMB #	3.2.1.1.
Production Strain	*Bacillus subtilis* AR-651

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 3.2.1.1 is for alpha-amylase.

2.2 Identity of the Source

2.2.1 Recipient Strain

The recipient strain used for the construction of the production strain is a genetically modified derivative of a classical *Bacillus subtilis* mutant strain.

The original *Bacillus subtilis*, which has been isolated from soil by the University of Osaka in the year 1974, was characterized as *Bacillus subtilis* by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ German Collection of Microorganisms and Cell Cultures). The strain was further developed by conventional mutagenesis for better yield. The resulting mutant has been used in AB Enzymes for the production of food enzymes since 2010.

For further development, targeted 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 alpha-amylase production strain AR-651.

The parental strain was identified by DSMZ by using the DuPont Identification Library with a similarity to DuPont ID DUP-12544 (*Bacillus subtilis*) of 1.00. The identity of the genetically modified recipient strain was confirmed by multi locus sequence typing (MLST) in 2020.

Therefore, the recipient can be described as follows:

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

2.2.2 Donor:

The alpha-amylase gene described in this application derives from the bacterium *Thermoactinomyces vulgaris* 94-2A (Hofemeister et al. 1994). *Thermoactinomyces vulgaris* is a Biosafety level 1 organism, that are generally regarded as safe to mankind and the environment. The *Thermoactinomyces vulgaris* alpha-amylase gene coding for the mature protein is fused to a signal sequence derived from *Bacillus spec.* and a transcription terminator from *T. vulgaris*. The promoter used for the alpha-amylase expression is from *Bacillus spec.* (Boer et al. 1994; Palva et al. 1981; Hofemeister et al. 1994).

2.3 Genetic modification

The *Bacillus subtilis* strain AR-651 was constructed for alpha-amylase production. The genetically modified *Bacillus subtilis* recipient strain (s.b.) was transformed with the plasmid pAA-A002 carrying the gene encoding an alpha-amylase. The plasmid pAA-A002 contains no genes conferring antibiotic resistance.

At AB Enzymes, *Bacillus subtilis* strains have been used and developed for a long period of time, for the production of various enzymes used in food industrial applications, including amylases. The reason for the genetic modification of the microorganism was to improve the production process and the enzyme yield. The resulting production strain AR-651 secretes high amounts of alpha-amylase into its culture supernatant, resulting in high alpha-amylase activity in the cultivation broth.

The strain **AR-651** was constructed by six genetic modification steps.

Steps 1-5: Markerless gene deletions from the genome of the parental strain:

The *B. subtilis* recipient strain was generated by targeted gene deletions from the genome of the parental *B. subtilis* strain. These deletions were carried out by the well described methods for markerless deletions from the genome of *Bacillus* species (Vehmaanperä et al. 1991; Iordănescu 1975; Rachinger et al. 2013) to get a host strain with improved production performance and an

intended host 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 deletions of native genes from the genome and are not present anymore in the final recipient strain. The deletions of the native genes from the genome of the original *Bacillus subtilis* parental strain 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 AR-651 by introduction of plasmid pAA-A002 into the *Bacillus subtilis* recipient strain:

In the sixth and final step, plasmid pAA-A002 containing the expression cassette for alpha-amylase was introduced into the recipient strain by protoplast transformation according to the method of Chang and Cohen (Chang and Cohen 1979). Transformants were plated on appropriate agar plates for selection of pAA-A002-carrying cells being able to complement the host's auxotrophy.

2.3.1 Genetic stability of the production strain

When implemented, the fermentation process always starts from identical replica of the AR-651 (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 WBC, 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 alpha-amylase activity in a number of fermentation batches performed for the AR-651 strain. The activity measurements from parallel fermentations showed that the productivity of the AR-651 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 AR-651 strain is presented in Appendix # 1.

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

The vector pAA-A002 consists of:

- Defined elements derived from well characterized plasmids pBC16-1 (Kreft et al. 1978) and pUB110 (Gryczan et al. 1978).
- pUB110 was isolated the first time by Gryczan et al. 1978. Ever since it has been used worldwide for the cloning in *Bacilli*. pUB110 is known to be stably maintained in *B. subtilis*, but also in *B. stearothermophilus*, *B. licheniformis*, *B. megaterium* and *B. pumilus* (Nugent 1989).
- The *Thermoactinomyces vulgaris* alpha-amylase gene coding for the mature protein was inserted in an expression cassette composed of a promoter derived from *Bacillus spec.*, a signal sequence from *Bacillus spec.,* and a transcriptional terminator from *T. vulgaris* (Palva et al. 1981; de Boer et al. 1994**,** Hofemeister et al. 1994).
- A native hydrolase derived from *Bacillus spec.*
- 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 plasmid pAA-A002.

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 pAA-A002 have been demonstrated over the full fermentation process.

Thermoactinomyces vulgaris and the *Bacillus* strains are all Biosafety level 1 organisms, that are generally regarded as safe to mankind and environment. In addition, all *Bacillus* strains used here qualify for QPS status. All mentioned donor strains have a safe history of use in biotechnology.

2.3.3 Demonstration of the absence of the GMM in the product

The absence of the GMM in the final enzyme preparation of AR-651 is achieved through filtering after the fermentation process. All viable cells of the production strain AR-651 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 alpha-amylase food enzyme preparation is free of detectable, viable production organism. The absence of the production strain is confirmed for every production batch. Three different samples were analyzed for absence of the production strain as summarized in Appendix #1.

Absence of the production strain in the final product is confirmed by a Roal[2] 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 AR-651 enzyme preparation is free from detectable, viable production organism as demonstrated in the chemical composition analysis report, 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* AR-651 enzyme preparation is produced by an aerobic submerged microbial fermentation using a genetically modified *Bacillus subtilis* strain. All viable cells of the production strain, AR-651, 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 enzymes (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.

2.3.6 Absence of Antibiotic Genes and Toxic Compounds

As mentioned above, the inserted DNA does not contain any antibiotic resistance genes. Furthermore, the production of known toxins according to the specifications elaborated both in Compendium of Food Additive Specifications by the Joint FAO/WHO Expert Committee on Food

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

Additives (Food and Agriculture Organization of the United Nations 2006) and the JECFA specifications for enzyme preparations[3] have been also tested for the fermentation products. Adherence to specifications of microbial counts is routinely analyzed. Three production batches produced by the production strain *Bacillus subtilis* AR-651 (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 Oy[4] by submerged fermentation of *Bacillus subtilis AR*-651 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 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.

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

[3] General Specifications and Considerations for Enzyme Preparations (fao.org)
[4] See footnote 2

- 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 ≤0.15% and ≤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 AR-651 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

After sufficient growth, the biomass is transferred to a seed fermentor, where further growth takes place under agitation and aeration.

