September 2, 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 Invertase (IUBMB 3.2.1.26) from a Genetically Modified *Trichoderma reesei* production strain produced by submerged fermentation is Generally Recognized as Safe; therefore, they are exempt from statutory premarket approval requirements.

The following information is provided in accordance with the proposed regulation:
Proposed 21C.F.R. § 170.36 (c)(i) *The name and address of notifier.*

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

Proposed 21C.F.R. § 170.36 (c)(ii) *The common or usual name of notified substance:*
Invertase (IUBMB 3.2.1.26) from a Genetically modified *Trichoderma reesei* production strain AR-996.

Proposed 21C.F.R. § 170.36 (c)(iii) *Applicable conditions of use:*
Invertase is to be used as a processing aid for catalyzing the breakdown of sucrose to fructose and glucose utilizing the hydrolysis of terminal non-reducing β-D-fructofuranoside residues in β-D-fructofuranosides for the production of short chain fructooligosaccharides (sc-FOS) and sugar reduction in fruit and vegetable processing (i.e., purees and juices). 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

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

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 do not contain data or information that is exempt from disclosure under the FOIA (Freedom of Information Act).

§170.225(c)(9) – Information included in the GRAS notification:
To the best of our knowledge, the information contained in this GRAS notification is complete, representative and balanced. It contains both favorable and unfavorable information, known to AB Enzymes and pertinent to the evaluation of the safety and GRAS status of the use of this substance.

Sincerely,

AB Enzymes GmbH

DocuSigned by:
[redacted]
452B3329EDDB45F...

i.V. Candice Cryne

Senior Global Regulatory Affairs Manager

02-Sep-2022 | 17:45 BST

DocuSigned by:
[redacted]
FD80C973075E42E...

Joab Trujillo

Regulatory Affairs Specialist

02-Sep-2022 | 17:46 BST

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

AB Enzymes GmbH – Feldbergstrasse 78 , D-6412 Darmstadt

GRAS Notification of an Invertase from a Genetically Modified *Trichoderma reesei*

AB ENZYMES GmbH

September 2, 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 Invertase (IUBMB# 3.2.1.26) from a Genetically Modified *Trichoderma reesei* production strain produced by submerged fermentation is Generally Recognized as Safe; therefore, they are exempt from statutory premarket approval requirements.

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

AB Enzymes Inc.[1]

8211 W. Broward Blvd. Suite 375

Plantation, FL 33324 USA

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

Invertase (IUBMB 3.2.1.26) from a Genetically modified *Trichoderma reesei* production strain AR-996.

§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:

Invertase is to be used as a processing aid for catalyzing the breakdown of sucrose to fructose and glucose utilizing the hydrolysis of terminal non-reducing β-D-fructofuranoside residues in β-D-fructofuranosides for the production of short chain fructooligosaccharides (sc-FOS) and sugar reduction in fruit and vegetable processing (i.e., purees and juices). The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements

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

under current Good Manufacturing Practices. There are no maximal limits set, just suggested dosages.

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

This GRAS determination is based upon scientific procedures.

§170.225(c)(6) – Premarket approval:

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

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

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

2.1.1 Common name of the enzyme

Name of the enzyme protein: Invertase

Synonyms: beta-fructofuranosidase, beta-(1-2)-fructofuranosidase, beta-D-fructofuranosidase,beta-D-fructofuranosidase fructohydrolase

2.1.2 Classification of the enzyme

IUBMB #	3.2.1.26
CAS #	9001-57-4

EC 3. is for hydrolases;

EC 3.2. is for glycosylases;

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

EC 3.2.1.26 is for beta-fructofuranosidase.

2.2 Strain Lineage Information

2.2.1 Production Strain

Production strain	*Trichoderma reesei* AR-996

Synopsis

Invertase from *Aspergillus niger* is produced from a genetically modified *Trichoderma reesei* production strain (AR-996). The genetic modifications conducted to develop the production strain are described in section 2.3 of the GRAS narrative along with confirmation on integration of expression cassettes in the *T. reesei* genome, the stability of the production strain, absence of DNA, antibiotic genes and toxic compounds. Information on the safety of the *Trichoderma reesei* production strain is provided in section 6 of the GRAS narrative. In short, safety of the production strain is substantiated by the safety of the genetic modifications, history of safe use for *Trichoderma reesei* as a food enzyme producer and the use of the safe strain linage concept described in Pariza and Johnson (2001).

AB Enzymes has submitted multiple GRAS notices to FDA in the past for enzymes produced from *Trichoderma reesei* production strains and have received letters of No Objection.

AB Enzymes' Previous GRAS Notices for Enzymes from *T. reesei production strains*

GRAS Notice	**Description**
GRAS Notice 524[2]	Phospholipase A2 enzyme preparation from *Trichoderma reesei*
GRAS Notice 557[3]	Polygalacturonase from produced in *Trichoderma reesei*
GRAS Notice 558[4]	Pectin esterase from produced in *Trichoderma reesei*

[2] GRN No. 524
[3] GRN No. 557
[4] GRN No. 558

GRAS Notice 566[5]	Mannanase produced in *Trichoderma reesei*
GRAS Notice 628[6]	Endo-1,4-beta-xylanase produced in Trichoderma *reesei*
GRAS Notice 631[7]	Triacylglycerol lipase produced in *Trichoderma reesei*
GRAS Notice 653[8]	Lysophospholipase produced in *Trichoderma reesei*
GRAS Notice 707[9]	Glucose oxidase produced in *Trichoderma reesei*
GRAS Notice 756[10]	Endo-1,4-beta-glucanase produced in *Trichoderma reesei*
GRAS Notice 817[11]	Serine endopeptidase produced in *Trichoderma reesei*
GRAS Notice 981[12]	Sterol esterase enzyme preparation produced in *Trichoderma reesei*

The *Trichoderma reesei* production strain AR-996 is deposited in the Westerdijk Fungal Biodiversity Institute, formerly known as the "Centraalbureau voor Schimmelcultures" (CBS) in the Netherlands with the deposit number CBS 148293.

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

Kingdom: Fungi

Division: *Ascomycota*

[5] GRN No. 566
[6] GRN No. 628
[7] GRN No. 631
[8] GRN No. 653
[9] GRN No. 707
[10] GRN No. 756
[11] GRN No. 817
[12] GRN No. 981

Class: Sordariomycetes

Order: Hypocreales

Family: Hypocreaceae

Genus: Trichoderma

Species: *Trichoderma reesei*

Strain: *Trichoderma reesei* AR-996

2.2.2. Recipient Strain

The recipient strain used in the genetic modification for the construction of the production strain is *Trichoderma reesei* AR-407. The recipient strain was created from parental strain AR-256 through a series of enzyme gene deletions, native to *Trichoderma reesei* to limit enzyme side activity expression. The recipient is negative in e. g. the genes encoding the four major *T. reesei* cellulases (cellobiohydrolases I and 2 and endoglucanases 1 and 2). The gene deletions have been done using a *T. reesei* gene counter selection method. The gene used was deriving from *T. reesei*, meaning that no heterologous marker genes were used in these gene deletions.

Therefore, the recipient can be described as followed:

Kingdom: Fungi

Division: *Ascomycota*

Class: *Sordariomycetes*

Order: *Hypocreales*

Family: *Hypocreaceae*

Genus: *Trichoderma*

Species: *Trichoderma reesei*

Strain: *Trichoderma reesei* AR-407

Commercial name: Not applicable. The organism is not sold as such.

2.2.2 Donor:

The donor for the invertase gene is *Aspergillus niger*. *Aspergillus niger* has a long and established history of use as an industrial enzyme production organism.

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

2.3 Genetic modification

T. reesei AR-996 was constructed for invertase production. The production strain differs from the recipient strain in its high invertase production capacity due to expression of the invertase gene from the expression cassette integrated into the genome of recipient strain. Besides the heterologous invertase production, no other changes in phenotype are made.

