November 30, 2022

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 endo-1,4-β-xylanase (IUBMB 3.2.1.8) 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 420
Plantation, FL 33324 USA

Proposed 21C.F.R. § 170.36 (c)(ii) *The common or usual name of notified substance:*
Endo-1,-4-β-xylanase (IUBMB 3.2.1.8) from a Genetically modified *Bacillus subtilis*

Proposed 21C.F.R. § 170.36 (c)(iii) *Applicable conditions of use:*
The endo-1,4-β-xylanase enzyme is to be used as a processing aid in baking processes, grain processing and brewing 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).

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

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

i.V. Candice Cryne

Senior Global Regulatory Affairs Manager

30-Nov-2022 | 22:30 GMT

Joab Trujillo

Regulatory Affairs Specialist

30-Nov-2022 | 22:31 GMT

AB Enzymes, Inc.
8211 W. Broward Blvd. Suite# 420
Plantation, Florida 33324

AB Enzymes GmbH – Feldbergstrasse 78 , D-6412 Darmstadt

GRAS Notification of an endo-1,4-β-xylanase from a Genetically Modified *Bacillus subtilis*

AB ENZYMES GmbH

November 30, 2022

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 **endo-1,4-β-xylanase** (IUBMB# 3.2.1.8) from a Genetically Modified *Bacillus subtilis* produced by fed-batch 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 420

Plantation, FL 33324 USA

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

Endo-1,4-β-xylanase (IUBMB 3.2.1.8) 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 **endo-1,4-β-xylanase** is to be used as a processing aid in baking processes, grain processing and brewing 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 (Germany) 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 **endo-1,4-β-xylanase from a genetically modified *Bacillus subtilis*.**

2.1.1 Common name of the enzyme

Name of the enzyme protein: endo-1,4-β-xylanase

Synonyms: endo-(1→4)-β-xylan 4-xylanohydrolase; endo-1,4-xylanase; xylanase; β-1,4-xylanase; endo-β-1,4-xylanase; endo-1,4-β-D-xylanase; 1,4-β-xylan xylanohydrolase; β-xylanase; β-1,4-xylan xylanohydrolase; β-D-xylanase

2.1.2 Classification of the enzyme

IUBMB #	3.2.1.8
CAS #	9025-57-4

EC 3. is for hydrolyases;

EC 3.2. is for glycosylases;

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

EC 3.2.1.8 is for endo-1,4-β-xylanase.

2.2 Strain Lineage Information

2.2.1 Production Strain

Production strain	*Bacillus subtilis* AR-153

Synopsis

Endo-1,4-β-xylanase from *Bacillus subtilis* is produced from a genetically modified *Bacillus subtilis* production strain (AR-153). The genetic modifications conducted to develop the production strain are described in section 2.3 of the GRAS narrative along with confirmation of the successful incorporation of the expression plasmid episomally in *Bacillus subtilis*, the stability of the production strain, absence of DNA, antibiotic genes, and toxic compounds. Information on the safety of the *Bacillus subtilis* production strain is provided in section 6 of the GRAS narrative. In this notice, we provide information on that the *Bacillus subtilis* production organism is non-pathogenic and non-toxigenic. In short, safety of production strain is substantiated by the safety of the genetic modifications, history of safe use for *Bacillus subtilis* as a food enzyme producer and the use of the safe strain lineage concept described in Pariza and Johnson (2001).

AB Enzymes has submitted GRAS notice to FDA in the past for enzymes produced from *Bacillus subtilis* production strain and has received 'No Questions' letters.

AB Enzymes' Previous GRAS Notices for Enzymes from *Bacillus subtilis* production strains

GRAS Notice	**Description**
GRAS Notice 746[2]	Maltogenic amylase from *Geobacillus stearothermophilus* produced in *Bacillus subtilis*
GRAS Notice 974[3]	Maltogenic alpha-amylase enzyme preparation produced by *Bacillus subtilis*

[2] GRN No. 746
[3] GRN No. 974

GRAS Notice 1011[4]	Alpha-amylase enzyme preparation produced by *Bacillus subtilis* AR-651 expressing the gene encoding alpha-amylase from *Thermoactinomyces vulgaris*

The *Bacillus subtilis* production strain AR-153 is deposited in the Westerdijk Fungal Biodiversity Institute, formerly known as the "Centraalbureau voor Schimmelcultures" (CBS) in the Netherlands with the deposit number CBS 147461.

Taxonomy*:* the production strain can thus be described as follows:

Kingdom:	Bacteria
Division:	*Firmicutes*
Class:	Bacilli
Order:	Bacillales
Family:	Bacillaceae
Genus:	Bacillus
Species:	*Bacillus subtilis*
Strain:	*Bacillus subtilis* AR-153

2.2.2. Recipient Strain

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

The original *Bacillus subtilis*, which had 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

[4] GRN No. 1011

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 section 2.3 for more details) to improve strain and production performance, resulting in the current recipient strain used for the construction of the xylanase production strain AR-153.

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.

Therefore, the recipient can be described as followed:

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

2.2.3 Donor

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

B. subtilis is a ubiquitous soil microorganism that contributes to nutrient cycling when biologically active due to the various enzymes produced by members of the species. *B. subtilis* is a Gram-positive bacterium which multiplies and disseminates by asexual processes. Well-established protocols for genetic manipulation of *B. subtilis* are one of the prime reasons why bacilli have

been extensively used in both applied and fundamental scientific research for more than 50 years. In 1990, a European-Japanese research collaboration was started with the aim to sequence the entire genome of *B. subtilis* strain 168. This has led to the publication of the entire annotated genome sequence in 1997 (Kunst et al. 1997).

B. 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, endo-1,4-β-xylanase, etc. 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 *B. subtilis* (natto). The production of natto dates back more than a thousand years and was first practiced in Japan (HARA and UEDA 1982).

2.3 Genetic modification

The *Bacillus subtilis* strain AR-153 was constructed for xylanase production. The genetically modified *Bacillus subtilis* recipient strain (s.b.) was transformed with the plasmid pXY-B001 carrying the gene encoding the endo-1,4-β-xylanase. The plasmid pXY-B001 contains no genes conferring antibiotic resistance.

Bacillus subtilis strains have been used and developed at AB Enzymes for a long period of time, to produce 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-153 secretes high amounts of xylanase into its culture supernatant, resulting in increased xylanase activity in the cultivation broth.

The strain **AR-153** was constructed by a number of genetic modification steps.

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 nor in the production 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.

Final step: Construction of production strain AR-153 by introduction of plasmid pXY-B001 into the *Bacillus subtilis* recipient strain:

In the final step, plasmid pXY-B001 containing the expression cassette for the xylanase 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 pXY-B001-carrying cells being able to complement the host's auxotrophy.

To clarify the statement, "complement the host's auxotrophy": The complementation of the host's auxotrophy was used instead of a plasmid selection system basing on 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-153 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.

Verification of Sequence Integration:

We confirm that the xylanase gene was correctly inserted into plasmid pXY-B001. The *B. subtilis* recipient strain was transformed with the plasmid resulting in the plasmid stably kept in the recipient cell. Both the plasmid and the whole genome of *Bacillus subtilis* AR-153 were sequenced using Whole Genome Sequencing (WGS). The xylanase cassette was not integrated into the chromosome of *Bacillus subtilis*, instead the genetic information was kept on the plasmid (extrachromosomally).

2.3.1 Genetic stability of the production strain

When implemented, the fermentation process always starts from identical replicas of the AR-153 (production strain) seed ampoule. Production preserves from the "Working Cell Bank" are used to start the fermentation process. A Working Cell Bank is a collection of ampoules containing a pure culture. The cell line history and the production of a Cell Bank, propagation, preservation and storage is monitored and controlled. The WCB is prepared from a selected strain. A WCB ampoule is only accepted for production runs if its quality meets the required standards. This is determined by checking identity, viability, microbial purity and productivity of the WCB ampoule. The accepted WCB ampoule is used as seed material for the inoculum.

The production starts from "Working Cell Bank" preserves. A Petri dish is inoculated from the culture collection preserve in such a way that single colonies can be selected. The 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). Subsequently the shake flasks are combined for the inoculation of the first process bioreactor.

Testimony to the stability of the strain is given by monitoring the growth behavior during fermentation and by comparable levels of xylanase activity in the AR-153 fermentations. The activity measurements from parallel fermentations showed that the productivity of the AR-153 strain remains similar. This clearly indicates that the strain is stable. The data of the analysis of

enzyme activities of enzyme preparations from different fermentation batches of the recombinant AR-153 strain is presented in Appendix #1.

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

The vector pXY-B001 contains the following components important to the *Bacillus subtilis* AR-153 production strain:

- The endo-1,4-β-xylanase gene, and signal sequence from *Bacillus subtilis* for overexpression of the notified enzyme
- The gene from the parental *B. subtilis* strain complementing the host's auxotrophy which was formerly introduced by deleting this gene from the recipient strain's genome (as mentioned section 2.3)
- A native hydrolase derived from *Bacillus* spec.

No genes conferring antibiotic resistance or encoding any transfer functions are present in plasmid pXY-B001.

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 pXY-B001 have been demonstrated over the full fermentation process.

The purpose of inserting the hydrolase gene was to aid in the manufacturing process of the final enzyme preparation as described in previous GRAS notices, GRN #974, GRN #1011.

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-153 is achieved by a number of down-stream processing steps (refer to sections 2.4.8 – 2.4.13) removing all viable cells of the production strain AR-153. The procedures are completed by trained staff based on documented standard operating procedures complying with the requirements of the quality system.

The endo-1,4-β-xylanase 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[5] 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

As the absence of the production strain is confirmed for every production batch (section 2.3.3), no additional information regarding the inactivation of the GMM cells is required.

2.3.5 Information on the possible presence of DNA

The *Bacillus subtilis* AR-153 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-153, are removed during the down-stream processing: the fermentation broth is filtered with pressure and sheet filters, and concentrated with ultra-filtration.

After this the final product does not contain any detectable number of colony forming units or recombinant DNA. Three discrete food enzyme samples (liquid enzyme concentrates) 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 Additives (Food and Agriculture Organization of the United Nations 2006) and the JECFA

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

specifications for enzyme preparations[6] has 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-153 (concentrates) were analyzed and no antibiotic or toxic compounds were detected (Appendix #1).

The genome of the *Bacillus subtilis* AR-153 production strain 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. We sequenced the production strain's genome (Whole Genome Sequencing WGS). The genome sequence was compared to databases on genes involved in antimicrobial resistance.

The comparison analysis revealed four genes which might be involved in antimicrobial resistance. It should be noted that the identification of genes on the genomic level does not give any information about the expression of the proteins and subsequent resistance phenotypes. The resistances identified are therefore hypothetical at this stage. Analysis of public databases showed that all four genes are consistently and widely present in the *Bacillus subtilis* group. None are foreign to *Bacillus subtilis*. All the identified genes and hypothetically conferred resistances are therefore intrinsic due to their distribution in the genus *Bacillus* and more precisely, in the species *Bacillus subtilis* and are therefore not a safety concern. *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.