2.4.7 Main Fermentation

Finally, the contents of the seed fermentor are transferred into the main fermentor, where enzyme production will take place. The main submerged fermentation is run under specified pH, temperature and aeration conditions, until sufficient enzyme production has taken place.

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 2.5 %.

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 taken into account 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 well-defined Master (MCB) and Working Cell Banks (WCB). The MCB contains the original deposit of the production strain. The WCB is a collection of ampoules containing a pure culture prepared from an isolate of the production strain in MCB. 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 would immediately result in 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 micro-organisms

- Cleaning and sterilization:
  - Validated standard cleaning and sterilization procedures of the production area and equipment
  - Sterilization of all fermentation media
  - Use of sterile air for aeration of the fermentors
- 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 takes 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 suitable 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 control system which reduces the probability of human errors in critical process steps.

These in-process controls comprise:

Microbial controls:

Absence of significant microbial contamination is analyzed by microscopy or plate counts before inoculation of both the seed and main fermentation 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'.

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.

The food enzyme preparation is tested by Quality Control for all related aspects, like expected enzyme activity and the general JECFA Specifications for Food Enzyme Preparations and released by Quality Assurance. The final product is packed in suitable food packaging material before storage. Warehousing and transportation are performed according to specified conditions mentioned on the accordant product label for food enzyme preparations.

2.4.16 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.	220 AZ/g
Appearance	Brown powder	
Particle size distribution	Max 1% > 250µm	

2.5.2. Formulation of a typical enzyme preparation

Enzyme concentrate	20-25
Sunflower oil	0.4
Wheat Flour	Remainder

2.5.3. Molecular mass and amino acid number of the enzyme

The alpha-amylase protein subject for this dossier consists of 453 amino acids with a calculated molecular mass of 52kDa.

2.5.4. Purity and identity specifications of the enzyme preparation

It is proposed that the food enzyme alpha-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: No more than 5 mg/kg

Salmonella sp.: Absent in 25 g of sample

Total coliforms:	Not more than 30 per gram
Escherichia coli:	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).

2.5.5. Composition of the enzyme preparation

For proof that the food enzyme complies with the specifications above, see the chemical composition report in Appendix #1 where 3 different batches were analyzed.

Other enzymatic activities: the food enzyme is standardized on target enzyme activity. Apart from it, the production organism *Bacillus subtilis* produces other endogenous proteins, e.g. amylase, protease. 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

Like any other enzyme, the alpha-amylase acts as a biocatalyst: with the help of the enzyme, a certain substrate is converted into a certain reaction product. It is not the food enzyme itself, but the result of this conversion that determines the effect in the food or food ingredient. After the conversion has taken place, the enzyme no longer performs a technological function.

Substrates: The substrates for alpha-amylase are starch, glycogen and related polysaccharides and oligosaccharides.

- Starch is the major reserve polysaccharide occurring naturally in cereal grains. Their main constituents are amylose which has linear polymers, and amylopectin which has branched polymers. Consequently, the substrate for alpha-amylase occurs naturally in vegetable-based foods.

The function of the alpha-amylase is to catalyze the hydrolysis of the α-(1,4) glycosidic linkages of the mentioned substrates in a random manner. The term α relates to the initial anomeric configuration of the free sugar group released and not to the configuration of the linkage hydrolyzed. Alpha-Amylases are endo-enzymes implying that they hydrolyze starch polymers internally, reducing rapidly the molecular weight of the polymers. The end products are oligosaccharides with varying length DP2-DP12 and branched oligosaccharides that are called α -limit dextrins (MacGregor et al. 2001) (figure 1). Alpha-amylases are also called liquefying amylases because they significantly reduce the viscosity of starch.

The substrate, the reaction products and the alpha-amylase are found by nature in cereal grains.

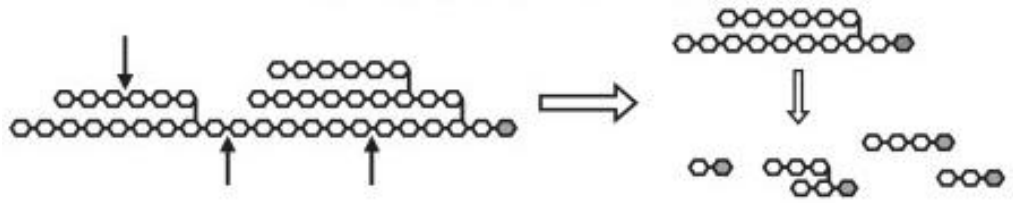


Figure: Schematic representation of the endo-type action of alpha-amylase enzymes on starch (amylopectin) polymers yielding branched and linear low molecular weight dextrins. The grey ring structure represents a reducing glucose residue (Goesaert et al. 2009b).

Reaction products: as a result of the catalytic activity of alpha-amylase, low levels of oligosaccharides are formed. These compounds are already present in the human diet.

Alpha-amylase is naturally present in cereal grains. The natural enzymatic conversion of starch in foods containing cereal grains (or derivatives such as flour) is of technological benefit in several industrial food manufacturing processes. But the levels of endogenous cereal alpha-amylases is

often too low (measured as a high falling number) and varies from batch to batch of raw material and the specificity of the enzyme may not be optimal to give the desired process advantages. Therefore, their content needs to be standardized.

The technological need of the enzymatic conversion of starch by alpha-amylases is the hydrolysis of the starch biopolymer to increase the level of low molecular weight dextrins. In this way, added alpha-amylase facilitates maltose production by the endogenous beta-amylase. Alpha-amylases are often used in combination with other types of enzymes such as maltogenic amylases, xylanases, lipases and proteases.

Alpha-amylase enzyme activity is widely present in nature and in particular in food ingredients. The substrates and the reaction products are themselves present in food ingredients. No reaction products which could be considered normal constituents of the diet are formed during the production or storage of the enzyme treated food. Consequently, no adverse effect on nutrients is expected.

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

Food enzymes preparations are known to have side activities in the form of other proteins i.e. other enzymes. This is because food enzymes preparations 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 alpha-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 alpha-amylase enzyme preparation.

2.7 Allergenicity

There have been reports of enzymes manufactured for use in food to cause inhalation allergy in sensitive workers exposed to the enzyme dust in manufacturing facilities. In the case of alpha-amylase, there is as any other enzymes, a theoretical possibility of causing such occupational allergy in sensitive individuals. However, the possibility of an allergic reaction to the alpha-amylase residues in food seems remote. 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.

- Previously, 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 (Dauvrin 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.
- Enzymes when used as digestive (Abad et al. 2010) aids are ingested daily, over many years, at much higher amounts when compared to enzymes present in food (up to 1 million times more).

