November 13, 2020

RE: GRAS Notification – Exemption Claim

Dear Sir or Madam:

Pursuant to the proposed 21C.F.R. § 170.36 (c)(1) AB Enzymes GmbH hereby claims that sterol esterase (IUBM 3.1.1.13) from a Genetically Modified *Trichoderma reesei* 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:*
Sterol Esterase (IUBMB 3.1.1.13) from a Genetically modified *Trichoderma reesei*

Proposed 21C.F.R. § 170.36 (c)(iii) *Applicable conditions of use:*
The sterol esterase is to be used as a processing aid for partial or extensive hydrolysis of lipids from plant sources and in bread making. The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements under current Good Manufacturing Practices.

Proposed 21C.F.R. § 170.36 (c)(iv) *Basis for GRAS determination:*
This GRAS determination is based upon scientific procedures.

Proposed 21C.F.R. § 170.36 (c)(v) *Availability of information:*
A notification package providing a summary of the information which supports this GRAS determination is enclosed with this letter. The package includes a safety evaluation of the production strain, the enzyme, and the manufacturing process, as well as an evaluation of dietary exposure. Complete data and information that are the basis for this GRAS determination are available to the Food and Drug Administration for review and copying at reasonable times (customary business hours) at a specific address set out in the notice or will be sent to FDA upon request (electronic format or on paper)

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

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

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]
72092C15A44C497...
13-Nov-2020 | 19:02 GMT

i.V. Candice Cryne

Regulatory Affairs Manager

DocuSigned by:
[redacted]
FD80C973075E42E...
13-Nov-2020 | 18:12 GMT

Joab Trujillo

Junior Regulatory Affairs Specialist

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GRAS Notification of a Sterol Esterase from a Genetically Modified *Trichoderma reesei*

AB ENZYMES GmbH

November 13, 2020

Contents

1 PART 1 §170.225 – SIGNED STATEMENTS AND CERTIFICATIONS

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

AB Enzymes GmbH hereby claims that sterol esterase (IUBMB 3.1.1.13) from a Genetically Modified *Trichoderma reesei* 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:

Sterol esterase (IUBMB 3.1.1.13) from a Genetically Modified *Trichoderma reesei*

§170.225(b) – Trade secret or confidential:

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

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

The sterol esterase is to be used as a processing aid for partial or extensive hydrolysis of lipids from plant sources and in bread making. The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements under current Good Manufacturing Practices.

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

This GRAS determination is based upon scientific procedures.

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

§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 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.

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

2.1 Identity of the notified substance

The dossier concerns a **sterol esterase from a genetically modified *Trichoderma reesei*.**

2.1.1 Common name of the enzyme

Name of the enzyme protein:	Sterol esterase
Synonyms:	Steryl ester acylhydrolase,
	Acylcholesterol lipase
	Cholesteryl ester hydrolase,
	Cholesterol esterase

2.1.2 Classification of the enzyme

IUBMB Enzyme Commission number	3.1.1.13

EC 3. is for hydrolases.

EC 3.1. is for hydrolases acting on ester bonds.

EC 3.1.1. is for carboxylic ester hydrolases.

EC 3.1.1.13 is for sterol esterase.

2.2 Identity of the Source

Production Strain	*Trichoderma reesei* AR-777

2.2.1 Recipient Strain

The recipient strain used for the genetic modification is *Trichoderma reesei* RF10310 strain, which is derived from a classical mutant originating from QM6a. This strain has been shown to be genetically stable for industrial production.

The classical mutant from *QM6a* was characterized by the Centraalbureau voor Schimmelcultures (CBS) in the Netherlands as *Trichoderma reesei*. It was identified based on the sequences of Internal Transcribed Spacer 1 and 2 and the 5.8S gene and Translation Elongation Factor 1a. The classical mutant from *QM6a* was deposited as a CBS culture (safe deposit) as CBS 114041.

Therefore, the recipient can be described as followed:

Genus:	*Trichoderma*
Species:	*Trichoderma reesei*
Subspecies (if appropriate):	not applicable
Generic name of the strain:	RF10310
Commercial name:	Not applicable. The organism is not sold as such.