2.4 ENZYME PRODUCTION PROCESS

2.4.1 Overview

The food enzyme is produced by ROAL Oy[7] by submerged fermentation of *Bacillus subtilis* AR-153 in accordance with current Good Manufacturing Practices for Food (GMP) and the principles

[6] General Specifications and Considerations for Enzyme Preparations (fao.org)
[7] See footnote 5

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
- Seed fermentation
- Main fermentation

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.

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, 13th edition, 2022 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 flocculant 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-153 is aseptically transferred to shake flasks containing fermentation medium.

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

2.4.6 Seed fermentation

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

2.4.7 Main Fermentation

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

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

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

2.4.8 Recovery

The purpose of the recovery process is:

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

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

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

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

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

2.4.9 Materials

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

- Flocculants
- Filter aids
- pH adjustment agents

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

2.4.10 Pre-Treatment

Flocculants and/or filter aids are added to the fermentation broth, in order to get clear filtrates, and to facilitate the primary solid/liquid separation. Typical amount of filter aids is 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 a well-defined Master (MCB) and Working Cell Bank (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 can result to decreased growth of the production organism, and consequently, in a low yield of the desired enzyme protein, resulting in a rejected product.

Measures utilized by ROAL OY to guarantee microbiological hygiene and prevent contamination with microorganisms ubiquitously present in the environment (water, air, raw materials) are as follows:

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

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

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

Chemical contaminants:

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

It is ensured that all raw materials used in production of food enzymes are of food grade quality or have been assessed to be fit for their intended use and comply with agreed specifications. In addition to these control measures in-process testing, and monitoring is performed to guarantee an optimal and efficient enzyme production process and a high-quality product (cGMPs). The whole process is controlled with a computer 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 the seed and main fermentations and at regular intervals and at critical process steps during fermentation and recovery.

Monitoring of fermentation parameters may include:

- pH
- Temperature
- Aeration conditions

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

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

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

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

2.4.15 Formulation and Packaging

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

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 quality related aspects, like expected enzyme activity and the general JECFA Specification for Food Enzyme Preparations and

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

2.4.16 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:

Enzyme preparation used for baking processes

Property	**Requirement**	
Activity	min.	100 XylH/g
Appearance	Light beige powder	
Particle size distribution	Max 1% > 250 µm	

Enzyme preparation used for grain processing and brewing

Property	Requirement	
Activity	min.	50,000 BXU/g
Appearance	Brown liquid	
Density	1.15-1.25 g/mL	

2.5.2 Formulation of a typical enzyme preparation

Enzyme preparation used for baking processes

Composition	
Constituent	**%**
Enzyme concentrate	2.0 – 5.0
Sunflower oil	0.4
Wheat Flour	Remainder

Enzyme preparation used for grain processing and brewing

Composition	
Constituent	**%**
Enzyme concentrate	30 – 40
Glycerol	45
Water	Remainder

2.5.3 Molecular mass of the enzyme

The endo-1,4-β-xylanase protein subject for this dossier has a calculated molecular mass of 20 kDa.

2.5.4 Purity and identity specifications of the enzyme preparation

It is proposed that the food enzyme endo-1,4-β-xylanase should comply with the internationally accepted JECFA specifications for chemical and microbiological purity of food enzymes (FAO/WHO 2006):

Lead:	Not more than 5 mg/kg[8]
Salmonella sp.:	Absent in 25 g sample
Total coliforms:	Not more than 30 per gram
Escherichia coli:	Absent in 25 g of sample
Antimicrobial activity:	Not detected
Mycotoxins:	Not applicable to bacteria

The proof that the food enzyme complies with these specifications is shown by the analyses on 3 different batches (see Appendix #1). The 3 samples do not contain any diluents.

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

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

2.6 Enzymatic Activity

The main activity of the enzyme preparation is endo-1,4-β-xylanase (EC 3.2.1.8). Like any other enzyme, the endo-1,4-β-xylanase 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

[8] JECFA's General Specifications and Considerations for Enzyme Preparations recommend the metal lead to be present no more than 5 mg/kg Food safety and quality: enzymes (fao.org)

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.

The substrates for endo-1,4-β-xylanase are cereal xylans, including arabinoxylans. Xylans are polysaccharides belonging to the pentosans (polymers of C5-sugars). Cereal xylans form part of the complex polysaccharides found in the cell walls of plant cells. Arabinoxylans are xylans branched with arabinose. They can be found in an array of different molecular weights in various plant materials including the cell walls and endosperm of cereals, such as wheat and barley. Consequently, the substrates for endo-1,4-β-xylanase occur naturally in foods.

The function of endo-1,4-β-xylanase is to catalyze the hydrolysis of (arabino)xylans into oligomers of 1,4-beta-xylan and 1,4-beta-arabinoxylan. They can be found in an array of different molecular weights in various plant materials including the cell walls and endosperm of cereals, such as wheat and barley. Like the substrate, the enzyme endo-1,4-β-xylanase is described to occur in various products from nature, such as papaya, wheat, and barley. Consequently, also the enzyme occurs naturally in foods.

The reaction products of the hydrolysis of (arabino)xylans catalyzed by endo-1,4-β-xylanase are oligomers of 1,4-beta-xylan and 1,4-beta-arabinoxylan. Consequently, also the reaction products occur naturally in foods and adverse effects on nutrients are not to be expected.

The method to analyse the activity of the enzyme is company specific and is capable of quantifying endo-1,4-β-xylanase activity as defined by its IUBMB classification. As demonstrated in section 2.5.1 of this notice, xylanase is measured with a different activity unit per enzyme preparation. The difference in the activity unit is based on the substrate in each of the respective food applications.

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

Food enzyme preparations are known to have side activities in the form of other enzymes. The reason is that food enzyme preparations are concentrates containing apart from the desired enzyme protein (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 gastrointestinal tract 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 and to gain energy.

Furthermore, the presence of such enzyme activities and the potential reaction products in food 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 activities come 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. AB Enzymes is not aware of any adverse effects from the side activities present in the **endo-1,4-β-xylanase** enzyme preparation.

Adverse effects from side activities are not expected from the **endo-1,4-β-xylanase** enzyme preparation. *Bacillus subtilis* has a long history of safe use in the food industry as described in the dossier (section 6.1.). The side activities that would arise in the enzyme preparation come from the production microorganism *Bacillus subtilis*. This microorganism is known to produce enzymatic side activities like proteases in low amounts. These side activities are considered to be normal and of no adverse consequence to human health.

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 **endo-1,4-β-xylanase**, 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 **endo-1,4-β-xylanase** 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 (Daurvin et al. 1998). The overall conclusion is that exposure to enzyme proteins by ingestion, as opposed to exposure by inhalation, are not potent allergens and that sensitization to ingested enzymes is rare.

- 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[9].
- Only a small amount of the food enzyme is used during food processing, which leads to very small amount of enzyme protein present in the final food. A high concentration generally equals a higher risk of sensitization, whereas a low level in final food equals a lower risk (Goodman et al. 2008).
- For cases where the proteins are denatured which is the case for this 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

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

that small protein fragments resulting from digestion are less likely to be allergenic (FAO/WHO 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

Alignments of the **endo-1,4-β-xylanase** mature amino acid sequence to the sequences in the allergen database were performed and results obtained were used to estimate the level of potential allergenicity of this enzyme. Similarity searches were performed to the sequences available in chosen public Allergen Online (FARRP) allergen database version 21 (last updated on February 14, 2021).

The alignment methods used in the searches are:

- Alignment (FASTA) of the entire query amino acid sequence to sequences in allergen online databases.
- Alignment (FASTA) of sliding 80-amino acid windows of the query protein to known protein allergens. Sliding window search means that every possible 80 amino acid segment of the query protein
- 8-mer sequence search

The comparison of query sequence with sequences of known allergens using the sliding 80-mer window was recommended by the FAO/WHO Expert panel already in 2001 and by the Codex Alimentarius Commission in 2003 (Joint FAO/WHO Codex Alimentarius Commission et al. 2009) as a method to evaluate the extent of which a protein is similar in structure to a known allergen

The identity limit set for the protein having an allergenic cross-reactivity is 35 % when alignment is performed using a full-length query sequence or an 80-mer sliding window. According to EFSA

(2010) even the set above 35 % identity is regarded conservative and above 50 % identity cut-off has been suggested.

Results of Allergenicity searches:

Type of Search	Outcome
Alignment of the endo-1,4-β-xylanase mature amino acid sequence to sequences in allergen online databases	No matches having greater than 35 % identity were found from the AllergenOnline database using the full-length search
Alignment of sliding 80-amino acid window of the query protein to known protein allergens	No matches having greater than 35% identity were found from the AllergenOnline database using the 80-mer sliding window search.
8-mer sequence search	Zero sequences with at least one 8-mer match were detected

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 **endo-1,4-β-xylanase** produced by *Bacillus subtilis* AR-153 is of no concern.

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

In general, the technological purpose of cereal xylans hydrolysis with the help of endo-1,4- β-xylanase can be described as: degradation of a component (the substrate cereal xylans) which causes technical difficulties in processing of raw materials containing this component.

As described in section 2.6 of this notice, endo-1,4-β-xylanase is naturally present in cereals. The natural enzymatic conversion of cereal xylans in such materials is of technological benefit in several industrial food manufacturing processes, like baking, grain processing and brewing. However, the levels of endogenous endo-1,4-β-xylanase are often inadequate and vary from

batch to batch of raw material, and the specificity of the enzyme may not be optimal to give desired process advantages. Therefore, industrial endo-1,4-β-xylanase is used during food processing.

As explained in section 2.6 of this notice, endo-1,4-β-xylanase catalyzes the hydrolysis of cereal xylans, resulting in the breakdown of cereal xylans into oligomers of 1,4-beta-xylan and 1,4- beta-arabinoxylan.

The use of endo-1,4-β-xylanases has been specifically approved for a number of years, which - together with the extensive use for decades in the US, Canada, Mexico, EU, Brazil, AUS/NZ and in the rest of the world - demonstrates the technological need of such food enzymes in food processes.

This dossier is specifically submitted for the use of endo-1,4-β-xylanase in **baking processes, in grain processing** and **brewing processes.**

In those processes, the use of endo-1,4-β-xylanase assists the food processing (improving processability, enhancing yields, …) therefore leading to better and/or more consistent product characteristics and helping to achieve more effective production processes, resulting in better production economy and environmental benefits such as the use of less raw materials, energy saving and production of less waste, being overall of high value for the food chain.

The benefits of the enzyme use in these processes are presented on the next page.

Baking Process

The process flow diagram (figure #1) shows the typical applications of the endo-1,4-β-xylanase in baking and the conditions under which the food enzyme is used.