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 majority of proteins are not 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[5].
- 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).

[5] The only enzyme protein used in food and known to have a weak allergenic potential is egg lysozyme

- For cases where the proteins are denatured which is the case for this enzyme due to the food process conditions, 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).

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 alpha-amylase cross reacting with known allergens and induce a reaction, the sequence homology 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.

A sequence homology comparison test was performed using a database of allergens from the Food Allergy Research and Resource Program (FARRP), University of Nebraska, Allergen Database

(Version 21, 14 February 2021), which contains the amino acid sequences of known and putative allergenic proteins. The amino acid sequence of the alpha-amylase subject to this dossier was scanned using two search methods. The first method was a FASTA alignment for the full-length alpha-amylase sequence to any allergenic proteins in the Allergen Online database. Some of the resulting alignments showed identities to allergenic proteins above the 35% identity threshold however, below 50% identity. The best hits of the FASTA alignment of the mature alpha-amylase protein to the database proteins showed an identity of 37.7% for Taka-amylase A (Taa-G1) produced by the fungal species *Aspergillus oryzae* and Alpha-amylase A type-1/2 precursor. Aalberse suggested "cross-reactivity is rare below 50% amino acid identity and in most situations requires more than 70% identity" (Aalberse 2000). The identity percentages of all the hits from FARRP were far below the suggested 50 % identity limit, making it unlikely that the alpha-amylase in question can be presumed to be allergenic based on full-length sequence relatedness to known allergens.

In the 80-mer sliding window analysis the alpha-amylase protein sequence did show degrees of identity from 36.2 % to 54.1 % with alpha-amylases from *Aspergillus oryzae* and *Periplaneta Americana*. As recommendation by the FAO/WHO, a possible cross-reactivity has to be considered, when there is more than 35% identity in the amino acid sequence of the expressed protein using an 80 amino acids window and a suitable gap penalty (Food and Agriculture Organization of the United Nations January/2001). This recommendation has however been challenged. According to Ladics et al. (2007) 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 (35% threshold) approach was supported recently by Goodman and Tetteh (2011) who suggested: *" 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 alpha-amylase in question would be just above threshold using the 80-mer sliding window approach for only the alpha-amylase from *A. oryzae*. Since this is a similar enzyme function, it is not surprising that there would be an alignment between the enzyme which is the subject of the current dossier and that of *A. oryzae*.

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 alpha-amylase produced by *Bacillus subtilis* AR-651 is of no concern.

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

As an enzyme, alpha-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 alpha-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 alpha-amylase are amylose, amylopectin and glycogen and related polysaccharides and oligosaccharides which occur naturally in nature and are part of the human diet.

The **function** of alpha-amylase is to catalyze the hydrolysis of the α-(1,4) glycosidic linkages of the mentioned substrates in a random manner.

Like most enzymes, the alpha-amylase performs its technological function during food processing. The alpha-amylase from *Bacillus subtilis* AR-651 object of this notice 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.

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 alpha-amylase in these food processes.

Below, the benefits of the use of industrial alpha-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:

Alpha-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).

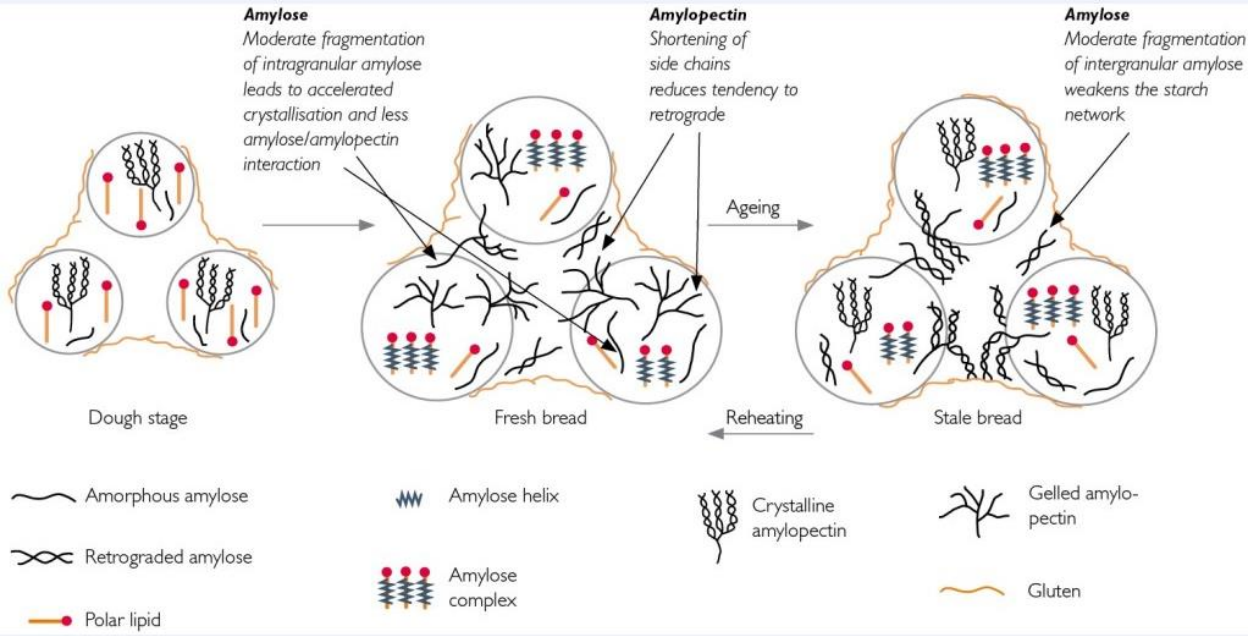
During the dough stages of baking, most of the starch in the flour is in semi-crystalline granules. As higher temperatures are reached in the oven the granular starch begins to gelatinize – to absorb water, swell and lose crystallinity. As the granules begin to rupture, much of the highly soluble amylose 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 maltooligosaccharides (maltose) during baking, alpha-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).

In general, the benefits of starch hydrolysis with the help of alpha-amylase in baking processing are:

- Helps to compensate and equalize the naturally occurring variations of wheat flours own endogenous amylase levels of different crop years due to geography and meteorology.
- Increase the level of fermentable and reducing sugars in dough
- Improves yeast fermentation
- Reduce dough viscosity during the start of starch gelatinization, resulting in a prolonged oven spring.
- Leads to different textual properties of the crumb due to the structural modification

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 alpha-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 on the next page.

The table below shows the range of recommended use levels for each application where the alpha-amylase from *Bacillus subtilis* AR-651 may be used:

Food Application	Raw material (RM)	Suggested recommended use levels (mg TOS/kg RM)
Baking	Flour	100

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.