T. reesei AR-996 secretes high amounts of invertase into its culture supernatant, resulting in high invertase activity in the cultivation broth. The secreted invertase is the main component of the enzyme mix produced by AR-996. In addition, the strain AR-996 produces endogenous *Trichoderma* enzymes in small amounts. These activities are not relevant from an application or safety point of view, due to the small amount and the fact that such activities are included in products which have been approved for decades in food processing.

The production strain AR-996 was constructed from the recipient strain in one genetic modification step. The inserted expression cassette contains the invertase gene under a *T. reesei* promotor and terminator. The inserted sequence also contains an *Aspergillus nidulans amd*S selection marker gene. The expression cassette used for transformation was cleaved from the pUC19 vector plasmid by restriction enzyme digestion followed by isolation of the expression cassette from agarose gel. A Southern blot hybridization experiment was performed on the

genomic DNA of the production strain AR-996 to confirm that no pUC19 vector DNA is included in the genome of AR-996.

Expression Cassette Components Table

Component	Description
***Aspergillus niger* invertase gene**	The invertase gene encodes the native *A. niger* invertase protein.
***Synthetic amdS* gene and promoter**	The marker gene has been isolated from *A. nidulans* VH1-TRSX6 (Kelly and Hynes 1985; Hynes et al. 1983). *A. nidulans* is closely related to *Aspergillus tubingensis*, which is used in industrial production of food enzymes. The gene codes for an acetamidase that enables the strain to grow on acetamide as a sole nitrogen source (Kelly and Hynes 1985). This characteristic has been used for selecting transformants. The product of the *amdS* gene, acetamidase, can degrade acetamide and is not harmful or dangerous. The *amdS* marker gene has been widely used as a selection marker in fungal transformations without any disadvantage for more than 30 years.
***Trichoderma reesei* promoter and terminator**	The invertase gene is fused to *T. reesei* native promoter. This promoter is strong and is used to overexpress invertase gene transcription, to obtain high yields of invertase. The

	transcription is terminated by the native terminator from *T. reesei*

Information relating to the genetic modification process

Standard DNA techniques were used in the construction and transformation of the expression cassettes. The transformation of the recipient strain with the invertase expression cassette was performed as described by Penttilä et al. 1987 with the modifications described in Karhunen et al. 1993. The transformants were selected according to their ability to grow on acetamide selection plates (*amd*S marker gene).

Genes of concern

The production organism does not contain any genes of concern. No antibiotic resistance marker genes were used during the construction of the production strain. The expression cassette was cleaved from the plasmid vector (pUC19) and isolated from agarose gel prior to transformation. The lack of plasmid vector derived DNA, including the ampicillin resistance gene (*bla*) was confirmed by Southern blot in which the genomic DNA digestions were probed using labelled pUC19.

Confirmation of Insertion:

Confirmation of insertion of two expression cassettes into the *T. reesei* genome was done via Whole Genome Sequencing.

2.3.1 Genetic stability of the production strain

The fermentation process always starts from identical replicas of the AR-996 (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. Ampoules in a WCB ampoule Bank are only accepted for production runs if their quality meets the required standards. This is determined by checking identity, viability,

microbial purity and productivity of the WCB ampoules. The accepted WCB ampoules are used as seed material for the inoculum.

Testimony to the stability of the strain is given by monitoring the growth behavior and by production of comparable levels of invertase activity in number of fermentation batches performed for the AR-996 strain. The activity measurements from parallel fermentations show that the productivity of the AR-996 strain remains similar. This clearly indicates that the strain is stable. In addition, to confirm the genetic stability of AR-996, a Southern blot was prepared using the genomic DNAs isolated from the mycelia collected from the end of three independent fermentations. Three different restriction digestions were performed, and the expression cassette transformed was used as a probe in the hybridization. The hybridization patterns were identical in all samples. The data of the analysis of enzyme activities from preparations deriving from different fermentation batches of the recombinant AR-996 strain is presented in Appendix #1.

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

No vector sequences were integrated.

A Southern blot hybridization experiment using plasmid vector as a labelled probe and genomic DNA of the production strain AR-996, digested with three different restriction enzymes was performed to confirm that no vector DNA is included in the genome of AR-996. It produced negative result (no hybridization), demonstrating that no part of the plasmid vector removed to generate the linear transforming DNA fragments was introduced into the *Trichoderma* production strain.

2.3.3 Demonstration of the absence of the GMM in the product

The down-stream process following the fermentation includes unit operations to separate the production strain. The procedures are executed by trained staff according to documented standard operating procedures complying with the requirements of the quality system.

The invertase enzyme production strain is recovered from the fermentation broth by a widely used process that results in a cell-free enzyme concentrate. The absence of the production strain is

confirmed for every production batch, using an internal Roal[13] method. This method has been validated in-house. The sensitivity of the method is 1 cfu/20 ml in liquid and 1 cfu/0.2 gram in dried semifinals.

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

The AR-996 enzyme preparation is free from detectable, viable production organism as demonstrated in Appendix #1. As the absence of the production strain is confirmed for every production batch, no additional information regarding the inactivation of the GMM cells is required.

2.3.5 Information on the possible presence of DNA

The invertase enzyme preparation is produced by an aerobic submerged microbial fermentation using a genetically modified *Trichoderma reesei* strain. All viable cells of the production strain, AR-996, are removed during the down-stream processing: the fermentation broth is filtered with pressure filters and subsequent sheet filters, concentrated with ultra-filtration, and optionally followed by sheet filtration(s).

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

2.3.6 Absence of Antibiotic Genes and Toxic Compounds

As noted above, the transformed DNA does not contain any antibiotic resistance genes. Further, the production of known mycotoxins according to the specifications elaborated by the General Specifications for Enzyme Preparations Used in Food Processing Joint FAO/WHO Expert Committee on Food Additives, Compendium of Food Additive Specifications, FAO Food and

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

Nutrition Paper[14] (FAO/WHO 2006) has been also tested from the fermentation product of the *Trichoderma reesei* strain AR-996. The Food Chemicals Codex ("FCC", 13th edition 2022), states the following: "Although limits have not been established for mycotoxins, appropriate measures should be taken to ensure that the products do not contain such contaminants." Adherence to specifications of microbial counts is routinely analyzed. The absence of antibiotic activities, according to the specifications recommended by JECFA (FAO/WHO 2006), was also confirmed from three AR-996 enzyme production batches in Appendix #1 and no antibiotic or toxic compounds were detected.

We confirm that the *T. reesei* AR-996 production strain is non-toxigenic. In regard to the mycotoxins produced by *Trichoderma reesei*, the composition report provided as Appendix #1 demonstrates mycotoxin values below the levels of quantification (LoQs) for the enzyme concentrate batches tested.

2.4 ENZYME PRODUCTION PROCESS

2.4.1 Overview

The food enzyme is produced by ROAL Oy[15] by submerged fermentation of *Trichoderma reesei* AR-996 in accordance with current Good Manufacturing Practices for Food (GMP) and the principles of Hazard Analysis of Critical Control Points (HACCP). As it is run in the EU, it is also subject to the Food Hygiene Regulation (852/2004).

The enzyme preparation described herein is produced by controlled fed-batch submerged fermentation. The production process involves the fermentation process, recovery (downstream

[14] In the General Specifications for enzyme preparations laid down by JECFA in 2006, the following is said: "Although nonpathogenic and nontoxigenic microorganisms are normally used in the production of enzymes used in food processing, several fungal species traditionally used as sources of enzymes are known to include strains capable of producing low levels of certain mycotoxins under fermentation conditions conducive to mycotoxin synthesis. Enzyme preparations derived from such fungal species should not contain toxicologically significant levels of mycotoxins that could be produced by these species." Additionally, no genes have been introduced that encode antimicrobial resistance to the parental or recipient organisms.