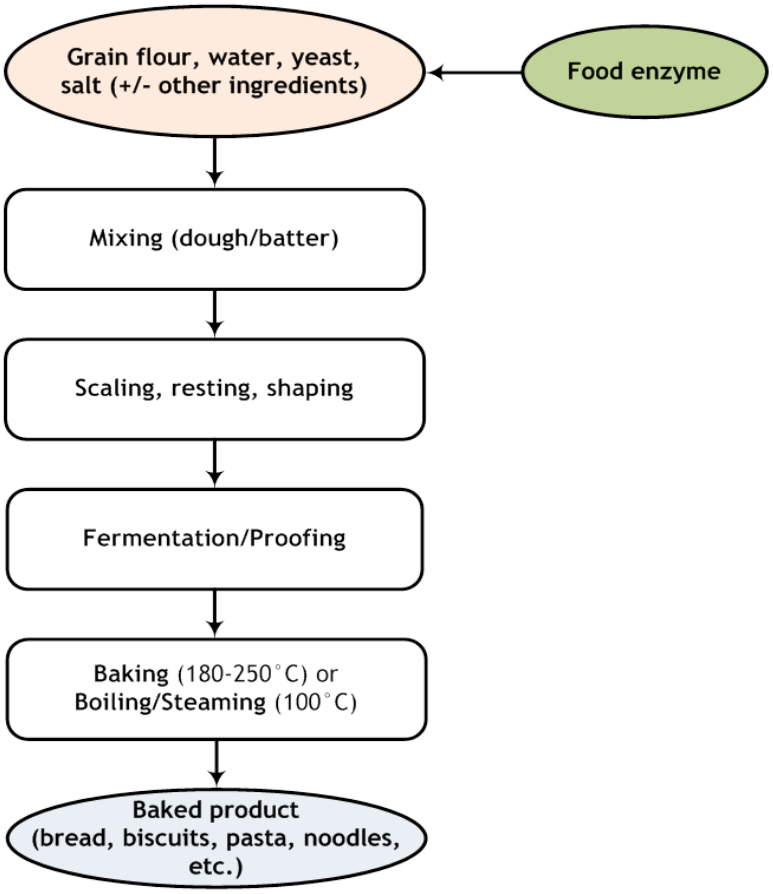


Figure #1: Baking process flow diagram

In general, the benefits of the hydrolysis of the cereal xylans, with the help of endo-1,4-β-xylanase in baking are:

- Improve / facilitate the handling of the dough (improved extensibility and stability, reduced stickiness leading to reduced losses of dough)
- Improve the dough's structure and behavior during the baking step
- Ensure a uniform and slightly increased volume and improve crumb structure of the bakery product, which might otherwise be impaired by industrial processing of the dough
- Reduce batter viscosity, beneficial in the production process for e.g. waffles, pancakes and biscuits
- Reduce manufacturers' reliance on natural crop and climate related changes of the flour: flour characteristics can vary from year to year and variety to variety and therefore benefit from being standardized with food enzymes such as xylanase.

Grain Processing

The process flow diagram (figure #2) shows the typical applications of the endo-1,4-β-xylanase in grain processing and the conditions under which the food enzyme is used.

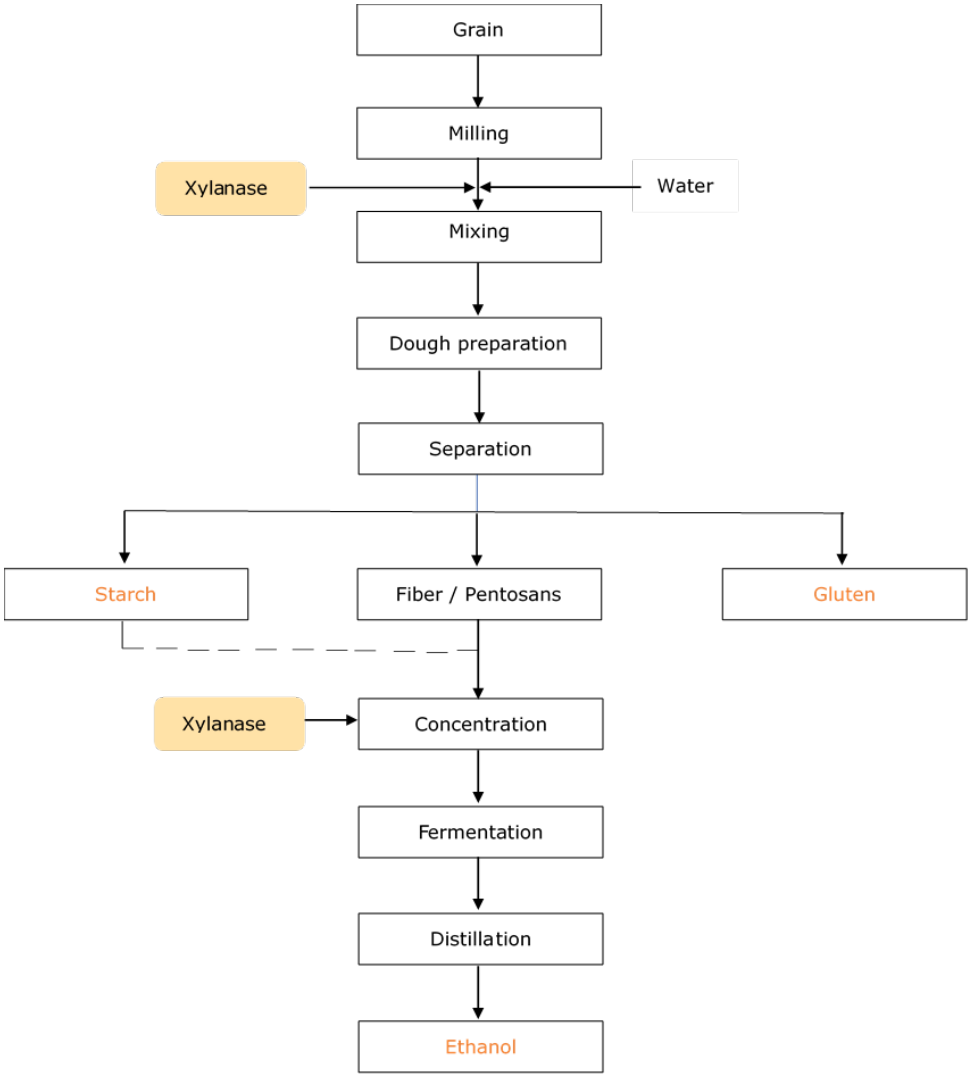


Figure #2: Grain Processing flow diagram

Cereals are highly complex structures that cause technical difficulties during processing when milled and when fractionated to starch, gluten and fibers.

Enzyme systems that act on the cereal components including xylans, are used at various stages in the starch – gluten separation process (including peeling and milling) to ensure smooth and efficient processing, facilitate the separation (by opening the grain structure) and ensuring high quality of the polysaccharide and gluten fractions.

The benefits of the conversion of (arabino)xylans with the help of xylanase in grain processing are the following:

- Reduced viscosity of the wheat flour batter, facilitating gluten and starch separation
- Improved gluten and starch purity due to greater extraction yield of the high value fraction and efficient removal of arabinoxylans
- Better degradation of cell wall components increasing effectiveness of the mechanical treatments such as milling and peeling
- Increased utilization of capacity as the result of rapid viscosity reduction and low fouling frequency of process equipment such as evaporators
- Higher energy savings due to less use of process water, lower evaporator costs and decreased production time.

Brewing Process:

The process flow diagram (figure #3) shows the typical applications of the endo-1,4-β-xylanase in brewing and the conditions under which the food enzyme is used.

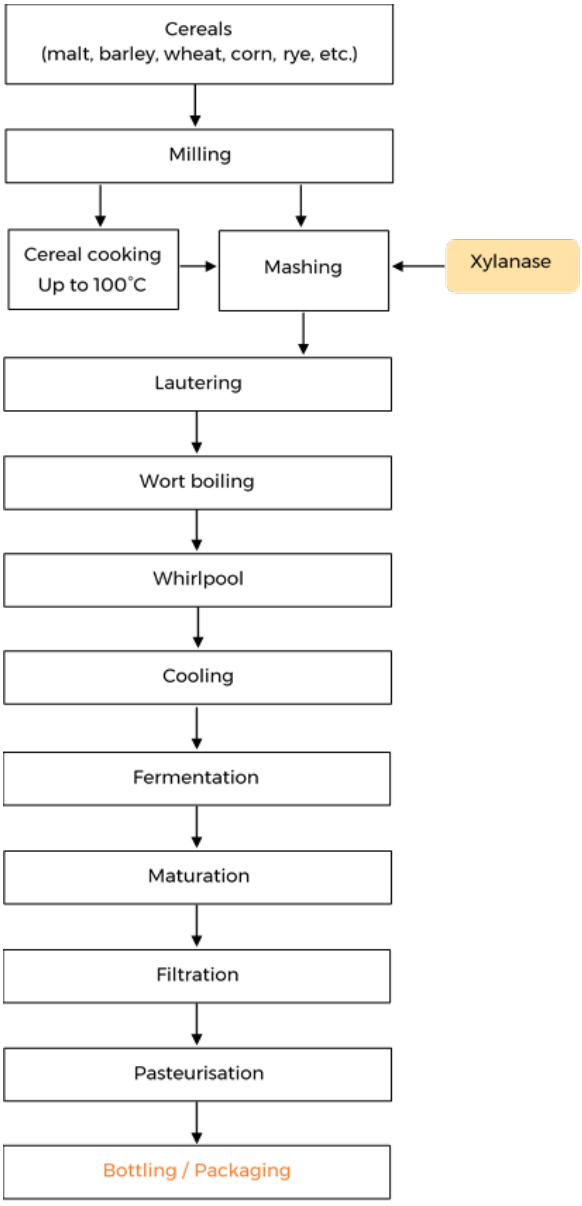


Figure #3: Brewing Process flow diagram

In general, the benefits of the hydrolysis of cereal xylans with the help of xylanases in brewing are:

- Decreased wort viscosity and beer turbidity
- Increased beer filtration rate and reduce need for beer filtration aids

- Improved beer colloid stability as the result of reduced haze caused by non-starch polysaccharides
- Higher brewing yield due to the improved processing, and thereby less use of raw materials
- Increased flexibility in the choice of raw materials: use of other raw materials which might otherwise lead to a more cumbersome process (e.g. wheat beer often leads to a more difficult and lengthier lautering).
- Improved solutions in meeting different customer's preferences for appearance and taste.

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 **endo-1,4-β-xylanase** 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 **endo-1,4-β-xylanase** from *Bacillus subtilis* AR-153 may be used:

Food Application	Raw material (RM)	Maximal recommended use levels (mg TOS/kg RM)
Baking	Flour	1
Grain processing	Cereals	50
Brewing	Cereals	38

2.10 Fate in food

As explained, 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.

Endo-1,4-β-xylanase performs its technological function during food processing. 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 endogenous endo-1,4-β-xylanase present in the fruit and vegetable raw materials and ingredients. In order to be able to perform a technological function in the final food, a number of conditions have to 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,** endo-1,4-β-xylanase typically performs its technological function during the dough or batter handling. Endo-1,4-β-xylanase is typically denatured at around 80ºC. Due to the temperature conditions of the baking (180 - 250ºC) or steaming step (100ºC) the enzyme is denatured by heat during this step.