Alpha-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 alpha-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 alpha-amylase, performs its technological function during the first steps of the baking process. The alpha-amylase is denatured by heat during baking (when higher temperatures above 80°C are reached) and has no further technological effect after baking.

Consequently, it can be concluded that the alpha-amylase does not exert any (unintentional) enzymatic activity in 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 TDMI of alpha-amylase enzyme preparation, the calculation used the suggested 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 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	flour	100	Baked products	0.71	71

* **Assumptions behind ratios of raw material to final food**:

Baking

- *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.*
- *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 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)
71 x 0.0125 = 0.8875	0 x 0.025 = 0	**0.8875**

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 alpha-amylase enzyme from *Bacillus subtilis* AR-651;
- 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.

The Margin of Safety (MoS) for human consumption can be calculated by dividing the NOAEL (see **Section 6**) by the Total Theoretical Maximal Daily Intake (TMDI). Total TMDI of the food enzyme is **0.8875** mg TOS/kg body weight/day. Consequently, the MoS is:

MoS= 1,000/**0.8875** = **1,127**

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 alpha-amylase from *Bacillus subtilis* AR-651 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 sub-sections, 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, inconsistent 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), Schallmey 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 amylases, proteases, glucanases, xylanases, 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 Dijl 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 $6x10^6$ 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 for 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 (Schallmey 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, 1997[6]). It was concluded that *B. subtilis* is not a human pathogen nor is it toxigenic.

[6] https://www.epa.gov/sites/production/files/2015-09/documents/fra009.pdf

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, 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 alpha-amylase) used *Bacillus subtilis*:

Authority	Food enzyme	Reference
JECFA	Maltogenic amylase	TRS 891-JECFA 51/18
	Alpha-Acetolactate decarboxylase	TRS 891-JECFA 51/17
	Carbohydrase and Protease	NMRS 50/TRS 488-JECFA 15/12
	Xylanase	TRS 928-JEFCA 63/42, TRS 928-JECFA 63/42
Australia/NZ	Alpha-Acetolactate decarboxylase Beta amylase Asparaginase Endo-1,4-β-xylanase Beta glucanase Hemicellulase multicomponent enzyme Maltogenic alpha-amylase Metalloproteinase Pullulanase Serine proteinase	Schedule 18 Processing aids
Canada	Alpha-Acetolactate decarboxylase Amylase (maltogenic) Asparaginase Glucanase Hemicellulase Lactase	5. List of Permitted Food Enzymes

	Pentosanase Protease Pullullanase Xylanase	
France	Alpha-Acetolactate decarboxylase Beta glucanase Asparaginase Beta galactosidase Endo-beta-glucanase Maltogenic exo-alpha amylase Glucosyltransferase Hemicellulase Protease Pullulanase Xylanase	Arrêté du 19 octobre 2006
USA[7]	Pullulanase Pectate lyase Branching glycosyltransferase 1,4-alpha branching enzyme Asparaginase Lactase Subtilisin Maltogenic amylase	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 GRAS Notice Inventory, GRN 746

[7] 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 (de Boer Sietske, A. and Diderichsen, B. 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 use[8]

[8] 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)[9]. The US EPA has added *B. subtilis* to the list of exempted organisms in 1997 (USA EPA, 1997)[10].
- 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)[11].

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* AR-651 qualifies for QPS status.

[9] https://osp.od.nih.gov/wp-content/uploads/2019_NIH_Guidelines.htm
[10] https://www.epa.gov/sites/production/files/2015-09/documents/fra009.pdf
[11] 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 in 1997 (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 oral 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 plasmid pAA-A002 results in recombinant strain AR-651. As mentioned before, the recipient strain belongs to a non-pathogenic species. The strain line has been used since 2010 for safe food enzyme production.

The production strain (AR-651) differs from its original parental strain in expressing alpha-amylase, featuring a set of defined genomic deletions and inclusion of hydrolase gene from

Bacillus spec. Besides this, AB Enzymes has not noticed any differences in the production strain AR-651 as compared to the parental strain.

Alpha-amylase (EC 3.2.1.1.) catalyzes the hydrolysis of the α-(1,4) glycosidic linkages of the substrates starch, glycogen and related polysaccharides and oligosaccharides. 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. Alpha-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 alpha-amylase enzyme preparations from various microorganisms (including genetically modified ones) are widely accepted and *Bacillus subtilis* – whether or not genetically modified[12] - 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.

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

Authority	Food enzyme	Reference
JECFA	Alpha-Amylase from *Bacillus subtilis*	710 WHO Food Additives Series 28, TRS 806-JECFA 37/10
	Alpha-Amylase from *Bacillus stearothermophilus* expressed in *Bacillus subtilis*	711 WHO Food Additives Series 28, TRS 806-JECFA 37/10
	Alpha-Amylase from *Bacillus megaterium* expressed in *Bacillus subtilis*	712 WHO Food Additives Series 28, TRS 806-JECFA 37/11

[12] *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).*

Australia/NZ	Alpha-Amylase from *Bacillus subtilis, Bacillus subtilis* containing the gene for α-amylase isolated from *Geobacillus stearothermophilus*[13]	Schedule 18 Processing Aids
Canada	Alpha-amylase from *Bacillus subtilis* var.; *Bacillus subtilis* B1.109 (pCPC800) (pCPC720) (ATCC 39, 705); *Bacillus subtilis* NBA (DS 68703)	5. List of Permitted Food Enzymes
France	Alpha-amylase from non-genetically modified strain of *Bacillus subtilis*; Alpha-amylase from *Bacillus subtilis*	Arrêté du 19 octobre 2006

The alpha-amylase protein overexpressed by AR-651 originates from *Thermoactinomyces vulgaris*. As the alpha-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 *Thermoactinomyces vulgaris* alpha-amylase gene for the production of alpha-amylase in *Bacillus subtilis* AR-651 does not lead to any particular safety concern.

Plasmid pAA-A002

Plasmid pAA-A002 contains no genes conferring antibiotic resistance and there is no transfer function present. The vector itself is fully characterized and free from potential hazards.

Genetic stability of the strain AR-651

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

[13] *Geobacillus stearothermophilus* – former name *Bacillus stearothermophilus*

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 AR-651 strain deposition to the culture collection.

The safety of the alpha-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, and the introduced genetic material does not encode and express any toxic substances, it is concluded that the use of the alpha-amylase from genetically modified *Bacillus subtilis* AR-651 is generally considered as safe.

We consider that the colonization capacity of AR-651 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* AR-651 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* AR-651 must be considered rather low or zero.