[15] See footnote 1

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

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.

AB Enzymes expects no major food allergens to be in the final enzyme preparation. A rigorous allergen risk assessment is routinely conducted during the manufacturing of the final ingredient (i.e., enzyme preparation) for the purpose of determining and avoiding cross-contamination of food allergens into the final enzyme concentrate (before formulation). AB Enzymes uses a wheat-

based fermentation ingredient for production of the invertase enzyme preparation from AR-996 production strain. We routinely test our enzyme products for gluten traces at an external testing partner using an R5 antibody-based ELISA (Codex Alimentarius specifies in Codex Standard 118-1979 (2008)) and recent analysis has detected gluten traces in the invertase enzyme preparation below the limit of quantification (LoQ) <5ppm.

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-996 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:

Property	**Requirement**	
Activity	min.	7000 GLU/g
Appearance	Brown liquid	
Density	1.20 g/ml	

2.5.2 Formulation of a typical enzyme preparation

Composition	
Constituent	**%**
Enzyme concentrate	2
Glycerol	50
Tri Sodium Citrate	2.26
Citric Acid	0.45
Water	Reminder

2.5.3 Purity and identity specifications of the enzyme preparation

It is proposed that the food enzyme invertase 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[16]

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: No significant levels[17]

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.

[16] 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)

[17] See JECFA specifications, ftp://ftp.fao.org/docrep/fao/009/a0675e/a0675e00.pdf, page 64: Although nonpathogenic and nontoxigenic microorganisms are normally used in the production of enzymes used in food processing, several fungal species traditionally used as sources of enzymes are known to include strains capable of producing low levels of certain mycotoxins under fermentation conditions conducive to mycotoxin synthesis. Enzyme preparations derived from such fungal species should not contain toxicologically significant levels of mycotoxins that could be produced by these species.

Other enzymatic activities: the food enzyme is standardized on enzyme activity. Apart from it, the production organism *Trichoderma reesei* produces other endogenous *Trichoderma reesei* proteins, e.g. **xylanase.** 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 *Trichoderma reesei* AR-996 enzyme preparation is invertase (IUBMB 3.2.1.26). The **function** of the invertase enzyme is to catalyse the breakdown of sucrose to fructose and glucose utilizing the hydrolysis of terminal non-reducing β-D-fructofuranoside residues in β-D-fructofuranosides (primary reaction). As a secondary reaction (side-activity of the invertase enzyme) in the production of sc-FOS, the **same enzyme molecule** catalyses fructotransferase reactions that means, that a fructose molecule from one sucrose molecule is transferred to another sucrose molecule to produce sc-FOS and glucose. This is ubiquitous for all invertase enzymes.

For the intended use of invertase in the production of short chain fructooligosaccharides and in fruit and vegetable processing, the substrate is sucrose. Consequently, the substrate for invertase occurs naturally and is therefore a part of the human diet.

The end products or reaction products for invertase are glucose and fructose or short chain fructooligosaccharides, depending on the reaction. All these reaction products are also found in many organisms and occur naturally in food for human consumption.

Enzyme reactions:

Primary[18]- Sucrose + H_2O ⇔ glucose + fructose

[18] Information on EC 3.2.1.26 - beta-fructofuranosidase - BRENDA Enzyme Database (brenda-enzymes.org)

Secondary[19]**-** Sucrose ⇔ FOS + glucose

The method to analyze the activity of the enzyme is company specific and is capable of quantifying invertase activity as defined by its IUBMB classification. The enzyme activity is usually reported in GLU/g.

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

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

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

Furthermore, the presence 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 activity

[19] See footnote 17

comes directly from the production strain. It is generally accepted that the enzyme proteins themselves do not pose any safety concern and are recognized to be generally considered as safe. AB Enzymes is not aware of any adverse effects from the side activities present in the **invertase** enzyme preparation.

Adverse effects from side activities are not expected from the **invertase** enzyme preparation. *Trichoderma reesei* has a long history of safe use in the food industry can described in the dosser (section 6.1.). The side activities that would arise in the enzyme preparation comes from the production microorganism *Trichoderma reesei*. The main enzyme activities produced by *T. reesei*, the four major cellulases, are not present in the invertase enzyme preparations as the encoding genes have been deleted from the recipient strain. This recombinant microorganism is known to produce enzymatic side activities of **xylanase** in low amounts. As noted in section 2.6, the invertase enzyme has a secondary reaction in the presence of two fructose molecules. These side activities are considered to be normal and of no adverse consequence to human health. Xylanases are considered globally as safe food enzymes and are part of the human diet.

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 **invertase**, 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 invertase 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[20].
- 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,

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

denatured proteins are much less immunogenic than the corresponding native proteins (Valenta and Kraft 2002; Valenta 2002; Takai et al. 2000; Nakazawa et al. 2005; Kikuchi et al. 2006).

- To add on, residual enzyme still present in the final food will be subjected to digestion in the gastro-intestinal system, that reduces further the risk of enzyme allergenicity. While stability to digestion is considered as a potential risk factor of allergenicity, it is believed that small protein fragments resulting from digestion are less likely to be allergenic (Joint FAO/WHO Codex Alimentarius Commission et al. 2009; 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 **invertase** 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.

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
- Search for 8 amino acid exact matches

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 Invertase 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 that 35 % identity were found from the AllergenOnline database using the 80-mer sliding window search
Search of 8 amino acid exact matches	The search modus used a number of 679 8mers as query. None of them delivered a hit in the database.

According to the results obtained from the alignments and taking into account the most recent scientific recommendations on the interpretation of such data lead us to conclude that the **invertase** enzyme is of no concern.

To summarize, the bioinformatics approach to estimate potential allergenicity and cross-reactivity based on relatedness to known allergens and taking into account the most recent scientific recommendations on the interpretation of such data leads us to conclude that the **invertase** produced by *Trichoderma reesei* AR-996 is of no concern.

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

Like any other enzyme, the invertase act as a biocatalyst: with the help of the enzyme, a certain substrate is converted into a certain reaction product. The technical effect on the food or food ingredient is caused by the conversion of the substrate to the reaction product caused by the enzymatic reaction involving invertase. Once the conversion occurs, the enzyme can no longer perform a technological function.

Like most enzymes, the invertase performs its technological function during food processing. The invertase from *Trichoderma reesei* AR-996 object of this notice is specifically intended to be used in **the production of short chain fructooligosaccharides (sc-FOS)** and **sugar reduction in fruit and vegetable processing**. In the production of sc-FOS, invertase is used as a processing aid in food manufacturing and is not added directly to final foodstuffs.

The production of the described sc-FOS (combination of sucrose and one to four fructose molecules) utilizes sucrose: the **substrate** for invertase is sucrose.

The **function** of the invertase enzyme is to catalyse the breakdown of sucrose to fructose and glucose utilizing the hydrolysis of terminal non-reducing β-D-fructofuranoside residues in β-D-fructofuranosides (primary reaction). As a secondary reaction (side-activity of the invertase enzyme), the **same enzyme molecule** catalyses fructotransferase reactions that means, that a fructose molecule from one sucrose molecule is transferred to another sucrose molecule to produce sc-FOS and glucose. This is ubiquitous for all invertase enzymes.

Please refer to Figure #1 below for the reaction diagram.