2.2.2 Donor:

The sterol esterase gene described in this application derives from *Melanocarpus albomyces*. *Melanocarpus* species are naturally found from soil and have worldwide distribution. *M. albomyces* is a thermophilic fungus and a biosafety level 1 microbe.

Genus:	*Melanocarpus*
Species:	*Melanocarpus albomyces*
Subspecies (if appropriate):	not applicable
Commercial name:	*Thielavia albomyces*

2.3 Genetic modification

Trichoderma reesei strain AR-777 was constructed for production of *Melanocarpus albomyces* sterol esterase. In constructing the strain AR-777, the expression cassette (sterol esterase gene under the control of the *T. reesei* promoter) was integrated into the genome of the *Trichoderma reesei* recipient strain.

The transformation of the recipient *T. reesei* strain with the expression cassette was performed as described in Penttilä et al. (1987) with the modifications described in Karhunen et al. (1993). The transformants were selected according to their ability to grow on acetamidase plates (*amd*S marker gene).

Standard DNA techniques were used in the construction and transformation of the plasmids. The constructs were characterized by restriction endonuclease digestion and verified by DNA sequencing.

Standard transformation techniques using protoplasts were used to integrate the expression cassettes into the genome of the *Trichoderma reesei* production strain.

The plasmid vector (pUC19) was only used in constructing the expression cassette but was not introduced into the *T. reesei* recipient strain in fungal transformation.

Expression cassette:

- ***Melanocarpus albomyces* sterol esterase gene:** the sterol esterase gene encodes the amino acid sequence of the *Melanocarpus albomyces* sterol esterase.
- ***Synthetic amdS* gene:** the synthetic *amd*S gene encodes the amino acid sequence of the *Aspergillus nidulans* acetamidase (Kelly and Hynes 1985; Hynes et al. 1983). The introns have been removed from the original *amd*S gene and selection of restriction sites has been removed by codon changes, to ease the cloning steps. The synthetic *amd*S gene is expressed from the native *A. nidulans amd*S promoter. The native *amd*S terminator is used as a transcription terminator. The encoded acetamidase enables the strain to grow on

plates containing acetamide as a sole nitrogen source. This characteristic has been used for selecting the *T. reesei* transformants. Acetamidase has been widely used as a selection marker in fungal transformations for more than 30 years without any disadvantage.

— ***Trichoderma reesei* promoter and terminator:** the *Melanocarpus albomyces* sterol esterase gene is fused to a strong *T. reesei* promoter and *T. reesei* carrier protein. For transcription termination the native *T. reesei* terminator is used.

2.3.1 Genetic stability of the production strain

In practice, the fermentation process always starts from identical replicas of the *T. reesei* AR-777 seed ampoule. Production preserves from the "Master Cell Bank" are used to start the fermentation process.

A Master Cell Bank is a collection of ampoules containing a pure culture. The cell line history and the production of a Cell Bank, propagation, preservation and storage is monitored and controlled. The MCB is prepared from a selected strain. A MCB ampoule is only accepted for production runs if its quality meets the required standards. This is determined by checking identity, viability, microbial purity and productivity of the MCB ampoule. The accepted MCB ampoule is used as seed material for the inoculum.

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

Trichoderma reesei AR-777 strain does not harbor any vector DNA. The expression cassettes used for transformations were cleaved from the pUC18 vector plasmids by restriction enzyme digestions followed by isolation of the expression cassettes from agarose gel.

A Southern blot hybridization experiment using plasmid with the pUC18 vector backbone as a labeled probe and genomic DNA of the production host AR-777 was performed to confirm that no vector DNA is included in the genome of AR-777. It produced negative result (no hybridization), demonstrating that the plasmid vector was completely removed to generate the linear transforming DNA fragment that was introduced into the *Trichoderma* production host.

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 AR-777 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 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 enzyme preparation is free from detectable, viable production organism (Appendix #1). As the absence of the production strain is confirmed for every production batch, no additional information regarding the inactivation of the GMM cells is required.