As demonstrated above, the alpha-amylase food enzyme from *Bacillus subtilis* AR-651 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 alpha-amylase produced by the genetically modified *Bacillus subtilis* AR-651 is based on the historical safety of 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 AR-651 production strain and recipient are derived from a classical *Bacillus subtilis* mutant parental strain which has been proven to be safe.

AB Enzymes performed toxicological studies on a strain within the strain lineage of AR-651 which produces the same alpha-amylase however does not include the hydrolase gene from *Bacillus spec.* Additionally, the AR-651 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 for the summary of the cytotoxicity study:

Cytotoxicity Study:

Bacillus subtilis AR-651 underwent an analysis of cytotoxicity of culture supernatant of the strain to Vero cells with LDH release assay. The study was conducted by BioSafe – Biological Safety

Solutions Ltd in Finland and was completed on February 3, 2021. The study complies with Good Laboratory Practices and under the current standards of the EU.

The bacterial cells, i.e. a cytotoxic strain *Bacillus cereus* DSM 31 (ATCC 14579), and a non-cytotoxic strain *Bacillus licheniformis* ATCC 14580, were grown in brain heart infusion broth for 6 h and 16 h and the supernatants were collected for cytotoxicity analysis. The cell free culture supernatant samples of *Bacillus* strain AR-651 were provided by AB Enzymes. The Vero cells were exposed to the bacterial supernatants for 3 h. Triton X-100 was used as a control for 100% LDH release. Vero cells exposed to cell culture medium without fetal bovine serum were used as a non-cytotoxicity control.

Results:

The cell-free supernatants of strain AR-651 (LDH release -0.5% after 6 h culture in brain heart infusion broth) and *Bacillus licheniformis* ATCC 14580 (1.0%) were not cytotoxic to Vero cells. The cell-free supernatant of *Bacillus cereus* DSM 31 (ATCC 14579) was extremely cytotoxic (73.0%).

Conclusion:

Bacillus strain AR-651 culture supernatant did not exceed the 20% toxicity threshold and was not hence cytotoxic to Vero cells.

The following studies were performed on a *Bacillus subtilis* alpha-amylase strain from the AR-651 strain lineage:

- Reverse Mutation Assay using Bacteria (*Salmonella typhimurium*) with Alpha-amylase produced with *Bacillus subtilis*
- *In vitro* Mammalian Micronucleus Assay in Human Lymphocytes with Alpha-amylase produced with *Bacillus subtilis*
- 90-Day Repeated Dose Oral Toxicity Study in Wistar Rats with Alpha-amylase produced with *Bacillus subtilis*

Alpha-amylase that has been tested is a liquid ultra-filtrated concentrate, before its formulation into a food enzyme preparation.

All tests were performed according to the principles of Good Laboratory Practices (GLP) and the current OECD and EU guidelines.

The full reports of the safety studies performed are summarized below.

Reverse Mutation Assay using Bacteria (*Salmonella typhimurium*) with Alpha-amylase produced with *Bacillus subtilis*

In order to investigate the potential of Alpha-amylase produced with *Bacillus subtilis* for its ability to induce gene mutations the plate incorporation test (experiment I) and the pre-incubation test (experiment II) were performed with the *Salmonella typhimurium* strains TA 98, TA 100, TA 1535, TA 1537 and TA 102.

In two independent experiments several concentrations of the test item were used. Each assay was conducted **with** and **without** metabolic activation) in experiment I and II.

No biologically relevant increases in revertant colony numbers of any of the five tester strains were observed following treatment with Alpha-amylase produced with *Bacillus subtilis* at any concentration level, neither in the nor absence of metabolic activation in experiment I and II.

In conclusion, it can be stated that during the described mutagenicity test and under the experimental conditions reported, Alpha-amylase produced with *Bacillus subtilis* did not cause gene mutations by base pair changes or frameshifts in the genome of the tester strains used.

Therefore, Alpha-amylase produced with *Bacillus subtilis* is considered to be non-mutagenic in this bacterial reverse mutation assay.

In vitro* Mammalian Micronucleus Assay in Human Lymphocytes with Alpha-amylase produced with *Bacillus subtilis

In order to investigate a possible potential of Alpha-amylase produced with *Bacillus subtilis* to induce micronuclei in human lymphocytes an *in vitro* micronucleus assay was carried out. The following study design was performed:

	Without S9		With S9
	Exp. 1	Exp. II	Exp. I
Exposure period	4 h	44 h	4 h
Cytochalasin B exposure	40 h	43 h	40 h
Preparation interval	44 h	44 h	44 h
Total culture period*	92 h	92 h	92 h

*Exposure started 48 h after culture initiation

The selection of the concentrations was based on data from the pre-experiment. In the main experiment I **without** and **with** metabolic activation 500 µg/mL test item and in experiment II 50 µg/mL test item was selected as the highest concentration for microscopic evaluation.

The following concentrations were evaluated for micronuclei frequencies:

Experiment I with short-term exposure (4 h):
without metabolic activation: 100, 150, 250 and 500 µg/mL
with metabolic activation: 100, 200, 400 and 500 µg/mL

Experiment II with long-term exposure (44 h):
without metabolic activation: 10, 25 and 50 µg/mL

If cytotoxicity is observed the highest concentration evaluated should not exceed the limit of 55% ± 5% cytotoxicity according to the OECD Guideline 487 (OECD 2016) [4]. Higher levels of cytotoxicity may induce chromosome damage as a secondary effect of cytotoxicity. The other concentrations evaluated should exhibit intermediate and little or no toxicity. However, OECD 487 does not define the limit for discriminating between cytotoxic and non-cytotoxic effects.

According to laboratory experience this limit is a value of the relative cell growth of 70% compared to the negative/solvent control which corresponds to 30% of cytostasis.

In both experiments an increase of the cytostasis above 30% was noted. In experiment I an increase of the cytostasis was noted at 250 µg/mL and higher (**without** metabolic activation) and at 400 µg/mL and higher (**without** metabolic activation). In experiment II an increase of the cytostasis was seen at 50 µg/mL (**without** metabolic activation).

In experiment I **without** and **with** metabolic activation and in experiment II **without** metabolic activation no biologically relevant increase of the micronucleus frequency was noted after treatment with the test item.

The nonparametric X^2 Test was performed to verify the results in both experiments. No statistically significant enhancement ($p<0.05$) of cells with micronuclei was noted in the dose groups of the test item evaluated in experiment I **with** and **without** metabolic activation and in experiment II **without** metabolic activation.

The X^2 Test for trend was performed to test whether there is a concentration-related increase in the micronucleated cells frequency in the experimental conditions. No statistically significant increase in the frequency of micronucleated cells under the experimental conditions of the study was observed in experiment I and II.