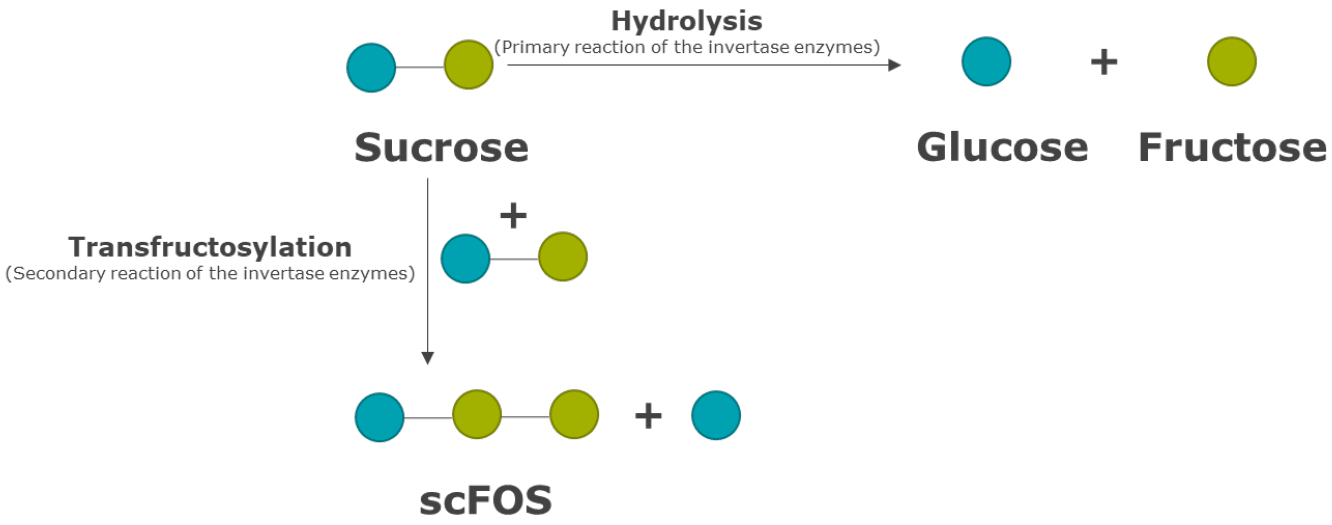

Figure #1 Hydrolysis and Transfructosylation reaction of the Invertase

Invertase from *Trichoderma reesei* AR-996 production strain is intended for use in the following applications:

- Production of sc-FOS from sucrose
- Sugar reduction in fruit and vegetable processing

sc-FOS Production

The sc-FOS production industry is relatively new and picking up traction due to the categorization of the substance as a prebiotic ingredient. Prebiotics fall under the functional food category which imply such foods have confirmed or potential health benefits for consumers due to their biofunctional properties (Ojwach et al. 2022; Mutanda et al. 2014). sc-FOS has the ability to stimulate gastrointestinal bacteria such as bifidobacteria (Martins et al. 2019). sc-FOS's history in food began in Japan with food producers adding FOS as a functional ingredient (Martins et al. 2019). Part of the interest in sc-FOS is the biochemical characteristics of the ingredient where compared to other saccharides, sc-FOS has smaller molecular weight, and polymerization (Ojwach et al. 2022). As an oligosaccharide, FOS is found in a number of fruits and vegetables which are part of the human diet, such as banana, barley, garlic, honey, onion, rye, chicory, Jerusalem

artichoke, yacon, cereal plants and tomato (Ojwach et al. 2022; Mutanda et al. 2014; Martins et al. 2019).

Use of enzymes in the industrial production of sc-FOS is an alternative to already established acid/chemical methods (Martins et al. 2019). The enzymatic production of sc-FOS in a simplified industrial conditions environment where the enzyme's ability to flip activity (between invertase and fructosyltransferase) streamlines the need to avoid additional control steps for sc-FOS production from sucrose.

Below, the benefits of the use of industrial invertase in those processes are described. The beneficial effects are of value to the food chain because they lead to better and/or more consistent product quality. Moreover, the applications lead to more effective production processes (such as reduction in complexity, enzymes are managed according to pH and temperature conditions), resulting in better production economy and environmental benefits such as the use of less raw materials (i.e., synthetic chemicals) and the production of less waste. Invertase is an established enzyme for the use of sc-FOS production for many years and its use in this growing industry is continuously increasing. The use of invertase has been recognized as acceptable in the production of sc-FOS for several years in the USA[21,22], Canada[23], and Australia/New Zealand[24,25] which demonstrates the technological need for such food enzymes in food processes.

In general, the benefits of invertase in sc-FOS production are:

- Higher yields of sc-FOS as final product
- No additional substrates as sucrose needed
- Lower production costs (enzyme synthesis vs. acid/chemical hydrolysis)

[21] GRAS Notice GRN 537: short chain fructo-oligosaccharides produced with invertase for use in infant formula
[22] GRAS Notice GRN 1006: short chain fructo-oligosaccharides produced with invertase for general use in food
[23] 5. List of Permitted Food Enzymes (Lists of Permitted Food Additives) - Canada.ca: Invertase is a permitted food additive enzyme for sucrose used in the production of fructooligosaccharides
[24] Application A1055 - Short-chain Fructo-oligosaccharides (foodstandards.gov.au): Invertase from *Aspergillus niger* was approved as a processing aid by FSANZ
[25] A1212 - Beta-fructofuranosidase enzyme from Aspergillus fijiensis (foodstandards.gov.au): Application to update Schedule 18 of FSANZ's Food Code entry for invertase from *Aspergillus niger* to *Aspergillus fijensis*

- Decreased chance in potential microbial contamination during manufacturing
- Less use of raw materials
- Energy savings and production of less waste products

Sugar Reduction in Fruit and Vegetable Processing:

In recent years, numerous studies have shown the negative health effects of high consumption of sugars and the positive health benefits of increasing the soluble dietary fiber in human diets. (Evans 2017; Deliza et al. 2021; Prada et al. 2022; Rippe and Angelopoulos 2016; Respondek et al. 2014; Nobre et al. 2022) In response to these studies and the recommendation to reduce the glycemic load (Augustin et al. 2015), the demand for lower glycemic index foods, which are less sugary and higher in soluble dietary fiber has been increased. To meet this demand, the replacement of traditional sugary carbohydrates like sucrose, glucose or fructose with substitutes like non-nutritive sweeteners, sugar alcohols, glucooligosaccharides and short chain fructooligosaccharides (sc-FOS) has been investigated and applied (Martins et al. 2019; Respondek et al. 2014). Particular interest has been directed to sc-FOS. These compounds impart mild sweetness, but also significantly, they are soluble dietary fibers with health benefits (Flores-Maltos et al. 2016; Respondek et al. 2014; Nobre et al. 2022). Some fruit and vegetable raw materials like banana, oranges, apples or carrots contain substantial amounts of sugars. The treatment of fruit and vegetable raw materials with invertase as part of the standard raw material processing leads to sucrose hydrolysis and sc-FOS formation and reduces the endogenous or naturally occurring sugar content. The addition of invertase is possible at several production steps depending on raw material, production process and final product.

In general, the benefits of invertase for sugar reduction in fruits and vegetable products are:

- Reduction of total sugars content, the sum of mono - and disaccharides like glucose, fructose and sucrose
- Increase of soluble dietary fiber content
- Decrease of glycemic index

- Decrease of energy value (calories)
- Less sweet taste
- In situ process for sugar reduction – no removal of valuable other substances like vitamins or organic acids

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 **invertase** 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 **invertase** from *Trichoderma reesei* AR-996 may be used:

Food Application	Raw material (RM)	Suggested recommended use levels (mg TOS/kg RM)
Short chain-fructooligosaccharides (sc-FOS) production	Sucrose	7
Sugar reduction in fruit and vegetable processing	Fruits and vegetables	14

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.