2.3.5 Information on the possible presence of recombinant DNA

The enzyme preparation is produced by an aerobic submerged microbial fermentation using a genetically modified *T. reesei* strain. All viable cells of the production strain, AR-777, 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 recombinant DNA. Two separate enzyme samples (concentrates from pilot scale fermentations) were tested for the presence of recombinant DNA using highly sensitive and specific PCR techniques. No recombinant DNA (recDNA) of the production strain was shown to be present above the detection limits.

2.3.6 Absence of Antibiotic Genes and Toxic Compounds

As 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 Nutrition Paper *(Food and Agriculture Organization of the United Nations 2006)* has been also tested from the fermentation products. Adherence to specifications of microbial counts is routinely analyzed. Two production batches produced by the production strain *T. reesei* AR-777 (concentrates) were analyzed and no antibiotic or toxic compounds were detected (Appendix #1).

2.4 ENZYME PRODUCTION PROCESS

2.4.1 Overview

The food enzyme is produced by ROAL Oy[2] by submerged fermentation of *Trichoderma reesei* AR-777 in accordance with current Good Manufacturing Practices for Food (GMP) and the principles of Hazard Analysis of Critical Control Points (HACCP). As it is run in the EU, it is also subject to the Food Hygiene Regulation (852/2004).

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

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

[2] See footnote 1

2.4.2 Fermentation

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

- Inoculum
- Seed fermentation
- Main fermentation

2.4.3 Raw materials

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

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

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

2.4.5 Inoculum

A suspension of a pure culture of strain AR-777 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 fermentor is run as normal submerged fed-batch fermentation. The content of the seed fermentor is aseptically transferred to the main fermentor 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 in order 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 is 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 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 a declared activity, standardized and preserved with food ingredients or food additives (food grade quality).

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

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 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.	5.000 SEU/g
Appearance	Solid, light beige	

Density	1.0 – 1.1 g/ml

2.5.2 Formulation of a typical enzyme preparation

Composition	
Constituent	**%**
Enzyme concentrate	8.0 – 10.0
Sunflower oil	0.4
Wheat flour	Remainder

2.5.3 Molecular mass and amino acid sequence of the enzyme

The mature enzyme protein subject for this dossier consists of 558 amino acid residues with a calculated molecular mass of 59 kDa (or 59,000 Da).

2.5.4 Purity and identity specifications of the enzyme preparation

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

Lead: Not more than 5 mg/kg

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[3]

[3] 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.

The proof that the food enzyme complies with the above specification is presented on 2 different batches, refer to Appendix #1. The 2 samples do not contain any diluents.

Other enzymatic activities: the food enzyme is standardized on enzyme activity. Apart from it, the production organism *Trichoderma reesei* produces other endogenous *Trichoderma* proteins, e.g. xylanases. 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.

The typical batch sizes range from 1 000 L to 150 000 L and are deeply depending on the market demand. Therefore, the frequency and the volume of production of the food enzyme vary. AB Enzymes is a small to mid-size company and this enzyme has only recently been developed. So far, the current market demand for this specific enzyme has not justified more than 2 fermentations and AB Enzymes is unfortunately not in the situation to have a wider range of batches available for comparisons. This explains the reduced number of samples that have been analyzed for the purpose of this dossier.

2.6 Enzymatic Activity

The main activity of the *Trichoderma reesei* AR-777 enzyme preparation is a sterol esterase (IUBMB 3.1.1.13). Sterol esterase's enzymatic function is to catalyze the hydrolysis of fatty acid esters. The substrates for the enzyme are lipids, explicitly fatty acid esters, specifically fatty acid esters of sterol and of glycerol (particularly triacylglycerols). Thus, the enzyme can also be referred to a lipid-hydrolyzing sterol esterase.

The end products or reaction products for sterol esterase are free fatty acids and partially hydrolyzed lipids, particularly mono- and diacylglycerols. All these reaction products are also found in many organisms and occur naturally in food for human consumption.

Enzyme reaction: Sterol ester + H_2O ⇔ a sterol + a fatty acid[4]

Consequently, the substrates for sterol esterase occur naturally and are therefore a part of the human diet.

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

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

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

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

[4] Source: https://www.brenda-enzymes.org/enzyme.php?ecno=3.1.1.13

nature, it also contains the very same enzymatic activities that break down such a substrate; e.g. to avoid its accumulation.