Methylmethanesulfonate (MMS, 50 µg/mL) and cyclophosphamide (CPA, 15 µg/mL) were used as clastogenic controls. Colchicine (Colc, 0.02 µg/mL and 0.4 µg/mL) was used as aneugenic control. All induced distinct and statistically significant increases of the micronucleus frequency. This demonstrates the validity of the assay.

In conclusion, it can be stated that during the study described and under the experimental conditions reported, the test item Alpha-amylase produced with *Bacillus subtilis* did not induce structural and/or numerical chromosomal damage in human lymphocytes.

Therefore, Alpha-amylase produced with *Bacillus subtilis* is considered to be non-mutagenic with respect to clastogenicity and/or aneugenicity in the *in vitro* Mammalian Cell Micronucleus Test.

90-Day Repeated Dose Oral Toxicity Study in Wistar Rats with Alpha-amylase produced with *Bacillus subtilis*

The aim of this study was to assess the possible health hazards which could arise from repeated exposure of Alpha-amylase produced with *Bacillus subtilis* via oral administration to rats over a period of 90 days.

The test item was administered daily in graduated doses to 3 groups of test animals, one dose level per group for a treatment period of 90 days. Animals of an additional control group were handled identically as the dose groups but received aqua ad injectionem (sterile water), the vehicle used in this study. The 4 groups comprised of 10 male and 10 female Wistar rats.

The following doses were evaluated:

Control: 0 mg/kg body weight

Low Dose: 100 mg/kg body weight

Medium Dose: 300 mg/kg body weight

High Dose: 1000 mg/kg body weight

The test item formulation was prepared at least every 10 days. The test item was dissolved in aqua ad injectionem and administered daily during a 90-day treatment period to male and female animals. Dose volumes were adjusted individually based on weekly body weight measurements.

During the period of administration, the animals were observed precisely each day for signs of toxicity. The animal that had to be sacrificed for animal welfare reasons was examined macroscopically and at the conclusion of the test, surviving animals were sacrificed and observed macroscopically.

Body weight and food consumption were measured weekly. At the conclusion of the treatment period, all animals were sacrificed and subjected to necropsy. The wet weight of a subset of tissues was taken and a set of organs/tissues was preserved.

A full histopathological evaluation of the tissues was performed on high dose and control animals. These examinations were not extended to animals of all other dosage groups. Only organs and tissues of the other dosage groups showing changes in the high dose group were examined.

Conclusion:

No mortality occurred in the control or any of the dose groups during the treatment period or recovery period of this study with exception of male animal no. 25 (MD group), which was euthanised in a moribund condition for animal welfare reasons. The animal was seen with abnormal breathing on study day 36. No findings were recorded at necropsy and according to histopathology evaluation, the cause of morbidity remained elusive for this animal and was considered most likely not test item related.

No clinical findings related to a systemic effect of the test item were observed in this study.

No test item related effect on body weight and food consumption was observed in females.
No test item related effect on haematology, coagulation parameters, urine and clinical chemistry parameters was observed.

Based on histopathological evaluation there were no gross lesions observed at necropsy that were considered to be related to treatment with test item.

No statistically significant differences in organ weight were found in male and female animals at any of the dose levels tested when compared to controls and no test item related changes in organ weight were observed.

No test item-related histopathological changes were observed in any organ. The no observed adverse effect level (NOAEL) of Alpha-amylase produced with *Bacillus subtilis* in this study is considered to be 1000 mg/kg body weight/day.

Comments on the safety of AR-651

The original 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. The hydrolase gene added to the production strain is minor and does not impact the function of the alpha-amylase as described in section 2.2.2.

It is concluded that the use of the alpha-amylase produced with the current genetically modified *Bacillus subtilis* AR-651 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 alpha-amylase from genetically modified *Bacillus subtilis* AR-651 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. AR-651 Chemical Composition Report
2. Flow Chart of the manufacturing process with control steps
3. Pariza and Johnson Decision Tree

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DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

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

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

FDA USE ONLY

GRN NUMBER	DATE OF RECEIPT
GRN 001011	May 5, 2021
ESTIMATED DAILY INTAKE	INTENDED USE FOR INTERNET
NAME FOR INTERNET	
KEYWORDS	

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

SECTION A – INTRODUCTORY INFORMATION ABOUT THE SUBMISSION

1. Type of Submission *(Check one)*

☒ New ☐ Amendment to GRN No. _____ ☐ Supplement to GRN No. _____

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

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

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

SECTION B – INFORMATION ABOUT THE NOTIFIER

1a. Notifier

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

City	State or Province	Zip Code/Postal Code	Country
Plantation	Florida	33324	United States of America

Telephone Number	Fax Number	E-Mail Address
+1 954 800 8606		joab.trujillo@abenzymes.com

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

Name of Contact Person	Position or Title
Organization *(if applicable)*	
Mailing Address *(number and street)*	

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

Alpha amylase (IUBMB 3.2.1.1) 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 _____

☐ 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 alpha amylase enzyme is to be used as a processing aid 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.

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 Alpha amylase (IUBMB 3.2.1.1) 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. agrees to make the data and information that are the basis for the conclusion of GRAS status available to FDA if FDA asks to see them; agrees to allow FDA to review and copy these data and information during customary business hours at the following location if FDA asks to do so; agrees to send these data and information to FDA if FDA asks to do so.
(name of notifier)

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 or his/her knowledge. Any knowing and willful misinterpretation is subject to criminal penalty pursuant to 18 U.S.C. 1001.

3. Signature of Responsible Official, Agent, or Attorney	Printed Name and Title	Date (mm/dd/yyyy)
Joab Trujillo Digitally signed by Joab Trujillo Date: 2021.05.05 09:43:44 -04'00'	Joab Trujillo Junior Regulatory Affairs Specialist	05/05/2021

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 Alpha Amylase.pdf	Administrative
	Cover Letter for Alpha Amylase GRAS Notice.pdf	Administrative
	GRAS Notice Alpha Amylase AB Enzymes.pdf	Submission
	1_ Alpha Amylase Composition Report AB Enzymes.pdf	Submission
	2_Generic Flow chart Manufacturing process.pdf	Submission
	3_Pariza and Johnson Decision Tree.pdf	Submission
	References for AB Enzymes GRAS Notice.zip	Submission

To: Dr. Katie Overbey

Division of Food Ingredients
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition

Regulatory Affairs
E-Mail info@abenzymes.com
Date: 2022-02-08

RE: Questions for Notifier of GRN 1011

1. We request that the notifier provide a Chemical Abstracts Service number for the alpha-amylase.

AB Enzymes' Response:

The Chemical Abstracts Service number for the alpha-amylase is the following, CAS # 9000-90-2

2. We request strain deposition information for the production strain *Bacillus subtilis* AR-651.

AB Enzymes' Response:

The *Bacillus subtilis* production strain AR-651 is deposited in the Westerdijk Fungal Biodiversity Institute, formerly known as the "Centraalbureau voor Schimmelcultures" (CBS) in the Netherlands with the deposit number CBS 147460.