Invertase 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 invertase 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

Fate of invertase in sc-FOS production:

In sc-FOS production, invertase is added during the enzyme reaction step after the preparation of sucrose solution. Considering previous information supplied to FDA on enzyme use in sc-FOS production (most recently GRN 1006[26]), invertase performs its technological function during the first steps of the sc-FOS production process. GRN 1006 also indicated the removal of the enzyme from the final sc-FOS product. To determine the removal of the enzyme in the production of sc-FOS, we have conducted laboratory trails replicating industrial conditions up to the ion exchange chromatography filtration step. When the sc-FOS solution passes through ion exchange chromatography, the enzyme binds to the exchange resin while the sc-FOS solution continues moving forward to the other manufacturing steps. The laboratory trials showed 98% removal of the enzyme via western blot analysis.

Summary of laboratory trials

In this study, the presence of the invertase enzyme used to produce sc-FOS was investigated. The invertase enzyme was purified and used as an antigen to produce polyclonal antibodies. By using these antibodies, a Western Blot was established for the sensitive detection of the invertase enzyme. The validation and limit of detection (LOD) of the Western Blot was determined internally with the LOD at 0.01 µg. The study is based on the enzymatic release of glucose from sucrose. Five times the recommended enzyme dosage was used to demonstrate that even at higher enzyme protein concentrations, as commonly used in industry, can be removed during the sc-FOS production process. The enzymatic reaction took place at 60 °C and pH 5.7 for 60 min followed by enzyme deactivation at 85 °C for 15 minutes. For the laboratory trial, a 70% sucrose solution was incubated with invertase enzyme at an activity of 37 GLU per gram of sucrose solution to generate sc-FOS.

In replicating the ion exchange chromatography step, a strong anion exchange resin was used for the purpose to bind negatively charged particles. An anion exchange resin is suitable for the

[26] See footnote 22

removal of the enzyme protein due to the negative charge of the protein at the pH value of 5.7. The isoelectric point (pI; protein has no net charge) of the invertase enzyme, based on the amino acid sequence of the mature protein, is calculated to be at pH=4.73. Thus, at a higher pH-value the protein has net negative charge. Due to the net negative charge of the enzyme protein it binds to the positive charge of the anion exchange chromatography resin. Less than 0.01 µg invertase enzyme was detected after the anion exchange chromatography step. The amount of 0.01 µg invertase enzyme corresponds to 2.1% of the applied 0.48 µg invertase enzyme which means that at least 98% invertase enzyme was removed by the anion exchange chromatography. Thus, we conclude that potential residual enzyme protein in the sc-FOS product is negligible because in the lab trials it was shown that even a 5-times higher amount of invertase enzyme (compared to AB Enzymes dose recommendation) can be removed to at least 98% already only by the anion exchange chromatography.

Additional Information on Enzyme Removal

Another enzyme removal step to consider is active carbon filtration. In short, the objective of using active carbon filtration is to remove organic substances from the substance undergoing filtration; enzymes are among the organic substance removed by activated carbon filtration as enzymes are composed of amino acids which are carbon based. Evidence of the ability for activated carbon to separate proteins was demonstrated to be based on three different types of activated carbon by both static treatment and by flowing through a packed column of activated carbon (Stone and Kozlov 2014).

In the case of active carbon trapping the enzyme, Kareem et al. (2011) demonstrated with activated charcoal the enzyme amylase is trapped for amylase recovery. To give an example outside of the food industry, active carbon can remove host cell protein from downstream processing (Slocum et al. 2021). Given that activated carbon filtration can remove enzymes from the filtrated solution then the following logically conclusion can be reached. Activated carbon filtration can effectively immobilize any enzyme residues missed by ion chromatography. This information adds to the weight of evidence of no or negligible residues of the invertase in the sc-

FOS syrup and powder (final foods) as there are two filtration steps that can remove the notified enzyme. With the evidence of enzyme removal in the production of sc-FOS, a calculation of margin of exposure is not required as the enzyme would be absent in the final food.

Fate of invertase in fruit and vegetable processing:

In fruit and vegetable processing, the invertase is denatured by a heat pasteurization step.

- Inactivation conditions in pasteurized products:
    - Invertase: >75°C / >2min

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 **invertase** 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)	Suggested recommended use level (mg TOS/kg RM)	Final food (FF)	Ratio RM/FF*	Suggested level in final food (mg TOS/kg food)
Liquid foods	Sugar reduction in fruit and vegetable processing	Fruits/Vegetables	14	Fruit and vegetable juices including citrus juices	1.3	**18.2**
Solid foods	Sugar reduction in fruit and vegetable processing	Fruits/Vegetables	14	Purees	1	**14**

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

- *For fruit juices, we assume that a RM/FF ratio of 1.3 kg fruit per L of fruit juice will be used (typically 0.75-0.9 l juice is produced per kg of fruit thus the range for RM/FF will be 1.1-1.3 kg fruit per L of fruit juice).*
- *For fruit purees, we assume a RR/FF of 1 (1 kg of fruits / kg of puree).*

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)
14 x 0.0125 = 0.175	18.2 x 0.025 = 0.455	**0.630**

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 invertase from *Trichoderma reesei* AR-996;
- 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 (Douglass et al. 1997).

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)[27] 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 invertase is calculated to be 0.560 mg TOS/kg body weight/day.

As a result, the MoE is:

MoE =1000/0.630 =**1587**.

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 invertase from *Aspergillus oryzae* AR-996 in the production of food is safe. Considering the high safety value determined by the MoS, 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.

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

All available known information has been reviewed and AB Enzymes GmbH is not aware of any data or information that is, or may appear to be, 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

The safety of *Trichoderma reesei* as an enzyme producer has been reviewed by Nevalainen et al.; Olempska-Beer et al.; Blumenthal (1994; 2006; 2004). T. *reesei* is regarded as a safe organism for production of industrial enzymes.

Food enzymes, including those derived from recombinant *Trichoderma reesei* strains, have been evaluated by JECFA and many countries which regulate the use of food enzymes, such as the USA, France, Denmark, Australia and Canada, resulting in the approval of the use of food enzymes from *Trichoderma reesei* in the production of various foods, such as baking, brewing, juice production, wine production and the production of dairy products.

Non exhaustive list of authorized food enzymes (other than invertases) produced by *Trichoderma reesei*		
Authority	**Food Enzyme**	**Reference**
JECFA	Cellulase Beta-glucanase Glucoamylase	FAS 30-JECFA 39/15 and FAS 22-JECFA 31/31 FAS 22-JECFA 31/25, JECFA monograph gluco amylase
Australia/New Zealand	Cellulase Glucan 1-3 beta-glucosidase Beta-glucanase Hemicellulase complex Gluco-amylase Endo 1,4-beta- xylanase Pectinases	Australia New Zealand Food Standards Code – Schedule 18 – Processing aids (legislation.gov.au)
Canada	Cellulase Glucanase Pentosanase Xylanase Protease Pectinase	5. List of Permitted Food Enzymes (Lists of Permitted Food Additives)
USA[28]	Pectin lyase Transglucosidase (GM) Glucoamylase Phospholipase A Polygalacturonase Pectin esterase Mannanase Endo-1,4-beta-xylanase	GRAS Notice Inventory, GRN 32 GRAS Notice Inventory, GRN 315 GRAS Notice Inventory, GRN 372 GRAS Notice Inventory, GRN 524 GRAS Notice Inventory, GRN 557 GRAS Notice Inventory, GRN 558 GRAS Notice Inventory, GRN 566 GRAS Notice Inventory, GRN 628

[28] GRAS affirmations and GRAS notifications

	Lipase Lysophospholipase Glucose oxidase Serine endopeptidase	GRAS Notice Inventory, GRN 631 GRAS Notice Inventory, GRN 653 GRAS Notice Inventory, GRN 707 GRAS Notice Inventory, GRN 817
France	Alpha-amylase (GM) Amyloglucosidase (GM) Beta-glucanase (GM) Xylanase Cellulase Lysophospholipase (GM)	Arrêté du 19 octobre 2006