In **grain processing**, during the process of creating starch, gluten and fiber, there are repeated washing steps used. Therefore, in the end, the presence of residual amounts of TOS (including the enzyme) after repeated washing during production is negligible.

In **brewing**, endo-1,4-β-xylanase typically performs its technological function in the mashing step or the adjunct before addition of the adjunct to the mash tun. Endo-1,4-β-xylanase is therefore denatured already in the consecutive lautering or mash filtration step. Endo-1,4-β-xylanase may also be added during the fermentation step. In this case, xylanase will be denatured during the pasteurization step.

3 Part 3 § 170.325- Dietary Exposure

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

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

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

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

To determine the TMDI of **endo-1,4-β-xylanase** enzyme preparation, the calculation used the maximum use levels. In addition, the calculation accounts for how much food or beverage is obtained per kg raw materials (as shown in the table below), All the TOS is assumed to be in the final product.

Applications		Raw Material (RM)	Maximal use level (mg TOS/kg RM)	Final Food (FF)	Ratio RM/FF*	Maximal level in final food (mg TOS/kg food)
Liquid Foods i.e., Beverages	Brewing	Cereals	38	Beer	0.17	6.46
	Grain Processing	Cereals	50	Soft drinks (starch derived syrups) and beverages (fibers)	1.1	55
Solid Foods	Baking and other cereal products	Flour	1	Baked products	0.71	0.71
	Grain Processing	Cereals	50	Starch, fibers	1.1	55

*<u>*Assumptions behind ratios of raw material to final food*</u>

<u>Baking:</u>

- *Bakery products fall in the category of solid foods.*

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

Brewing and cereal drinks add to the class of liquid foods:

- *Raw materials used in brewing and cereal drink processes are various kinds of grist (e.g. malt, barley, wheat, sorghum and maize). Yields will vary dependent on the type of grist, process used, and the type of drink produced.*
- *Beer production has a range of RM/FF from 14-28 kg of grist per 100 L of beer, with 80-90 % of all beers produced at a RM/FF ratio of 14-20 kg of grist per 100 L of beer. The same RM/FF ratio holds true for cereal beverage.*
- *The assumption used for calculation of dietary exposure is a yield of 100 L of drink per 17 kg of cereal corresponding to a RM/FF ratio of* ***0.17 kg grist per L of beer or cereal beverage****.*

Grain Processing

Food ingredients obtained from grain processing are typically Starch, Fiber, Gluten and Flour. These food ingredients can be use in the making of both solid and liquid final foods.

Grain processes might start with cereals (grains or grist) or flour as the raw material. Cereals contain starch in a range of 55-65%, fiber in the range of 6-18%[10] *and gluten in the range of 10-15%.*

[10] http://wholegrainscouncil.org/whole-grains-101/fiber-in-whole-grains

- *Starch: Typically, 0.55 kg starch is produced per 1 kg cereal. The most considerable final food application is dairy and bakery with a maximum added starch content of 5%. Starch is also used in the less voluminous application area of confectionary, where it is used up to a content of 12%. Based upon the most considerable applications (bakery), the corresponding RM/FF ratio is 0.09 kg cereal per kg final food (same for dairy).*

Starch can also be further processed into syrups (e.g. High Fructose Corn Syrup, HFCS), sweeteners and modified starch (Starch processing). Syrups and sweeteners are mainly used in liquid foods (soft drinks). With the assumptions expressed above (typically 0.55 kg starch is produced per 1 kg cereal) and assuming that typically 1 kg of sweetener is produced per 1 kg starch, and that soft drinks typically contain 10-14% w/v HFCS so on average 120 g HFCS per L, it can be concluded that the typical ratio of RM/FF is 0.21 kg cereals per L final beverage.

- *Fiber: Typically, 0.12 kg fiber is produced per 1 kg cereal. Fiber is used in bakery and beverage products with a maximum added fiber content of 13% (total fiber content max. 25%). The corresponding RM/FF ratio is 1.1 kg cereal per kg final food.*

- *Gluten: Typically, 0.10 kg gluten is produced per 1 kg cereal. Gluten is used in the production of bakery products with a maximum added gluten content of 10% in the final food. The corresponding RM/FF ratio is 1 kg cereal/kg final food.*

In respect to dietary exposure calculation, the worst-case scenario, both in respect to solid and liquid food, is food ingredient Fiber with a RM/FF ratio of ***1.1 kg cereal per kg final food.***

The Total TDMI can be calculated using the maximal values found in food and beverage, multiplied by the average consumption of food and beverage/kg body weight/day. The Total TMDI is the following:

TMDI in food (mg TOS/kg body weight/day)	TDMI in beverage (mg TOS/kg body weight/day)	Total TMDI (mg TOS/kg body weight/day)
0.688	1.375	**2.063**

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 (and beverages) use specific endo-1,4-β-xylanase from *Bacillus subtilis* AR-153;
- It is assumed that ALL producers apply the HIGHEST use level per application;
- For the calculation of the TMDI's in food, only the above foodstuffs were selected containing the highest theoretical amount of TOS. Therefore, foodstuffs containing lower theoretical amounts were not included;
- 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 (Add reference)

Dietary exposure is calculated on the basis of the total organic solids (TOS) content in the final (commercial) enzyme preparation and is usually expressed in milligrams or micrograms of TOS per kilogram of body weight per day. TOS encompasses the enzyme component and other organic material originating from the production organism and the manufacturing process, while excluding intentionally added formulation ingredients.

The Margin of Exposure (MoE)[11] for human consumption can be calculated through the division of the NOAEL (no-observed adverse effect) value by the TMDI (Total Theoretical Maximal Daily Intake). Total TMDI of the food enzyme 2.063 mg TOS/kg body weight/day.

As a result, the MoE is:

MoE =1000/2.063 = **485**.

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

Conclusion:

To conclude, the use of the food enzyme endo-1,4-β-xylanase from *Bacillus subtilis* AR-153 in the production of food is safe. Considering the high safety value determined by the MoE, even when calculating using means of overestimation of intake via the Budget method, there is no need to restrict the use of the enzyme in food. The suggested dosage for food manufacturers is not a restrictive value and could be higher or lower depending on usage within cGMPs.

[11] JECFA considers the estimated dietary exposure to an enzyme preparation based on the proposed uses and use levels in food and relates it to the no-observed-adverse-effect level (NOAEL) in its hazard assessment in order to determine a margin of exposure (MOE) section9-1-4-2-enzymes.pdf (who.int)

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 provide 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, not consistent with our conclusion of the notified substance GRAS status.

6.1 Safety Risk Assessment for Production Strain

6.1.1 History of Production Microorganism in Food

B. subtilis-like organisms are ubiquitous in the environment (soil, water, plants and animals) and as a result can be also found in food (Boer and Diderichsen 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 (Boer and Diderichsen 1991).

B. 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, endo-1,4-β-xylanase, etc.

In addition to *Bacillus licheniformis*, *B. subtilis* has become one of the most well-established cell factories in biotechnology especially for the production of exo-proteins like proteases and alpha-amylases (Westers et al. 2004; Pohl and Harwood 2010; van 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 *B. subtilis* (natto). Some $6x10^6$ kg of natto are consumed annually in Japan.

Furthermore *B. 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, grain processing and brewing 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 dehairing and batting in the leather industry, and for the production of xylanases as bread improver (Harbak and Thygesen 2002).

Non-exhaustive list of authorized food enzymes (other than endo-1,4-β-xylanase) used *Bacillus subtilis*:

Authority	Food enzyme	Reference
JECFA	Alpha amylase	WHO Food Additives Series 28
	Mixed microbial carbohydrase and protease	JECFA Evaluation
	Riboflavin	JECFA Evaluation
	Maltogenic amylase	JTRS 891-JECFA 51/18
Australia/NZ	α-acetolactate decarboxylase	Australia New Zealand Food Standards Code – Schedule 18 – Processing aids (legislation.gov.au)
	α-amylase	
	β-amylase	
	β-glucanase	
	Hemicellulase multicomponent enzyme	

	Maltogenic α-amylase	
	Metalloproteinase	
	Pullulanase	
	Serine proteinase	
Canada	Amylase	List of Permitted Food Enzymes Health Canada
	Glucanase	
	Hemicellulase	
	Protease	
France	α-acetolactate decarboxylase	Arrêté du 19 octobre 2006
	α-amylase	
	Beta glucanase	
	Asparaginase	
	Beta-galactosidase	
	Maltogenic exo-alpha amylase	
	Glucosyltransferase	
	Hemicellulase	
	Protease	
	Pullulanase	
USA[12]	Pullulanase	GRAS Notice Inventory, GRN 20 , GRAS Notice Inventory, GRN 205
	Pectate lyase	GRAS Notice Inventory, GRN 114
	Branching glycosyltransferase	GRAS Notice Inventory, GRN 274
	1,4-alpha branching enzyme	GRAS Notice Inventory, GRN 406
	Asparaginase	GRAS Notice Inventory, GRN 476

[12] GRAS affirmations and GRAS notifications

	Lactase	GRAS Notice Inventory, GRN 579
	Subtilisin	GRAS Notice Inventory, GRN 714
	Maltogenic amylase	GRAS Notice Inventory, GRN 746

Non-exhaustive list of authorized endo-1,4-β-xylanase from production organisms other than *Bacillus subtilis*		
Authority	**Production Organism**	**Reference**
Australia/NZ	*Aspergillus niger* *Aspergillus oryzae* *Bacillus amyloliquefaciens* *Humicola insolens* *Trichoderma reesei*	Australia New Zealand Food Standards Code – Schedule 18 – Processing aids (legislation.gov.au)
France	*Aspergillus niger* *Aspergillus oryzae* *Trichoderma longibrachiatum*	Arrêté du 19 octobre 2006
USA[13]	Xylanase derived from *Fusarium venenatum* carrying a gene encoding xylanase from *Thermomyces lanuginosus* Xylanase from *Trichoderma reesei*	GRAS Notice Inventory, GRN 54 GRAS Notice Inventory, GRN 567

[13] The United States uses a "Generally Considered as Safe" documentation analysis for the acceptance of use for marketing the product

	Xylanase from *Aspergillus niger* Endo-1,4-beta-xylanase from *Trichoderma reesei*	GRAS Notice Inventory, GRN 589 GRAS Notice Inventory, GRN 628
Canada	*Aspergillus acidus*[14] *Aspergillus oryzae*[15] *Bacillus licheniformis*[16] *Trichoderma reesei*[17]	List of Permitted Food Enzymes Health Canada
JECFA	*Humicola insolens* *Fusarium venenatum*	JECFA Evaluations JECFA Evaluations

6.1.2 Safety of the genetic modification

The genetic modification, i.e., the transformation of the recipient strain *Bacillus subtilis* with the plasmid pXY-B001 results in recombinant strain AR-153. 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-153) differs from its original parental strain in overexpressing endo-1,4-β-xylanase, featuring a set of defined genomic deletions, and inclusion of a hydrolase gene. Besides this, AB Enzymes has not noticed any differences in the production strain AR-153 as compared to the parental strain.