3. Please confirm that you verified sequence integration and state the method used.

AB Enzymes' Response:

We confirm that the alpha-amylase gene was correctly inserted into the plasmid. The *B. subtilis* recipient strain was then transformed with the plasmid. The expression plasmid is stably kept in the recipient cell. Both the plasmid and the whole genome of AR-651 *Bacillus subtilis* were sequenced using Whole Genome Sequencing (WGS). The alpha-amylase cassette was not integrated into the genome of *Bacillus subtilis*, instead the genetic information was kept on the plasmid (extrachromosomally).

4. Please state if the production strain genome, not just the plasmid, contains any antibiotic resistance genes and the method used to determine this. Additionally, please clarify the statement "complements the host's auxotrophy" (p8).

AB Enzymes' Response:

Bacillus subtilis AR-651 production strain genome does not contain any acquired antibiotic resistance genes from the genetic modification process (i.e., construction of the production strain). *Bacillus subtilis* does contain inherent genes which potentially could be involved in antibiotic resistance as part of the bacterium's defense system. As mentioned in our response to question #3, we sequenced the production strain genome using Whole Genome Sequencing (WGS). The sequence of the genome was compared to a database on genes involved in antimicrobial resistance. We want to highlight the following, just because potential genes were detected: the presence of a gene does not give information on the gene being expressed at all.

The comparison analysis revealed three genes which might be involved in antimicrobial resistance however these genes are intrinsic to *Bacillus subtilis* and are therefore not a safety concern. These genes are highly conserved in *Bacillus subtilis and its close relatives* and are not transferred. *Bacillus subtilis* and its close relatives have been used for the manufacture of food products for decades. In the USA, *Bacillus subtilis* has been recognized to be a GRAS organism by FDA.

To clarify the statement, "complements the host's auxotrophy," this statement replaces antibiotic resistance markers. Antibiotic resistance markers were used as selection markers in the past all over the world for keeping a plasmid stably in a cell. The cells were not able to grow in the presence of the corresponding antibiotic if the plasmid was lost. To note, the *Bacillus subtilis* AR-651 production strain does not contain any antibiotic resistance markers. Instead, for keeping the plasmid in the cell, a gene encoding an essential protein (for the cell's metabolism) was deleted from the host's genome and provided by the plasmid. The cells which have lost the plasmid cannot grow anymore (if the metabolite is not provided by the cultivation medium) because they do not have this essential gene. They are auxotrophic. Only

cells, containing the plasmid which provides the essential gene, i.e., complements the host's auxotrophy, can grow.

5. Based on your description of the construction of the production strain, this enzyme preparation contains both non-native alpha amylase and hydrolase. Please provide the purpose of inserting the hydrolase gene.

AB Enzymes' Response:

The purpose of inserting the hydrolase gene was to aid in the manufacturing process of the final preparation. The hydrolase aids in the recovery step of the manufacturing process by reducing and/or preventing an increase in thickness of the fermentation broth after the fermentation process.

6. Based on the narrative you have provided, the unpublished studies support the safety of the alpha amylase enzyme produced by AR-651 but not the hydrolase. Please provide a short narrative on the safety of the production strain that makes the article of commerce, i.e., the alpha-amylase and the hydrolase.

AB Enzymes' Response:

The safety narrative for the *Bacillus subtilis* AR-651 production strain that expresses the alpha-amylase and co-expresses the hydrolase can be concluded from the following points:

- The genetic modifications used to create the production strain are well characterized
- History of use of *Bacillus subtilis and close relatives* as an enzyme producer in food
- Supplemental safety data presented in the GRAS narrative

In the GRAS narrative we have provided information on the genetic modifications that took place to create the *Bacillus subtilis* AR-651 production strains. A series of native gene deletions were conducted from the genome of the original *Bacillus subtilis* parental strain. The deletions were carefully monitored by PCR and sequencing revealing that no DNA-fragments of the deletion vectors remained in the cell. We confirmed that the alpha-amylase gene was inserted correctly into the pAA-002 vector. We sequenced the vector and the genome of *Bacillus subtilis* AR-651 to confirm genetic stability and the correct sequence of the plasmid containing the genes of the target enzyme, alpha-amylase, and the co-expressed hydrolase. As noted in section 2.3.2 of the GRAS narrative the components of the pAA-002 vector, including elements derived from vectors pBC16-1 and pUB110, can be regards as safe. pAA-002 vector does not contain any antibiotic resistance genes and we explained in our response to question #4 that the production strain does not have any acquired antibiotic resistance genes resulting from the genetic modifications.

B. subtilis as a production organism 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 (Boer and Diderichsen 1991). As mentioned in the GRAS narrative under section 6.1. "safety of the production strain" *Bacillus subtilis* as a production organism for food enzymes is generally recognized as safe. In the case of alpha-amylase enzyme preparations *B. subtilis* has been used as a production host for decades, starting in 1929 for the manufacture of chocolate syrup to reduce viscosity.

The source organism of the hydrolase gene is closely related to *B. subtilis*. Taxonomically, it belongs to the *B. subtilis* species complex (Ngalimat et al. 2021; Fan et al. 2017; Fritze 2004). A number of members of this taxonomical group, like *B. subtilis*, have a long history of safe use. It is not uncommon for the *Bacillus species* to natively produce different types of hydrolases including the one present in the *Bacillus subtilis* AR-651 production strain. In section 6.2.1. of the narrative, we provided a summary of a cytotoxicity study conducted on *Bacillus subtilis* AR-651 production strain using Vero cells. The conclusion of the cytotoxicity study was the production strain is not cytotoxic. In section 2.4 "Enzyme Production Process" of the GRAS narrative, we have demonstrated that the manufacturing of the commercial

enzyme preparation containing the enzymes from AR-651 is done in accordance with current Good Manufacturing Practices.

Even with the co-expression of the hydrolase from *Bacillus spec.* the production strain still qualifies for QPS status. If 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* AR-651 qualifies for QPS status.

7. Based on the description of the construction of the production strain, it appears that the hydrolase activity is distinct from activity of native hydrolase. Please provide a short narrative on how the presence of the hydrolase in the final enzyme preparation will affect the intended use of the article of commerce.