Non exhaustive list of authorized invertase from production organisms other than *Trichoderma reesei*		
Authority	**Production organisms**	**Reference**
Australia/NZ	*Aspergillus niger* *Aspergillus fijiensis ATCC 20611* *Saccharomyces cerevisiae*	Australia New Zealand Food Standards Code – Schedule 18 – Processing aids (legislation.gov.au)
France	*Aspergillus niger*	Arrêté du 19 octobre 2006
USA	*Saccharomyces cervisiae*	GRAS Notice Inventory, GRN 88
Brazil	*Aspergillus niger* *Bacillus subtilis* *Kluyveromyces fragilis* *Saccharpmyces carlsbergensis*	RDC N 53 October 7, 2014, RDC Nº 728 July 1, 2022

	Saccharomyces cerevisiae	
Canada	*Aspergillus fijiensis* *Saccharomyces sp.*	5. List of Permitted Food Enzymes (Lists of Permitted Food Additives)
South Korea	*Aspergillus aculeatus & variants* *Aspergillus awamori & variants* *Aspergillus niger & variants* *Bacillus genus* *Kluyveromyces lactis & variants* *Saccharomyces cerevisiae & variants*	South Korean Food Code Invertase
JECFA	*Saccharomyces cerevisiae*	JECFA Evaluations-INVERTASE FROM SACCHAROMYCES CEREVISIAE-(inchem.org)

6.1.2 Safety of the genetic modification

The recipient strain used for the genetic modification is a *Trichoderma reesei* mutant strain, which originates from QM6a. This mutant strain has been shown to be genetically stable for industrial production.

Trichoderma reesei has a long history (more than 30 years) of safe use in industrial-scale enzyme production (Blumenthal 2004), and (Olempska-Beer et al. 2006) with a conclusion that the organism *T. reesei* is non-pathogenic and non-toxigenic and can be considered as a safe organism to be used as a host for production of enzymes for food and feed processing (as well as for other industrial applications) based upon the decision tree (Appendix #3) (Pariza and Johnson 2001; Nielsen 2010).

The gene encoding for invertase produced by *Trichoderma reesei* AR-996 originates from *Aspergillus niger*. *Aspergillus niger* has a long and established history of use as an industrial enzyme production organism. AB Enzymes limits the possibilities of mutations in the product enzyme, as well as in the production strain, through the inoculation of the seed culture for the fermentation with controlled spore stocks that have been stored at -80°C.

Our evaluation of the genetically modified *T. reesei* strain is comparable to that of the recipient strain and the produced food enzyme is non-pathogenic for healthy humans and animals.

The synthetic acetamidase encoding *amd*S *gene* of *Aspergillus nidulans* is used as a selectable marker. *A. nidulans* is closely related to *Aspergillus niger* which is used in industrial production of food enzymes. The product of the *amd*S gene, acetamidase (AmdS), can degrade acetamide which enables the strain to grow on media without any other nitrogen sources. The AmdS is not harmful or dangerous; the *amd*S marker gene has been widely used as a selection marker in fungal transformations without any disadvantage for more than 30 years.

The transformed expression cassette, fully characterized and free from any harmful sequence or any potential hazards, is stably integrated into the fungal genome, and is no more susceptible to any further natural mutations than any other genes in the fungal genome.

No additional growth/mutagenesis cycles have been performed after the AR-996 strain has been constructed and thereafter deposited to the culture collection (Master Cell Bank, MCB).

Therefore, it can be concluded that the *Trichoderma reesei* strain AR-996 can be regarded as safe as the recipient or the parental organism to be used for production of enzymes for food processing.

Enzyme Safety Narrative:

For determining safety of an enzyme preparation used in food processing, the primary consideration is safety of the production strain (Pariza and Johnson 2001). The safety of the enzyme itself, the invertase subject to this GRAS notice can also be considered safe for use in food processing based on:

- History of safe use in food
- Well known and monitored manufacturing conditions of the commercial enzyme preparation
- Low risk of allergenic potential confirmed by bioinformatics
- Fate of the enzyme in food

Invertases are not harmful, invertases are safe for use in food and they are readily biodegradable in the environment. Fungal (*Saccharomyces cereus*) invertase preparation is listed in FDA´s GRAS *Notice* Inventory for manufacturing of food (see table above). Entries of invertase from the donor *Aspergillus niger* (see table above) are listed in other countries' enzyme food positive lists or regulations, adding to the weight of evidence that invertase is safe for use in food. In addition, use of invertase in fructooligosaccharides production has been recognized in USA and other countries (see section 2.8). Several different types of invertases are also naturally produced by organisms in nature, even though in much lower relative amounts compared to the production of the recombinant invertase by *Trichoderma reesei* AR-996 production strain.

To add on, the manufacturing conditions of the enzyme are relevant to consider in regard to safety. The invertase commercial enzyme preparation is manufactured using GMP (good manufacturing practice) with raw materials conforming to the specifications in the 13th and current edition of the Food Chemicals Codex. The commercial enzyme preparation complies with the requirements in JECFA's General Specifications of Food Enzyme Preparations as demonstrated by the specifications of the enzyme batches in section 2.5 of the notice.

Furthermore, an allergenic potential was not detected for this invertase. As explained in section 2.7.1. the allergen searches did not detect matches of concern for any of the searches.

Lastly, the fate of the enzyme in the final food is relevant to consider with regard to the safety of the enzyme. Invertase is used in the production of short chain fructooligosaccharides for the breakdown of sucrose into fructose and glucose and in fruit and vegetable processing to reduce sugars. Sections 2.8 and 2.10 demonstrate that invertase does not perform any technical function in the final product. Based on detailed assessment, including the high safety factor calculated by means of an overestimation of the intake, the overall conclusion is that the use of this enzyme in the production of food is safe.

The recipient strain used for the genetic modification is a *Trichoderma reesei* mutant strain, which originates from QM6a. This strain has been shown to be genetically stable for industrial production.

6.1.3 Toxicological testing

The safety of the invertase produced by the genetically modified *Trichoderma reesei* AR-996 from a toxicological perspective is supported by the historical safety of strain lineage. Toxicological studies were performed on a strain (AR-700) which derives from the same recipient strain within the strain lineage of AR-996.

The following studies were performed on a *Trichoderma reesei* AR-700 from the AR-996 strain lineage:

- Reverse Mutation Assay using Bacteria (*Salmonella typhimurium and Escherichia Coli*) with *Trichoderma reesei* produced phytase
- Micronucleus Assay in Bone Marrow Cells of the Rat with *Trichoderma reesei* produced phytase

- *Trichoderma reesei* produced phytase: 90-Day Oral Toxicity (Gavage) Study in the Wistar Rat

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

Please refer to below to the summary of each of the toxicological studies in Appendix #3.

As mentioned above both the AR-996 and AR-700 have been developed from the same recipient. Expression constructs 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, the expression cassettes are very similar and are stably integrated into the genome of the strains without any additional growth/mutagenesis cycles thereafter, differences in the genetic modification of AR-996 and AR-700 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-996 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-996.