[14] Strain specific
[15] Strain specific
[16] Strain specific
[17] Strain specific

Safety of the Production Strain Narrative:

The safety narrative for the *Bacillus subtilis* AR-153 production strain that overexpresses endo-1,4-β-xylanase and co-expresses a hydrolase can be concluded from the following points:

- The genetic modifications used to create the production strain are well characterized
- History of safe use of *Bacillus subtilis* and close relatives as an enzyme producer in food
- Safety of the co-expressed hydrolase
- Supplementation safety data presented in the GRAS narrative (sections 6.1.3 and 6.1.4)

Genetic modifications safety description:

Section 2.3 of this notice contains information on the genetic modifications that took place to create the *Bacillus subtilis* AR-153 production strain. 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. The endo-1,4-β-xylanase gene was inserted into the pXY-B001 vector. Both the vector and genome of *Bacillus subtilis* AR-153 production strain were sequenced to confirm genetic stability and the correct sequence of the plasmid containing the genes of the target enzyme, endo-1,4-β-xylanase, and the co-expressed hydrolase. Plasmid pXY-B001 does not contain any antibiotic resistance genes and the production strain does not have any acquired antibiotic resistance genes resulting from the genetic modifications.

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 (Boer and Diderichsen 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 (Boer and Diderichsen 1991). 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[18]). It was concluded that *B. subtilis* is not a human pathogen nor is it toxigenic. In the EU, the *Bacillus subtilis* AR-153 production strain qualifies for QPS status even with the co-expression of the hydrolase (for more details refer to section 6.1.4).

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. (see table #1 in section 6.1.1 for more details). In the case of history of use for xylanase produced by *Bacillus subtilis* there are entries in enzyme positive lists of other international jurisdictions such as Brazil[19] and Mexico[20].

Safety of co-expressed hydrolase:

Regarding the co-expressed hydrolase, we have demonstrated safety of the enzyme in GRN #974. 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-153 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 hydrolase activity in three independent pilot batches was comparable to that in the negative control. SDS PAGE confirmed a very low content of the

[18] https://www.epa.gov/sites/production/files/2015-09/documents/fra009.pdf
[19] RESOLUÇÃO - RDC Nº 728, DE 1° DE JULHO DE 2022 - RESOLUÇÃO - RDC Nº 728, DE 1° DE JULHO DE 2022 - DOU - Imprensa Nacional (in.gov.br)
[20] Aditivos Alimentarios Anexo VI. | Comisión Federal para la Protección contra Riesgos Sanitarios | Gobierno | gob.mx (www.gob.mx)

hydrolase (no evidence of corresponding gel bands). 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 in the function of the final enzyme preparation in the intended food processes (baking, grain processing, and brewing). As mentioned previously, the function of the hydrolase is to aid in the recovery step of manufacturing the final preparation.

Supplemental Safety information:

In the following section of this notice (section 6.1.3), we further substantiate the safety of the production strain through the use of safe strain lineage with the toxicological studies of the reference strain, *Bacillus subtilis* RF13018 from GRN #974. Apart from the toxicological studies from the reference strain, we include a summary of the cytotoxicity study conducted on *Bacillus subtilis* AR-153 indicating that the production strain is not cytotoxic.

6.1.3 Toxicological testing

The safety of the endo-1,4-β-xylanase produced by the genetically modified *Bacillus subtilis* AR-153 from a toxicological perspective is supported by the historical safety of strain linage. *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. AB Enzymes performed toxicological studies on a strain within the strain lineage of AR-153, the *Bacillus subtilis* RF13018 production strain from GRN #974[21]. Both AR-153 and RF13018 share an intermediate strain in the strain lineage. Additionally, the AR-153 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.

[21] Safe strain lineage was used in GRN #974 to substantiate the safety of *Bacillus subtilis* RF13018. Since filing GRN #974 we have conducted toxicological studies on the strain.

Please refer below for the summary of the cytotoxicity study:

Cytotoxicity Study:

Bacillus subtilis AR-153 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. 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-153 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-153 and *Bacillus licheniformis* ATCC 14580 were not cytotoxic to Vero cells. The cell-free supernatant of *Bacillus cereus* DSM 31 (ATCC 14579) was extremely cytotoxic.

Conclusion:

Bacillus strain AR-153 culture supernatant did not exceed the toxicity threshold and was not hence cytotoxic to Vero cells.

Toxicological Studies from Reference Strain

The following studies were performed for *Bacillus subtilis* strain RF13018:

- Reverse Mutation Assay using Bacteria (*Salmonella typhimurium and Escherichia coli*) with Maltogenic amylase from *Bacillus subtilis*
- *In vitro* Mammalian Micronucleus Assay in Human Lymphocytes with Maltogenic amylase from *Bacillus subtilis*

- 90-Day Repeated Dose Oral Toxicity Study in Wister Rats with Maltogenic amylase from *Bacillus subtilis*

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

The summaries for each of the toxicological studies is found in Appendix #3 of this notice.

As mentioned above both AR-153 and RF13018 have been developed from the same intermediate strain. Expression plasmids are very similar, only differing by the expression cassette/enzyme gene of interest. As both production strains are free of any harmful sequences or any potential hazards, differences in the genetic modification of AR-153 and RF13018 are not a safety concern.

Safety of the Production strain (SSL)

- **Pariza and Johnson Decision Tree** (Appendix #3)
- **JECFA Safe Progeny Strain statement** (Appendix #4)
- **Differences between tox tested strain and AR-153 production strain** (Appendix #4)
- **Diagram on Strain Lineage** (Appendix #4)

6.1.4 Pathogenicity and Toxigenicity

Bacillus subtilis strains are non-pathogenic for healthy humans and animals (Olempska-Beer et al. 2006; Boer and Diderichsen 1991). Apart from the well-established pathogenicity of *B. anthracis*, a pathogen of humans and some animals, *B. cereus*, which causes gastroenteritis, and the group of insect pathogens related *to B. thuringiensis*, most other species of *Bacillus* are regarded as nonpathogenic or cause only opportunistic infections, often in compromised patients. The lack of pathogenicity among strains of *B. subtilis* or any of its close relatives has resulted in the Food and Drug Administration granting the organism GRAS (generally regarded as safe) status.

Pathogenic *B. subtilis* strains are not described in the Bergey's Manual or in the ATCC and other catalogues. The species *B. subtilis* does not appear on the list of pathogens in Annex III of Directive 2000/54/EC[22] 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 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)[23]. The US EPA has added *B. subtilis* to the list of exempted organisms in 1997 (USA EPA, 1997)[24].
- 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[25]
- 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)[26].

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

[22] Directive 2000/54/EC - biological agents at work | Safety and health at work EU-OSHA (europa.eu)
[23] https://osp.od.nih.gov/wp-content/uploads/2019_NIH_Guidelines.htm
[24] https://www.epa.gov/sites/production/files/2015-09/documents/fra009.pdf
[25] http://www.ec.gc.ca/lcpe-cepa/default.asp?lang=En&n=5AE12597-1&offset=2&toc=show
[26] https://www.biosafety.be/content/tools-belgian-classification-micro-organisms-based-their-biological-risks

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. "*In the case of GMMs being used as production organisms for which the recipient strain qualifies for the QPS status, and for which the genetic modification does not give rise to safety concerns, the QPS approach can be extended to the genetically modified production strain*" (EFSA 2018).

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). Similarly, other *Bacillus* species have been assessed as production organism by EFSA and also accorded QPS status provided that the qualification requirements are met (EFSA 2020): this approach requires the identity of the strains to be conclusively established and evidence that they do not harbour acquired antimicrobial resistance genes, and that the strains lack toxigenicity.

The production organism does not show cytotoxicity (see section 6.1.3). As described below in section 4.1.3., the genetic modifications were introduced using well established methods, the specific modifications are well characterized, and the introduced genetic material does not encode or express any toxic substances. The manufacturing process has been extensively described and did not lead to the introduction of any risks. The antimicrobial activity has been tested (see section 2.3.6) without any observed growth inhibition of the test organisms, proving the lack of antimicrobial production of the strain. Therefore, the production strain fully qualifies for QPS status.

The US Food and Drug Administration reviewed the safe use of food-processing enzymes from recombinant microorganisms, including *B. subtilis* and closely related *B. amyloliquefaciens* (Olempska-Beer et al. 2006).

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[27]) and not appearing on the list of pathogens from Belgium (Belgian Biosafety Server[28]).

In conclusion, the production organism fulfils all the specific qualifications for the QPS status, the genetic modification does not raise any safety concerns and neither does the manufacturing, therefore the production strain *B. subtilis* AR-153 can be considered safe.

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).

[27] https://www.baua.de/DE/Angebote/Rechtstexte-und-Technische-Regeln/Regelwerk/TRBA/pdf/TRBA-466.pdf?__blob=publicationFile&v=11

[28] H_A_bacteries_webCORR.xls (biosafety.be)

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.

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-153 Composition Report
2. Flow Chart of the manufacturing process with control steps
3. Summary of Toxicological Studies and Decision Tree Xylanase AR-153
4. Safe Strain Lineage Narrative AR-153

Publication bibliography

Abad, Ana; Fernández-Molina, Jimena Victoria; Bikandi, Joseba; Ramírez, Andoni; Margareto, Javier; Sendino, Javier et al. (2010): What makes Aspergillus fumigatus a successful pathogen? Genes and molecules involved in invasive aspergillosis. In *Revista iberoamericana de micología* 27 (4), pp. 155–182. DOI: 10.1016/j.riam.2010.10.003.

Bindslev-Jensen, Carsten; Skov, Per Stahl; Roggen, Erwin L.; Hvass, Peter; Brinch, Ditte Sidelmann (2006): Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry. In *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association* 44 (11), pp. 1909–1915. DOI: 10.1016/j.fct.2006.06.012.

Boer, A. S. de; Diderichsen, B. (1991): On the safety of Bacillus subtilis and B. amyloliquefaciens. A review. In *Applied Microbiology and Biotechnology* 36 (1), pp. 1–4.

Daurvin, T.; Groot, G.; Maurer, K. H.; Rijke, D. de; Ryssov-Nielsen, H.; Simonsen, M.; Sorensen T.B. (1998): Working Group on Consumer Allergy Risk from Enzyme Residues in Food. AMFEP. Copenhagen.

Douglass, J. S.; Barraj, L. M.; Tennant, D. R.; Long, W. R.; Chaisson, C. F. (1997): Evaluation of the budget method for screening food additive intakes. In *Food additives and contaminants* 14 (8), pp. 791–802. DOI: 10.1080/02652039709374590.

EFSA (2007): Introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA. In *The EFSA Journal* 587, pp. 1–16.