AB Enzymes' Response:

The hydrolase is not expected to be in the final preparation in significant amounts. We consider the hydrolase to be a minor side activity in the final enzyme preparation based on our internal analysis for hydrolase activity. The analysis consisted of testing the protein content of three independent pilot fermentation batches from *Bacillus subtilis* AR-651 production strain via SDS Page and hydrolase activity analysis. The hydrolase activity in the three fermentation samples was determined and compared using fermentation samples of a *Bacillus* strain overexpressing the hydrolase enzyme as a positive control and a *Bacillus subtilis* tox tested strain as a negative control. The negative control strain does not have the hydrolase co-expression but possesses a native hydrolase like all B. subtilis strains. The summaries of the toxicological studies presented in the GRAS narrative demonstrate that the *Bacillus subtilis* tox tested strain is non-mutagenic with a NOAEL of 1000 mg/kg body weight/day. The hydrolase activity in the three independent pilot batches was not

higher than that in the negative control. SDS PAGE confirmed a very low content of the hydrolase. Based on these results it could be concluded that the hydrolase is only a minor enzyme side activity.

We do not anticipate the presence of these minor amounts of the hydrolase in the final enzyme preparation to have a significant impact or play a subsidiary (supportive) role for the function of the final enzyme preparation in the intended food processes (baking). As mentioned in our reply to question #5, the function of the hydrolase was to aid in the recovery step of manufacturing the final preparation. The hydrolase is a minor side activity produced by the production microorganism, *Bacillus subtills*.

8. Please state if the fermentation medium contains allergens and if these allergens are removed in the final product.

AB Enzymes' Response:

The fermentation medium does not contain any allergens. It is important to note that the commercial enzyme preparation does contain wheat flour which has gluten.

9. On p.28, you state: "… the bioinformatics approach to estimate potential allergenicity and cross-reactivity based on relatedness to known allergen and taking into account the most recent scientific recommendations on the interpretation of such data lead us to conclude that the alpha-amylase produced by Bacillus subtilis AR-651 is no concern."

   We note that the two citations you provided to justify the use of a 50% threshold over a 35% threshold in the 80-mer analysis (Ladics et al., 2007 & Goodman and Tetteh, 2011) reflect only the opinions of several groups of scientists, and it appears there is not supportive evidence that their views are generally recognized and accepted by the scientific community.

We further note that you state on p28: "In the 80-mer sliding window analysis the alpha-amylase protein sequence did show degrees of identity from 36.2% to 54.1% with alpha-amylases from *Aspergillus oryzae* and *Periplaneta Americana*."

It is not clear how you arrived at the conclusion that identities detected "just above" the "recommended" 50% threshold to aforementioned alpha-amylases are not a safety concern.

AB Enzymes' Response:

To provide more context to our conclusion on allergenicity from the GRAS narrative, we present the following additional information to add to the weight of evidence. To start off, the hits above 35% homology were related to the target enzyme, alpha-amylase. As the amino acid sequence of the alpha amylase from *Bacillus subtilis* AR-651 production strain was used to run the searches, hits for other alpha amylases from other sources is not unreasonable. For the 80-mer search we presented the percentage ID of the three hits 36.2-54.1 %, the full alignment ID% for the hits is slightly lower, please refer to the table below.

Hit #	Name of Hit	Species	Best %ID	Hits > 35%	Full Alignment E-value	Full Alignment % ID	Full Alignment length
1	Alpha-amylase A type ½ precursor	*Aspergillus oryzae*	54.10%	240 of 374	8.9 e-048	37.70%	470
2	Taka-amylase A (Taa-G1) precursor	*Aspergillus oryzae*	54.10%	242 of 374	5.5 e-048	37.70%	469
3	Alpha-amylase [Periplaneta American	*Periplaneta americana*	36.20%	4 of 374	1.4	24.30%	452

Regarding hits 1 and 2 in the table, alpha-amylase from *A. oryzae* is known as an occupational respiratory allergen associated with baker's asthma. However, several studies have shown that adults with occupational asthma to a food enzyme (like α-amylase from *A. oryzae*) may be able to ingest the corresponding enzyme without acquiring clinical symptoms of food allergy (Cullinan et al. 1997; Poulsen 2004; Armentia et al. 2009). Considering this information and the wide use of α-amylase as a food enzyme without major reported issues, the risk of allergic sensitization and elicitation reactions via the consumption of the enzyme subject for this dossier, under the intended conditions of use, can be excluded. Truly, quantifying the risk for allergenicity is not possible in view of the individual susceptibility to food allergens.

Allergen hit 3 is an alpha amylase identified from the American cockroach, analysis has shown there is a possibility that the α-amylase protein of *P. americana* is also a dust allergen associated with the cockroach species. Cockroaches are found in flour and it is highly possible that the dust allergen, α-amylase, is transferred from the flour to the cockroaches[1].

No information is available on oral and respiratory sensitization or elicitation reactions of this alpha-amylase. When describing the fate of the enzyme in the food manufacturing of baking products in the GRAS narrative, the enzyme serves as a processing aid and is inactivated doing the manufacturing process. In the case that the enzyme is digested by consumers of the final food, the optimum pH of the enzyme is 4.5 where the digestive acids of the stomach is pH of 2, the enzyme cannot survive in such conditions. Therefore, the hits shown above in table 3 are inhalation allergens and the risk of allergic sensitization and elicitation reactions are considered to be rare.

[1] In Silico Identification of Potential American Cockroach (Periplaneta americana) Allergens (nih.gov)

Publication bibliography

Armentia, A.; Díaz-Perales, A.; Castrodeza, J.; Dueñas-Laita, A.; Palacin, A.; Fernández, S. (2009): Why can patients with baker's asthma tolerate wheat flour ingestion? Is wheat pollen allergy relevant? In *Allergologia et immunopathologia* 37 (4), pp. 203–204. DOI: 10.1016/j.aller.2009.05.001.

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Fritze, Dagmar (2004): Taxonomy of the genus bacillus and related genera: the aerobic endospore-forming bacteria. In *Phytopathology*® 94 (11), pp. 1245–1248. DOI: 10.1094/PHYTO.2004.94.11.1245.

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Division of Food Ingredients
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition

Regulatory Affairs
E-Mail info@abenzymes.com
Date: 2022-03-24

RE: Questions for Notifier of GRN 1011

1. Please confirm that the levels and activity of the non-native hydrolase are not expected to have a safety profile different from the native hydrolase of the production strain, and thus do not impact the GRAS conclusion of your enzyme preparation. We note that the FDA had a similar question in GRN 974, and would like to confirm if the use of the hydrolase is the same as in that notice.

AB Enzymes' Response:

We confirm that the levels and activity of the non-native hydrolase are not expected to have a safety profile different from the native hydrolase of the production strain, and thus do not impact the GRAS conclusion of our enzyme preparation. We also confirm that the use of the hydrolase is the same as in GRN 974.

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