Safety of the Production strain (SSL):

For more details on the safety of the *Trichoderma reesei* AR-996 production strain, we refer to the following appendices:

- **Pariza and Johnson Decision Tree** (Appendix #3)
- **JECFA Safe Progeny Strain statement** (Appendix #4)
- **Differences between tox tested strain and AR-996 production strain** (Appendix #4)
- **Diagram on Strain Lineage** (Appendix #4)

6.1.4 Pathogenicity and Toxigenicity

Trichoderma reesei strains are non-pathogenic for healthy humans and animals. As mentioned above, *Trichoderma reesei* is not present on the list of pathogens in the EU (Directive Council Directive 2000/54/EC) and is present in major culture collections worldwide, as it is globally regarded as a safe microorganism:

Trichoderma reesei is globally regarded as a safe microorganism:

- In the USA, *Trichoderma reesei* is not listed as a Class 2 or higher Containment Agent under the National Institute of Health (NIH, 1998) Guidelines for Recombinant DNA Molecules. Data submitted in Generally Recognized as Safe (GRAS) petitions to the Food and Drug Administration (FDA) for numerous enzyme preparations from *T. reesei* for human and animal consumption demonstrate that the enzymes are nontoxic. The Environmental Protection Institute (EPA) completed a risk assessment on *T. reesei* in 2011 resulting in a Proposed Rule in 2012, concluding that it is appropriate to consider *T. reesei* as a recipient microorganism eligible for exemptions from full reporting requirements[29], if this fungus was to be used in submerged standard industrial fermentation for enzyme production. To add on in March 2020, the EPA issued a final rule on **Microorganisms; General Exemptions From Reporting Requirements; Revisions to Recipient Organisms**

[29] reporting procedures in place under the Toxic Substances Control Act (TSCA) for new micro-organisms that are being manufactured for introduction into the commerce

Eligible for Tier I and Tier II Exemptions[30] as part of the 40 Code of Federal Regulations Part 725 where *Trichoderma reesei* is classified as a Tier I organism.

As a result, AR-*996* can be used under the lowest containment level at large scale, GILSP, as defined by OECD (OECD 1992).

The genus *Trichoderma* contains filamentous fungi which are frequently found on decaying wood and in soil. Industrial *T. reesei* strains have a long history of safe use and several of the *Trichoderma* based products have been approved for food and feed applications[31]. *T. reesei* is listed as a "Risk Group 1" organism according to German TRBA classification (Federal Institute for Occupational Safety and Health, www.baua.de) and as "Biosafety Level 1" organism by the American Type Culture Collection (www.atcc.org). *Trichoderma reesei* strains are non-pathogenic for healthy humans and animals. The DNA based identification methods have shown that *T. reesei* is taxonomically different from the other *Trichoderma* species of the section *Longibrachiatum* (Druzhinina et al. 2005).

Some species belonging to *Trichoderma* genus are able to secrete various types of antibiotics in laboratory cultures. However, strains of *T. reesei* used in industrial applications are proven to be devoid of antibiotic activities (Coenen et al. 1995; Hjortkjaer et al. 1986). The absence of antibiotic activities, according to the specifications recommended by JECFA (FAO/WHO 2006), was also confirmed for AR-996. The analyzed data are presented in Appendix #1.

Additionally, the original *T. reesei* host and the genetically modified *T. reesei* strain do not carry any acquired antimicrobial resistance genes.

The production strain is non-toxigenic for the following reasons:

- Results of the toxicological studies provided in the narrative (Appendix #4);

[30] https://www.regulations.gov/document?D=EPA-HQ-OPPT-2011-0740-0018

[31] AMFEP. 2009. Association of Manufacturers and Formulators of Enzyme Products List of enzyme products on markets;
http://amfep.drupalgardens.com/sites/amfep.drupalgardens.com/files/Amfep-List-of-Commercial-Enzymes.pdf

- Safety and history of use of the production organism *Trichoderma reesei;*
- Mycotoxin testing results presented in the composition report (Appendix #1).

With the use of safe strain lineage, we have substantiated the safety of the AR-996 *Trichoderma reesei* production strain via three toxicological studies on the *Trichoderma reesei* AR-700 production strain to demonstrate non-toxigenicity of the strain lineage. The toxicological studies conducted include, a reverse mutation assay using bacteria, a Micronucleus Assay in Bone Marrow Cells of the Rat and a 90-day repeated dose oral toxicity study in Wister rats. All three toxicological studies showed negative findings demonstrating the AR-700 production strain to be non-mutagenic, to not induce structural and/or numerical chromosomal damage, and to not cause toxigenic effects on the Wister rats tested in the 90-day oral toxicity study. For more details on the results of the toxicological studies conducted on the production strain, please refer to Appendix #3.

To add on, as mentioned in in this section of the dossier, the *Trichoderma reesei* as a production organism has a long history of use for the production of industrial food enzymes. Food enzymes, including those derived from recombinant *Trichoderma reesei* strains, have been evaluated by JECFA and many countries which regulate the use of food enzymes such as France Denmark, Australia, and Canada, apart from the USA. Also, AB Enzymes has used *Trichoderma reesei* strains for food enzyme production for many years without any safety problems. Lastly, we have demonstrated the low presence of the mycotoxins produced by the *Trichoderma reesei* microorganism. The composition report provided as an appendix to this GRAS notification demonstrates mycotoxin values below the levels of quantification (LoQs) for the enzyme concentrate batches tested.

Conclusion: Based on the above-mentioned available data, it is concluded that the organism *T. reesei*, has a long history of safe use in industrial-scale enzyme production and can be considered as a safe production organism for enzymes for food as well as feed processing and numerous other industrial applications. As an example, *T. reesei* strains have been cultivated in the production plant of Alko Oy/Roal Oy since 1987. During this time, genetic engineering techniques

have been used to improve the industrial production strains of *Trichoderma reesei* and considerable experience on the safe use of recombinant *Trichoderma reesei* strains at industrial scale has accumulated. From above, secondary metabolites are of no safety concern in fermentation products derived from *Trichoderma reesei*. Thus, *Trichoderma reesei* and its derivatives can be considered generally safe not only as a production organism of its natural enzymes, but also as a safe host for heterologous gene products. In section 6.1.2 we also provided a short narrative on the safety of the invertase enzyme.

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-996 Composition Report
2. Flow Chart of the manufacturing process with control steps
3. Summary of Toxicological Studies and Decision Tree
4. Safe Strain Lineage Narrative AR-996

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.

Augustin, L. S. A.; Kendall, C. W. C.; Jenkins, D. J. A.; Willett, W. C.; Astrup, A.; Barclay, A. W. et al. (2015): Glycemic index, glycemic load and glycemic response: An International Scientific Consensus Summit from the International Carbohydrate Quality Consortium (ICQC). In *Nutrition, metabolism, and cardiovascular diseases : NMCD* 25 (9), pp. 795–815. DOI: 10.1016/j.numecd.2015.05.005.

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.

Blumenthal, Cynthia Z. (2004): Production of toxic metabolites in Aspergillus niger, Aspergillus oryzae, and Trichoderma reesei: justification of mycotoxin testing in food grade enzyme preparations derived from the three fungi. In *Regulatory toxicology and pharmacology : RTP* 39 (2), pp. 214–228. DOI: 10.1016/j.yrtph.2003.09.002.

Coenen, T. M.; Schoenmakers, A. C.; Verhagen, H. (1995): Safety evaluation of beta-glucanase derived from Trichoderma reesei: summary of toxicological data. In *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association* 33 (10), pp. 859–866.

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.

Deliza, Rosires; Lima, Mayara F.; Ares, Gastón (2021): Rethinking sugar reduction in processed foods. In *Current Opinion in Food Science* 40, pp. 58–66. DOI: 10.1016/j.cofs.2021.01.010.

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.

Druzhinina, Irina S.; Kopchinskiy, Alexei G.; Komoń, Monika; Bissett, John; Szakacs, George; Kubicek, Christian P. (2005): An oligonucleotide barcode for species identification in Trichoderma and Hypocrea. In *Fungal genetics and biology : FG & B* 42 (10), pp. 813–828. DOI: 10.1016/j.fgb.2005.06.007.

Evans, Charlotte Elizabeth Louise (2017): Sugars and health: a review of current evidence and future policy. In *The Proceedings of the Nutrition Society* 76 (3), pp. 400–407. DOI: 10.1017/S0029665116002846.

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/.