EFSA (2018): Update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA 7. Suitability of taxonomic units notified to EFSA until September 2017. In *EFSA Journal* 16 (1), p. 2931. DOI: 10.2903/j.efsa.2018.5131.

EFSA (2020): Update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA 12. Suitability of taxonomic units notified to EFSA until March 2020. In *EFSA Journal* 18 (7), e06174. DOI: 10.2903/j.efsa.2020.6174.

FAO/WHO (2001): Evaluation of allergenicity of genetically modified foods. Food and Agriculture Organization of the United Nations. Rome, Italy. Available online at http://www.fao.org/3/y0820e/y0820e.pdf.

FAO/WHO (2006): Compendium of food additive specifications. Joint FAO/WHO Expert Committee on Food Additives : 67th Meeting 2006. Rome: FAO (FAO JECFA monographs, 1817-7077, 3). Available online at http://www.fao.org/documents/card/en/c/a6fe72dc-82fb-437c-81cc-bc4d739043a5/.

Goodman, Richard E.; Vieths, Stefan; Sampson, Hugh A.; Hill, David; Ebisawa, Motohiro; Taylor, Steve L.; van Ree, Ronald (2008): Allergenicity assessment of genetically modified crops--what makes sense? In *Nature biotechnology* 26 (1), pp. 73–81. DOI: 10.1038/nbt1343.

Hansen, S. C. (1966): Acceptable daily intake of food additives and ceiling on levels of use. In *Food and cosmetics toxicology* 4 (4), pp. 427–432.

HARA, Toshio; UEDA, Seinosuke (1982): Regulation of polyglutamate production in Bacillus subtilis (natto). Transformation of high PGA productivity. In *Agricultural and Biological Chemistry* 46 (9), pp. 2275–2281. DOI: 10.1271/bbb1961.46.2275.

Harbak, L.; Thygesen, H. V. (2002): Safety evaluation of a xylanase expressed in Bacillus subtilis. In *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association* 40 (1), pp. 1–8.

Iordănescu, S. (1975): Recombinant plasmid obtained from two different, compatible staphylococcal plasmids. In *Journal of bacteriology* 124 (2), pp. 597–601.

Joint FAO/WHO Codex Alimentarius Commission; World Health Organization; Food and Agriculture Organization of the United Nations (2009): Foods derived from modern biotechnology. 2nd edition. Rome: World Health Organization : Food and Agriculture Organization of the United Nations (Codex alimentarius, 0259-2916).

Kikuchi, Yuko; Takai, Toshiro; Kuhara, Takatoshi; Ota, Mikiko; Kato, Takeshi; Hatanaka, Hideki et al. (2006): Crucial commitment of proteolytic activity of a purified recombinant major house dust mite allergen Der p1 to sensitization toward IgE and IgG responses. In *Journal of immunology (Baltimore, Md. : 1950)* 177 (3), pp. 1609–1617. Available online at https://pubmed.ncbi.nlm.nih.gov/16849469/.

Kunst, F.; Ogasawara, N.; Moszer, I.; Albertini, A. M.; Alloni, G.; Azevedo, V. et al. (1997): The complete genome sequence of the Gram-positive bacterium Bacillus subtilis. In *Nature* 390 (6657), pp. 249–256.

Logan, N. A. (1988): Bacillus species of medical and veterinary importance. In *Journal of medical microbiology* 25 (3), pp. 157–165. DOI: 10.1099/00222615-25-3-157.

Nakazawa, Takuya; Takai, Toshiro; Hatanaka, Hideki; Mizuuchi, Eri; Nagamune, Teruyuki; Okumura, Ko; Ogawa, Hideoki (2005): Multiple-mutation at a potential ligand-binding region decreased allergenicity of a mite allergen Der f 2 without disrupting global structure. In *FEBS letters* 579 (9), pp. 1988–1994. DOI: 10.1016/j.febslet.2005.01.088.

Olempska-Beer, Zofia S.; Merker, Robert I.; Ditto, Mary D.; DiNovi, Michael J. (2006): Food-processing enzymes from recombinant microorganisms--a review. In *Regulatory toxicology and pharmacology : RTP* 45 (2), pp. 144–158. DOI: 10.1016/j.yrtph.2006.05.001.

Pariza, M. W.; Johnson, E. A. (2001): Evaluating the safety of microbial enzyme preparations used in food processing: update for a new century. In *Regulatory toxicology and pharmacology : RTP* 33 (2), pp. 173–186. DOI: 10.1006/rtph.2001.1466.

Pohl, Susanne; Harwood, Colin R.: Heterologous Protein Secretion by Bacillus Species. In, vol. 73, pp. 1–25.

Rachinger, Michael; Bauch, Melanie; Strittmatter, Axel; Bongaerts, J.; Evers, Stefan; Maurer, Karl-Heinz et al. (2013): Size unlimited markerless deletions by a transconjugative plasmid-system in Bacillus licheniformis. In *Journal of biotechnology* 167 (4), pp. 365–369. DOI: 10.1016/j.jbiotec.2013.07.026.

Schallmey, Marcus; Singh, Ajay; Ward, Owen P. (2004): Developments in the use of Bacillus species for industrial production. In *Can. J. Microbiol.* 50 (1), pp. 1–17. DOI: 10.1139/W03-076.

Takai, T.; Ichikawa, S.; Yokota, T.; Hatanaka, H.; Inagaki, F.; Okumura, Y. (2000): Unlocking the allergenic structure of the major house dust mite allergen der f 2 by elimination of key intramolecular interactions. In *FEBS letters* 484 (2), pp. 102–107.

Valenta, Rudolf (2002): The future of antigen-specific immunotherapy of allergy. In *Nature reviews. Immunology* 2 (6), pp. 446–453. DOI: 10.1038/nri824.

Valenta, Rudolf; Kraft, Dietrich (2002): From allergen structure to new forms of allergen-specific immunotherapy. In *Current opinion in immunology* 14 (6), pp. 718–727.

van Dijl, Jan Maarten; Hecker, Michael (2013): Bacillus subtilis: from soil bacterium to super-secreting cell factory. In *Microb Cell Fact* 12 (1), p. 3. DOI: 10.1186/1475-2859-12-3.

Vehmaanperä, J.; Steinborn, G.; Hofemeister, J.; Vehmaanperä, Jari; Steinborn, Gerhard; Hofemeister, Jürgen (1991): Genetic manipulation of Bacillus amyloliquefaciens. In *J. Biotechnol.* 19 (2-3), pp. 221–240. DOI: 10.1016/0168-1656(91)90061-Y.

Westers, Lidia; Westers, Helga; Quax, Wim J. (2004): Bacillus subtilis as cell factory for pharmaceutical proteins: a biotechnological approach to optimize the host organism. In *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* 1694 (1-3), pp. 299–310. DOI: 10.1016/j.bbamcr.2004.02.011.

Objective: **Chemical Composition Analysis of Xylanase from *Bacillus subtilis*, Strain AR-153**

Samples:
1. Liquid enzyme concentrate batch C22005-006 LIMS ID: 1-22-01882-002
2. Liquid enzyme concentrate batch C21097+98, LIMS ID: 1-21-02301-001
3. Liquid enzyme concentrate batch C21090+91, LIMS ID: 1-21-02302-001

Table 1. Main activity

	Batch		
	C22005-006	**C21097+98**	**C21090+91**
XylH6.0/g	1020	654	743

XylH6.0: Assay of Xylanase activity, Roal internal method B081

Table 2. Antimicrobial activity, presence of production strain, microbiological quality and lead

	Batch		
	C22005-006	**C21097+98**	**C21090+91**
Antimicrobial activity	not detected	not detected	not detected
Presence of production strain	not detected	not detected	not detected
Escherichia coli (/25 g)	not detected	not detected	not detected
Salmonella (/25 g)	not detected	not detected	not detected
Total coliforms (cfu*/g)	<30	<30	<30
Lead (mg/kg)	<0.03	<0.05	<0.05

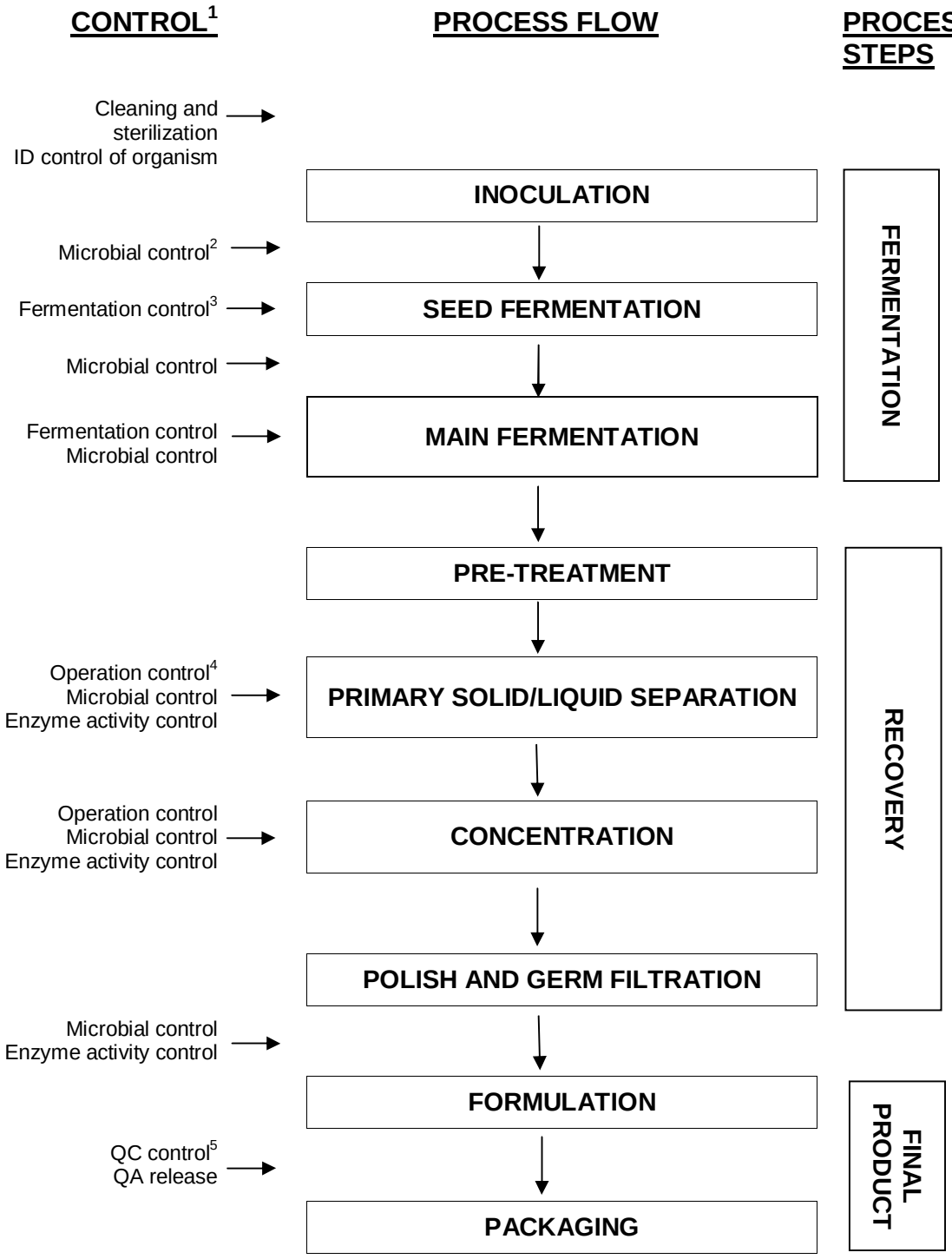
Antimicrobial activity: Specifications for Identity and Purity of Certain Food Additives, FAO Food and Nutrition Paper 65 (2006), Rome, Vol.4, p. 122.
E. coli: ISO 16649-3:2015, mod.
Salmonella: NMKL 71:1999, mod.
Total coliforms: ISO 4832:2006, mod. *cfu: colony forming units
Lead: ISO 17294-2

25.11.2022 Rajamäki, Finland

ROAL OY
The Art of Enzymes roal
Valta-akseli 6, FI-05200 Rajamäki

Quality Information Specialist
Roal Oy

Production Process of Food Enzymes from Fermentation



[1] The controls shown on the flow chart may vary depending on the production set-up. Controls are conducted at various steps throughout the production process as relevant.