Furthermore, the presence in food of such enzyme activities and the potential reaction products is not new and should not be of any safety concern. During the production of food enzymes, the main enzyme activity contains several other enzymes excreted by the microbial cells or derived from the fermentation medium. As in the case of the enzyme for this application, the side activity comes directly from the production strain. It is generally accepted that the enzyme proteins themselves do not pose any safety concern and are recognized to be generally considered as safe along with known not to cause adverse effects. Apart from sterol esterase, the food enzyme also contains other enzymatic side activities in small amount which are naturally and typically produced by the production organism *Trichoderma reesei,* mainly xylanases. AB Enzymes is not aware of any adverse effects from the side activities present in the sterol esterase enzyme preparation.

2.7 Allergenicity

As some enzymes manufactured for use in food have been reported to cause inhalation allergy in workers exposed to enzyme dust in manufacturing facilities, sterol esterase may also cause such occupational allergy in sensitive individuals. However, the possibility of an allergic reaction to the residues of sterol esterase in food seems remote. To address allergenicity by ingestion, it may be taken into account that:

- 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 comprised enzymes produced by wild-type and genetically modified strains as well as wild-type enzymes and protein engineered variants and comprised 400 patients with a diagnosed allergy to inhalation allergens, food allergens, bee or wasp. It was concluded from this study that

ingestion of food enzymes in general is not likely to be a concern with regard to 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 products (Daurvin et al. 1998). The overall conclusion was that – as opposed to exposure by inhalation – there are no scientific indications that the small amounts of enzymes in food can sensitize or induce allergy reactions in consumers.
- 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).

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

Additional considerations supporting the assumptions that the ingestion of an enzyme protein is not a concern for food allergy should also be considered:

- The majority of proteins are not food allergens and based on previous experience, the enzyme industry is not aware of enzyme proteins used in food being homologous to known food allergens[5].
- The food enzyme is used in small amounts during food processing resulting in very small amounts of the enzyme protein in the final food. A high concentration generally equals a higher risk of sensitization, whereas a low level in the final food equals a lower risk (Goodman et al. 2008).
- In the case where 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. In general, these alterations in conformation are associated with decrease in the antigenic reactivity in humans: in the vast majority of investigated cases, denatured proteins are much less immunogenic than the corresponding native proteins (Valenta 2002; Valenta

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

and Kraft 2002; Takai et al. 1997; Takai et al. 2000; Nakazawa et al. 2005; Kikuchi et al. 2006).

- In addition, residual enzyme proteins still present in the final food will be subjected to digestion in the gastro-intestinal system, which reduces further the risk of enzyme allergenicity. While stability to digestion is considered as a potential risk factor of allergenicity, it is believed that small protein fragments resulting from digestion are less likely to be allergenic (Food and Agriculture Organization of the United Nations January/2001; Goodman et al. 2008).
- Finally, enzymes have a long history of safe use in food processing, with no indication of adverse effects or reactions. Moreover, a wide variety of enzyme classes (and structures) are naturally present in food. This is in contrast with most known food allergens, which are naturally present in a narrow range of foods.

2.7.1 Allergenicity Search

To specifically evaluate the risk that the enzyme sterol esterase would cross react with known allergens and induce a reaction in an already sensitized individual, sequence homology testing to known allergens was performed. This test used an 80 amino acids sliding window search as well as conventional FASTA alignment (overall homology), with the threshold of 35% homology as recommended in the most recent literature (Food and Agriculture Organization of the United Nations January/2001; Ladics et al. 2007; Goodman and Tetteh 2011).

A sequence homology comparison test, which is accessible through the webpage http://www.allergenonline.org/, was performed using a database of allergens from the Food Allergy Research and Resource Program (FARRP), University of Nebraska, Allergen Database (Version 19, February 10, 2019), which contains the amino acid sequences of known and putative allergenic proteins.