Flores-Maltos, Dulce A.; Mussatto, Solange I.; Contreras-Esquivel, Juan C.; Rodríguez-Herrera, Raúl; Teixeira, José A.; Aguilar, Cristóbal N. (2016): Biotechnological production and application of fructooligosaccharides. In *Critical reviews in biotechnology* 36 (2), pp. 259–267. DOI: 10.3109/07388551.2014.953443.

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.

Hjortkjaer, R. K.; Bille-Hansen, V.; Hazelden, K. P.; McConville, M.; McGregor, D. B.; Cuthbert, J. A. et al. (1986): Safety evaluation of Celluclast, an acid cellulase derived from Trichoderma reesei. In *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association* 24 (1), pp. 55–63.

Hynes, M. J.; Corrick, C. M.; King, J. A. (1983): Isolation of genomic clones containing the amdS gene of Aspergillus nidulans and their use in the analysis of structural and regulatory mutations. In *Mol Cell Biol* 3 (8), pp. 1430–1439.

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).

Kareem, S. O.; Akpan, I.; Popoola, T. O. S.; Sanni, L. O. (2011): Activated charcoal-a potential material in glucoamylase recovery. In *Enzyme research* 2011, p. 483943. DOI: 10.4061/2011/483943.

Karhunen, T.; Mantyla, A.; Nevalainen, K. M.; Suominen, P. L. (1993): High frequency one-step gene replacement in Trichoderma reesei. I. Endoglucanase I overproduction. In *Molecular and General Genetics MGG* 241 (5-6), pp. 515–522.

Kelly, J. M.; Hynes, M. J. (1985): Transformation of Aspergillus niger by the amdS gene of Aspergillus nidulans. In *The EMBO journal* 4 (2), pp. 475–479.

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/.

Martins, Gonçalo N.; Ureta, Maria Micaela; Tymczyszyn, E. Elizabeth; Castilho, Paula C.; Gomez-Zavaglia, Andrea (2019): Technological Aspects of the Production of Fructo and Galacto-Oligosaccharides. Enzymatic Synthesis and Hydrolysis. In *Frontiers in nutrition* 6, p. 78. DOI: 10.3389/fnut.2019.00078.

Mutanda, T.; Mokoena, M. P.; Olaniran, A. O.; Wilhelmi, B. S.; Whiteley, C. G. (2014): Microbial enzymatic production and applications of short-chain fructooligosaccharides and inulooligosaccharides: recent advances and current perspectives. In *Journal of industrial microbiology & biotechnology* 41 (6), pp. 893–906. DOI: 10.1007/s10295-014-1452-1.

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.

Nevalainen, H.; Suominen, P.; Taimisto, K. (1994): On the safety of Trichoderma reesei. In *Journal of biotechnology* 37 (3), pp. 193–200.

Nielsen, Per Munk (2010): Enzymes in Protein Modification. In Robert J. Whitehurst, Maarten van Oort (Eds.): Enzymes in food technology. 2nd ed. West Sussex, U.K., Ames, Iowa: Wiley-Blackwell, pp. 292–319.

Nobre, Clarisse; Simões, Lívia S.; Gonçalves, Daniela A.; Berni, Paulo; Teixeira, José A. (2022): Fructooligosaccharides production and the health benefits of prebiotics. In : Current Developments in Biotechnology and Bioengineering: Elsevier, pp. 109–138.

OECD (1992): Safety Considerations for Biotechnology. With assistance of Organisation for Economic Co-operation and Development.

Ojwach, Jeff; Adetunji, Adegoke Isiaka; Mutanda, Taurai; Mukaratirwa, Samson (2022): Oligosaccharides production from coprophilous fungi: An emerging functional food with potential health-promoting properties. In *Biotechnology Reports* 33, e00702. DOI: 10.1016/j.btre.2022.e00702.

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.

Penttilä, M.; Nevalainen, H.; Rättö, M.; Salminen, E.; Knowles, J. (1987): A versatile transformation system for the cellulolytic filamentous fungus Trichoderma reesei. In *Gene* 61 (2), pp. 155–164.

Prada, Marília; Saraiva, Magda; Garrido, Margarida V.; Sério, Ana; Teixeira, Ana; Lopes, Diniz et al. (2022): Perceived Associations between Excessive Sugar Intake and Health Conditions. In *Nutrients* 14 (3). DOI: 10.3390/nu14030640.

Respondek, F.; Hilpipre, C.; Chauveau, P.; Cazaubiel, M.; Gendre, D.; Maudet, C.; Wagner, A. (2014): Digestive tolerance and postprandial glycaemic and insulinaemic responses after consumption of dairy desserts containing maltitol and fructo-oligosaccharides in adults. In *European journal of clinical nutrition* 68 (5), pp. 575–580. DOI: 10.1038/ejcn.2014.30.

Rippe, James M.; Angelopoulos, Theodore J. (2016): Relationship between Added Sugars Consumption and Chronic Disease Risk Factors: Current Understanding. In *Nutrients* 8 (11). DOI: 10.3390/nu8110697.

Slocum, Ashley; Santora, Steven; Ly, Mellisa; Zhang, Junyan; Castano, Juan; Becerra-Arteaga, Alejandro (2021): Development of an activated carbon filtration step and high throughput screening method to remove host cell proteins from a recombinant enzyme process. In *Biotechnology Progress* 37 (4), e3151. DOI: 10.1002/btpr.3151.

Stone, Matthew T.; Kozlov, Mikhail (2014): Separating proteins with activated carbon. In *Langmuir : the ACS journal of surfaces and colloids* 30 (27), pp. 8046–8055. DOI: 10.1021/la501005s.

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.

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

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

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

FDA USE ONLY

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

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

SECTION A INTRODUCTORY INFORMATION ABOUT THE SUBMISSION

1. Type of Submission *(Check one)*

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

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

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

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

SECTION B INFORMATION ABOUT THE NOTIFIER

1a. Notifier

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

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

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

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

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

City	State or Province	Zip Code/Postal Code	Country

Telephone Number	Fax Number	E-Mail Address

SECTION C GENERAL ADMINISTRATIVE INFORMATION

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

Invertase enzyme preparation produced by Trichoderma reesei

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 1006

☐ b) GRAS Affirmation Petition No. GRP _____

☐ c) Food Additive Petition No. FAP _____

☐ d) Food Master File No. FMF _____

☐ e) Other or Additional *(describe or enter information as above)* _____

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

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

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

☐ Yes *(Proceed to Item 8*

☒ No *(Proceed to Section D)*

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

☐ Yes, information is designated at the place where it occurs in the submission

☐ No

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

☐ Yes, a redacted copy of the complete submission

☐ Yes, a redacted copy of part(s) of the submission

☐ No

SECTION D INTENDED USE

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

Intended for use as an enzyme at a maximum level of 7 mg total organic solids/kg sucrose during the production of short chain fructooligosacchrides and at a maximum level of 14 mg total organic solids/kg sucrose in fruit and vegetable processing for sugar reduction.

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 Invertase enzyme preparation produced by Trichoderma reesei
(name of notified substance)

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

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

8211 W. Broward Blvd. Suite 375 Plantation, Florida 33324 USA
(address of notifier or other location)

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

3. Signature of Responsible Official, Agent, or Attorney	Printed Name and Title	Date (mm/dd/yyyy)
Joab Trujillo Digitally signed by Joab Trujillo Date: 2022.09.02 13:04:57 -04'00'	Joab Trujillo Regulatory Affairs Specialist	09/02/2022

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 Invertase	Administrative
	AB Enzymes Invertase GRAS Notice	Submission
	1_AR-996 Composition Report	Submission
	2_Flow Chart of the manufacturing process with control steps	Submission
	3_Summary of Toxicological Studies and Decision Tree	Submission
	4_Safe Strain Lineage Narrative AR-996	Submission
	References for AB Enzymes' Invertase GRAS Notice.zip	Submission
	AB Enzymes Cover Letter for Invertase GRAS Notice	Submission