[2] Microbial control: Absence of significant microbial contamination is analyzed by microscope or plate counts

[3] During fermentation parameters like e.g. pH, temperature, oxygen, CO_2, sterile air overflow are monitored / controlled.

[4] Operation control in downstream processes cover monitoring and control of parameters like e.g. pH, temperature

[5] Final QC control will check that product does live up to specifications like e.g. enzyme activity as well as chemical and microbial specification.

TOXICOLOGICAL STUDIES SUMMARY

Reverse Mutation Assay using Bacteria (*Salmonella typhimurium* and *Escherichia coli*) with Maltogenic amylase from *Bacillus subtilis* RF13018

The test performed is based on OECD Guidelines No. 471. This study was performed to investigate the potential of the food enzyme to induce gene mutations according to the treat and plate method, using the *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and tester strain *E. coli* WP2 uvrA (pKM 101), in the absence and presence of metabolic activation.

Concurrent positive controls demonstrated the sensitivity of the assay and the metabolising activity of the S9 mix. The assay was performed at different concentrations of the solution supplied, in 2 independent experiments both with and without metabolic activation. Each concentration, including the controls, was tested in triplicate. There were no issues of concern, no items which would impact the scoring, the positive and negative controls were acceptable and therefore all criteria of validity were met.

The results obtained in both experiments were very similar. No mutagenic activity was observed in any of the 5 strains used, in any condition.

In conclusion, it can be stated that during the described mutagenicity test and under the experimental conditions reported, Maltogenic amylase from *Bacillus subtilis* did not cause gene mutations by base pair changes or frameshifts in the genome of the tester strains used. Therefore, Maltogenic amylase from *Bacillus subtilis* is considered to be non-mutagenic in this bacterial reverse mutation assay.

***In vitro* Mammalian Micronucleus Assay in Human Lymphocytes with Maltogenic amylase from *Bacillus subtilis* RF13018**

The test was based on OECD Guidelines No. 487. The food enzyme was tested and evaluated for clastogenic potential in human peripheral blood lymphocytes in vitro in two independent experiments (scoring numerical and structural chromosomal aberrations). The tests were

performed both in the presence and absence of a post-mitochondrial supernatant fluid preparation (S9 mix). The food enzyme was tested at different concentrations.

The data showed no cytogenicity. It was therefore concluded that the test item is classified as non-clastogenic, when tested at the highest concentration as suggested by the guidelines and adjusted to TOS.

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

Therefore, Maltogenic amylase from *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 Maltogenic amylase from *Bacillus subtilis* RF13018

The test was performed according to OECD No. 408. In this subacute toxicity study, the test item was administered daily by oral gavage to a group of rats of both sexes at 3 different dose levels of enzyme preparation / kg body weight for a period of 13 weeks. A control group was treated similarly with the vehicle only.

Animals were sacrificed after 13 weeks of treatment and were given a detailed post mortem examination and a full list of tissues places in fixative. Histological examination of these tissues was carried out for all animals treated at the highest dose, all control animals and all premature decedents.

There were no test item-related findings of toxicological relevance at any dose level. No treatment related mortality was observed. No treatment-related effects on body weight and food /water consumption were seen. There was no test item-related ophthalmoscopic changes at any dose level. It was concluded that there were no consistent changes in clinical chemistry

parameters which could be contributed to the treatment. There were no histopathological findings which could be related to the treatment. At necropsy, there were no findings in either sex that were considered to be treatment related.

In conclusion, the no-observed-adverse-effect-level (NOAEL) for the test item was determined at 1000 mg TOS / kg body weight / day, the highest dose tested.

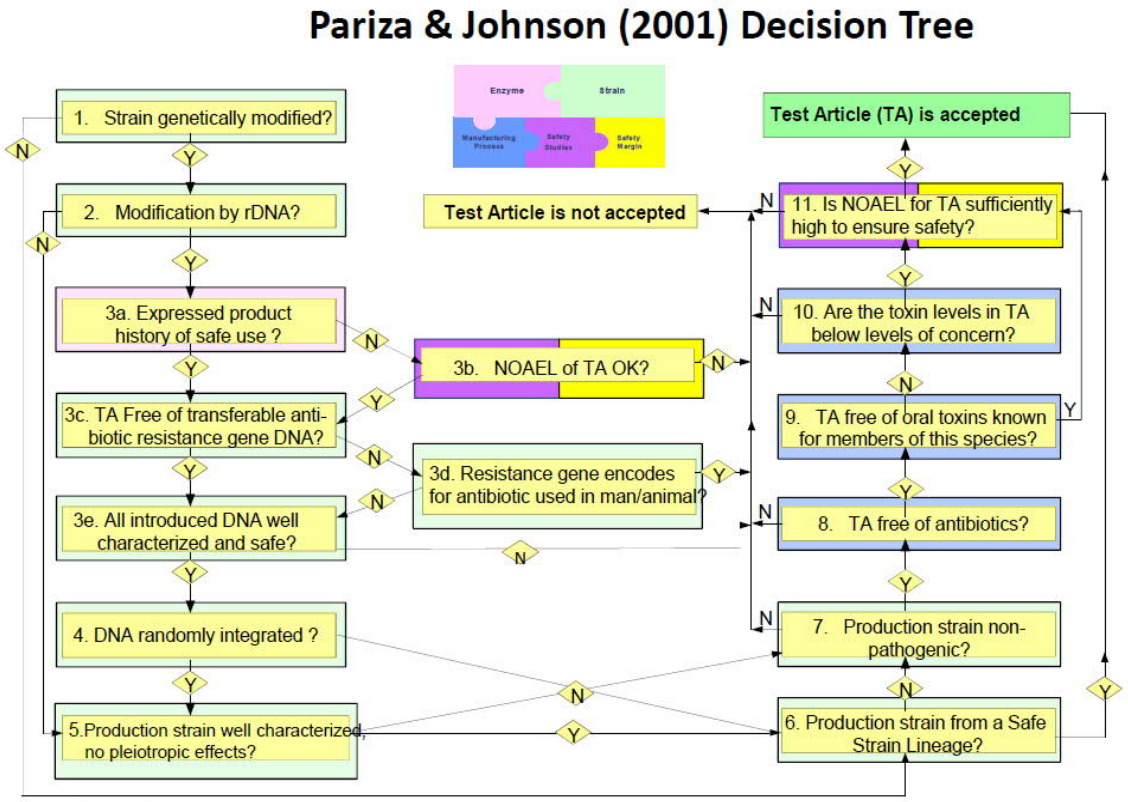
ANALYSIS OF SAFETY BASED ON PARIZA AND JOHNSON DECISION TREE

Pariza and Johnson have published updated guidelines for the safety assessment of microbial enzyme preparations (2001)[1] from the IFBC Decision Tree[2]. The safety assessment of a given enzyme preparation is based upon an evaluation of the toxigenic potential of the production organism. The responses below follow the pathway indicated in the decision tree as outlined in Pariza and Johnson, 2001. The outcome of this inquiry is that *Bacillus subtilis* AR-153 production strain producing xylanase is "ACCEPTED" as safe for its intended use.

[1] Pariza M.W. and Johnson E.A. Reg. Toxicol. Pharmacol. Vol. **33** (2001) 173-186

[2] IFBC (International Food Biotechnology Committee), Chapter 4: Safety Evaluation of Foods and Food Ingredients Derived from Microorganisms in Biotechnologies and Food: Assuring the Safety of Foods Produced by Genetic Modification, Regulatory Toxicology and Pharmacology. Vol. **12**:S1-S196 (1990).

Decision Tree:



1. Strain genetically modified? (Yes, AR-153 strain is genetically modified, see section 2.3 for genetic modification description). Go to #2

2. Modification by rDNA? (Yes, AR-153 strain is modified by rDNA) Go to #3a

3.

   3a. Expressed product history of safe use (Yes, please refer to section 6.1 on the safety of the production strain and section 2.8 technological purpose for evidence) Go to #3c

   3c. TA Free of transferable antibiotic resistance gene DNA? (Yes, refer to section 2.3.6. for further details) Go to #3e

   3e. All introduced DNA well characterized and safe (Yes, all introduced DNA is well characterized as safe) Go to #4

4. DNA randomly integrated? (No, the pXY-B001 plasmid was introduced into the host cell by protoplast transformation. The introduced DNA (i.e., plasmid pXY-B001) is kept episomally and not integrated into the chromosome of *Bacillus subtilis*. Go to #6

6. Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure? (Yes, *Bacillus subtilis* has been demonstrated as a safe production host and methods of modification have been well documented. Safety of this organism has been evaluated and confirmed through toxicological testing as described herein). If yes, the test article is **ACCEPTED**.

Thus, AB Enzymes concludes that the decision tree shows that the *Bacillus subtilis* production strain AR-153 is **ACCEPTED.**

Safe Strain Lineage:

Industrial production microorganisms are regularly improved by conventional or recombinant DNA methods. If strains from a certain strain lineage have been tested and used for several years, and further improved by e. g. mutagenesis or deleting genes, then one must conclude at a certain point in time that a strain from this strain lineage can be declared safe for use without further testing by extensive programs including animal testing. This strain should be designated as "parental strain" of a "Safe Strain Lineage" and be used as a starting point for further development and improvement for production strains.

Enzyme preparations meet the JECFA definition of Safe Food Enzyme Production Strain[1] or a Presumed Progeny Strain[2] when appropriate toxicological testing (i.e. repeated-dose toxicity and genotoxicity testing) is conducted on enzymes from closely related strains derived from the same parental organism.