The protein sequence of the sterol esterase had no matches of greater than 35% identity to any known or putative allergen when searching through 80 amino acids sliding window. Furthermore,

the sequence of the sterol esterase had no exact matches when searching through 8 amino acids sliding window. The full-length sequence alignment had no matches with an E-value (expectation value) smaller than 0.5. The E-value denotes the degree of similarity of a query protein to its matches. The smaller the E-value the closer is their relationship. An E-value of 1 or larger indicates no relation in evolution or structure. An E-value smaller than 0.02 might be considered related in terms of three-dimensional structure (Pearson 1996 and http://www.allergenonline.org/databasehelp.shtml) for a database like AllergenOnline. Nevertheless, even with an E-value of 0.02 two protein structures are not likely sufficiently similar for antibody cross-recognition. Matches with E-values larger than 0.0000001 are not likely to identify immunologic or allergic cross-reactivity. Just with E-values smaller than 10^{-30} this becomes much more likely in at least some allergic individuals (Hileman 2002 and http://www.allergenonline.org/databasehelp.shtml).

Conclusion:

Based on the results obtained from the bioinformatics approach to estimate potential allergenicity on relatedness to known allergens and taking into account the most recent scientific recommendations on the interpretation of such data, and furthermore because the enzyme is typically denatured during its application as processing aid and because any residual enzyme possibly ingested will be subject to digestion in the gastro-intestinal system, it is not likely that the enzyme under evaluation, sterol esterase produced by *Trichoderma reesei* AR-777, will cause allergic reactions after ingestion.

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

Like any other enzyme, the sterol esterase is a biological catalyst, which accelerates the conversion of certain substrates into specific reaction products. Not the enzyme itself, but the result of this conversion causes a desired effect in the manufacturing process and potentially also in the final consumer product. After the conversion has taken place, the enzyme no longer performs a

technological function. The enzyme effect remains, irrespective of whether the enzyme is still present or absent in the final consumer product. Like most enzymes, the sterol esterase performs its technological function during the manufacturing process. The sterol esterase is added during the manufacturing process and does not perform any technological function in the final product. The reasons why the enzyme does not exert any (unintentional) enzymatic activity in the final food can be due to a combination of various factors, depending on the application and the process conditions used by the individual manufacturer. These factors include depletion of the substrate, denaturation of the enzyme during processing, low water activity, unsuitable pH or temperature, etc. In some cases (e.g. after alcohol distillation), the enzyme may no longer be present in the final food.

The **substrates** for the sterol esterase are lipids, explicitly fatty acid esters, specifically fatty acid esters of sterol and of glycerol, particularly triacylglycerols. Thus, the enzyme can also be referred as a "lipid hydrolyzing sterol esterase".

The **function** of the lipid-hydrolyzing sterol esterase is to catalyze the hydrolysis of fatty acid esters.

The **reaction products** of the lipid-hydrolyzing sterol esterase are free fatty acids and partially hydrolyzed lipids, particularly mono- and diacylglycerols. All of these reaction products are also naturally present in many organisms, and also occur already naturally in food for human consumption.

In general, the technical purpose of the enzymatic conversion of lipids, particularly triacylglycerols, with the help of lipid-hydrolyzing sterol esterase is partial digestion of a lipid component, particularly hydrolysis of the substrate triacylglycerols. Lipids are naturally present in most living organisms, and consequently also occur naturally in food and food raw materials, e.g. wheat flour.

Lipid-hydrolyzing sterol esterase are also naturally present in most living organisms, and consequently also occur in many plant and animal food raw materials, including wheat grains.

The natural enzymatic conversion of triacylglycerols in such materials would theoretically be of technological benefit in several industrial food manufacturing processes. However, the levels of endogenous sterol esterase are often inadequate and vary from batch to batch of raw material, and the specificity of the enzyme may not be optimal to give desired process advantages. Therefore, extra added sterol esterase is used during food processing. The enzyme is used as a processing aid in food manufacturing and is not added directly to the final food.

This dossier specifically describes the use the sterol esterase in baking application, particularly in bread making. Different types of lipid specific hydrolases (carboxylic-ester hydrolases, such as esterases, triacylglycerol lipases, ….) have been used in the food industry (please see section 6.1.) including baking for the last 30 year and their use in the bakery industry is continuously increasing. This demonstrates their technological need in these food processes.