As of 2020, JECFA has evaluated over 80 food enzyme preparations from a variety of microorganisms and has never recorded a positive result in any toxicity study, suggesting either that toxins were not present or that toxins were present at levels that were below the limit of detection of the bioassays. JECFA concluded provided that the genetic modification (either recombinant DNA or chemical mutagenesis) is well characterized, additional toxicological testing would not be required.

The use of safe strain lineage concept is only a waiver for toxicological studies, however it does not exempt the enzyme product from other safety requirements, such as allergenicity, cytotoxicity, toxin analysis and other safety parameters.

Based on the concept of safe strain lineage, we utilized the toxicological studies from production strain RF13018 to substantiate the safety of AR-153.

[1] A “Safe Food Enzyme Production Strain” is a non-pathogenic, non-toxigenic microbial strain with a demonstrated history of safe use in the production of food enzymes. Evidence supporting this history of safe use includes knowledge of taxonomy, genetic background, toxicological testing, other aspects related to the safety of the strain and commercial food use (Principles Related to Specific Groups of Substances, of Environmental Health Criteria 240 (EHC 240), 2020).

[2] A “Presumed Safe Progeny Strain” is developed from a Safe Food Enzyme Production Strain or from the parent of that Safe Food Enzyme Production Strain. The progeny strain is developed through specific well-characterized modifications to its genome; the modifications must be thoroughly documented, must not encode any harmful substances and must not result in adverse effects. This concept also applies to multiple generations of progeny. Evidence supporting their safety includes knowledge of taxonomy, genetic background and toxicological testing (including read-across of toxicological studies) (Principles Related to Specific Groups of Substances, of Environmental Health Criteria 240 (EHC 240), 2020).

The endo-1,4-β-xylanase is produced by *Bacillus subtilis* AR-153. The transformation of the recipient strain *Bacillus subtilis* with plasmid pXY-B001 results in recombinant strain AR-153 (see Table 1 below).

The production organism *Bacillus subtilis* has been genetically engineered by the deletion of genes from the chromosome and by transformation of the strain with xylanase expression plasmid pXY-B001 to improve the xylanase production. All genetic modifications are well characterized and as such the safe strain lineage concept was employed as the *Bacillus subtilis* intermediate strain, AR-300, was similarly used for the RF13018 strain which has been assessed in several toxicological studies as presented below in **Table 1**. AR-300 is the last common intermediate strain after which the lineage separates. Both AR-153 and RF13018 derive from AR-300. The recipient strains for AR-153 and RF13018 differ only slightly in the deletions of genes from the chromosome applied. The safe strain lineage flow chart is present in **Figure 1** illustrating the relationship between the different strains in the lineage. The differences between the two strains (AR-153 and RF13018) are the inserted expression cassettes containing the enzyme genes of interest and the additional deletion applicable to AR-153.

Table 1: Comparison of the AR-153 and Reference strain

Production Strain	TOX TESTED	Plasmid	Enzyme
AR-153 *B. subtilis*	NO	pXY-B001	Xylanase
RF13018[3] *B. subtilis* (GRN #974)	YES[4]	pAA-A001	Maltogenic amylase

[3] For GRN #974 we substantiated the safety of RF13018 production strain with the toxicological studies from GRN #746 utilizing safe strain lineage.

[4] Since submitting GRN #974 we have conducted toxicological studies on the strain, again with no findings s. Table 2.

Table 2: Toxicological Test Summaries

Production Strain	Enzyme	Toxicology Test	Result
RF13018 *B. subtilis*	Maltogenic amylase	Cytotoxicity study	Non-cytotoxic
		90-day sub-chronic study in rats	No adverse effects
		Reverse Mutation Assay using Bacteria (*Salmonella typhimurium*)	Non-mutagenic
		In-vitro mammalian micronucleus assay in human lymphocytes	Non-clastogenic
AR-153 *B. subtilis*	Xylanase	Cytotoxicity study	Non-cytotoxic

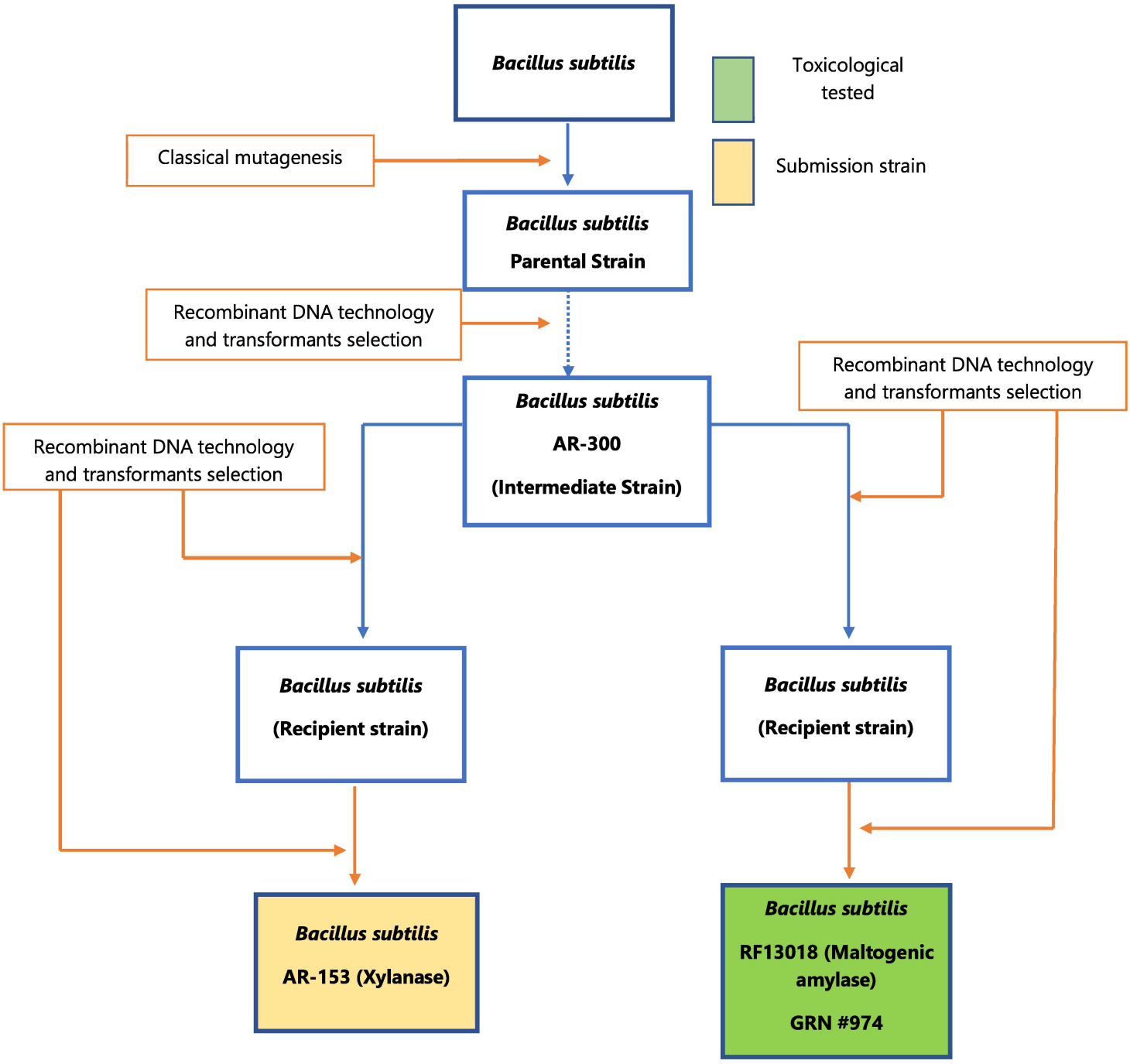


Figure 1: *Bacillus subtilis* Safe Strain Lineage of AR-153

Safety Narrative:

As mentioned above both the AR-153 and RF13018 have been developed from the same parental strain (*Bacillus subtilis*). They are very similar. The recipient strains differ only slightly in the gene deletion pattern. The similarity is also true for the expression plasmids, only differing by the expression cassette for the enzyme of interest. Both AR-153 and RF13018 contain the same gene encoding a hydrolase which is of no safety concern.

As the production strains (including the expression plasmids) are free of any harmful sequences or any potential hazards, differences in the genetic modification of AR-153 and RF13018 are not a safety concern.

Furthermore, the manufacturing conditions between the two production strains are very similar. The slight changes in pH levels and fermentation medium (food-grade) have been thoroughly assessed. They are considered minor (common industry practice) and do not trigger any additional safety issue.

To add on, enzyme product from AR-153 production strain complies with JECFA specifications for chemical and microbiological purity of food enzymes (Food and Agriculture Organization of the United Nations 2006) which confirms the safety of the production strain AR-153.

Based on the rationale provided above as per JECFA, 2020, as well as on the review of the strains meeting the requirements of Pariza and Johnson Decision Tree (Appendix #3), AB Enzymes concludes endo-1,4-β-xylanase produced by *Bacillus subtilis* AR-153 to be safe and does not pose a significant risk to human health.

Margin of Exposure:

According to the Safe Strain Lineage concept, the Margin of Exposure (MoE) for human consumption can be calculated by dividing the NOAEL by the Total Theoretical Maximal Daily Intake (TMDI). In the case of the safe strain lineage concept for AR-153, there is no NOAEL. However, the NOAEL from the 90-day toxicological study from the closely related production strain *Bacillus subtilis* RF13018 is used to calculate the MoE and support the safety of the endo-1,4-β-xylanase *Bacillus subtilis* AR-153.

FDA FORM 3667

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
001111	Nov 30, 2022
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

	Name of Contact Person: Joab Trujilo	Position or Title: Regulatory Affairs Specialist	
	Organization *(if applicable)*: AB Enzymes Inc.		
	Mailing Address *(number and street)*: 8211 W. Broward Blvd. Suite 420		
City: Plantation	State or Province: Florida	Zip Code/Postal Code: 33324	Country: United States of America
Telephone Number: +1 954 800 8606	Fax Number:	E-Mail Address: joab.trujillo@abenzymes.com	

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

Xylanase enzyme preparation 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 974

☐ 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)* GRAS Notice No. 1011

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 **endo-1,4-β-xylanase** is to be used as a processing aid in baking processes, grain processing and brewing 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 gested 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 Xylanase enzyme preparation 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 420 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: 2022.11.30 18:36:17 -05'00'	Joab Trujillo Regulatory Affairs Specialist	11/30/2022

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 Xylanase 2022	Administrative
	AB Enzymes' Cover Letter for Xylanase GRAS Notice 2022	Submission
	1_AR-153 Composition Report	Submission
	2_Flow Chart of the manufacturing process with control steps	Submission
	3_Summary of Toxicological Studies and Decision Tree Xylanase	Submission
	4_Safe Strain Lineage Narrative AR-153	Submission
	References for AB Enzymes' Xylanase GRAS Notice.zip	Submission
	AB Enzymes Xylanase GRAS Notice 2022	Submission