Below, the benefits of sterol esterase 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 characteristics. Moreover, the applications lead to more effective production processes, resulting in better production economy and environmental benefits such as the use of less raw materials and the production of less waste.

Sterol esterase can be used in the manufacturing of bakery products such as, but not limited to, bread, biscuits, steamed bread, cakes, pancakes, tortillas, wafers and waffles.

Wheat flour contain approximately 2.0 to 2.5 % lipids. The wheat flour lipids contain 49.1% polar lipids (including 26.4% glycolipids, particularly digalactosyl diglycerides and monogalactosyl diglycerides, and 22.7% phospholipids) and 50.9% non-polar lipids, including 20.8%

triacylglycerols.

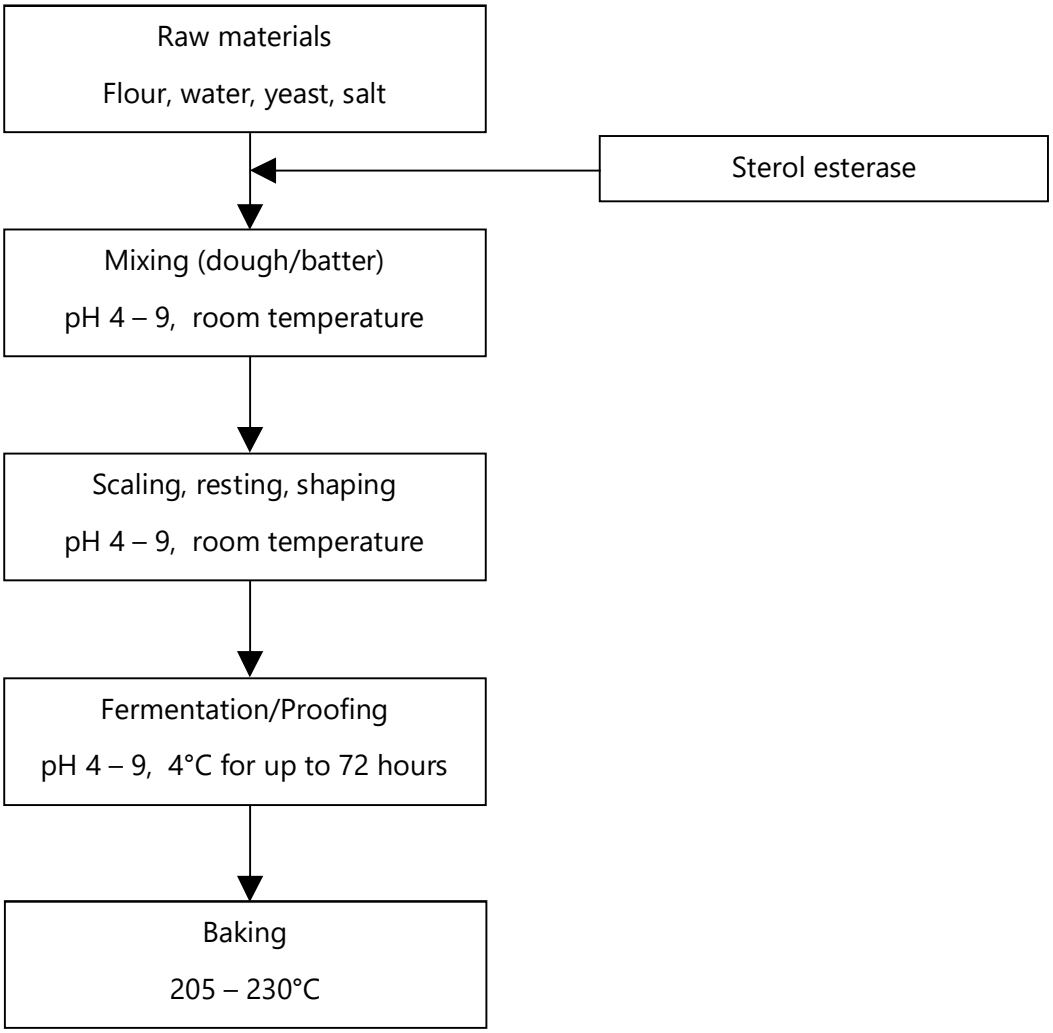
During mixing, free lipids become bound or trapped within the gluten fraction. Limited hydrolysis of the triacylglycerols with the help of sterol esterase result in an improved natural ratio of polar lipids. Increased proportion of polar lipids has a positive effect on gas retention, as they can align at the interface of the gas cells formed in the dough and therefore increase the stability of the gas cells, whereas endogenous wheat non-polar lipids destabilize gas cells in dough and therefore limit bread volume. The use of sterol esterase help removing this negative effect.

In addition, the degradation of the substrate triacylglycerols with the help of sterol esterase results in the formation of monoacylglycerols, that interacts with gelatinizing starch, in particular with amylose to form irreversible monoacylglycerol-amylose-complexes.

The use of sterol esterase can therefore influence the interactions between the different constituents of the dough, i.e. gluten proteins and lipids, starch and lipids as well as gluten and starch. The benefits of the conversion of triacylglycerols (non-polar lipids) with the help of sterol esterase in baking can therefore be summarized as follows:

- Facilitate the handling of the dough
- Improve dough stability and strength which results in processing tolerance
- Improve the dough's structure and behavior during the baking steps
- Regulate batter viscosity, beneficial in the production process for e.g. waffles, pancakes and biscuits

The process flow of baking process is presented below:



The fate of the enzyme protein during the flour processing:

In baking, the sterol esterase performs its technological function during dough or batter handling in order to contribute to an improved and consistent baking process. The sterol esterase is denatured by heat during the baking step.

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 his 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 sterol esterase 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 actually 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 more 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 the 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 following page.

The Table below shows the range of recommended use levels for each application where sterol esterase from *Trichoderma reesei* AR-777 may be used, are shown in the table below:

Food Application	Raw material (RM)	Suggested recommended use levels (mg TOS/kg RM)
Baking	Flour	10

2.10 Fate in food

Not the enzyme itself, but the result of the enzymatic conversion causes a desired effect in the manufacturing process and potentially also in the final consumer product. After the conversion has taken place, the enzyme no longer performs a technological function. The enzyme effect remains, irrespective of whether the enzyme is still present or absent in the final consumer product. Like most enzymes, sterol esterase performs its technological function during the manufacturing process. The sterol esterase is added during the manufacturing process and does not perform any technological function in the final product. The reasons why the enzyme does not exert any (unintentional) enzymatic activity in the final food can be due to a combination of various factors, depending on the application and the process conditions used by the individual manufacturer. These factors include depletion of the substrate, denaturation of the enzyme during processing, low water activity, unsuitable pH or temperature, etc.

To be able to perform a technological function in the consumer product, many conditions have to be fulfilled simultaneously:

- the enzyme protein must be in its 'native' (non-denatured, active) 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

The main intension of adding sterol esterase in baking is to improve the baking process, particularly to facilitate the handling of the dough, improve dough stability and behavior, regulate batter viscosity during the baking steps. Such beneficial effects can be linked with positive effects on the final product. However, such positive effects are not exclusively obtainable by addition of sterol esterase but could be achieved without the addition of an enzyme, for example through modified, potentially more expensive production processes, use of chemicals or changes in recipe or enzymes already present in food raw materials and ingredients. Addition of such an enzyme as sterol esterase in baking ensures a maximum compatibility with modern industrial processes (also leading to less production variations, thereby ensuring standardized quality products), which technologically justifies addition of the enzyme.

The reasons why the sterol esterase does not exert any (unintentional) enzymatic activity in the final food are:

- the sterol esterase is denatured by heat during the manufacturing process, particularly the sterol esterase is inactivated during regular baking processes, which results in temperatures between 95°C and 100°C inside the dough for at least 10 to 15 minutes.
- the remaining water content (water activity) within baked goods is much too low to enable any hydrolytic enzyme activity in the final product.

Consequently, it can be concluded that the sterol esterase does not exert any (unintentional) enzymatic activity in the final foods.

3 PART 3 § 170.325 - DIETARY EXPOSURE

The most appropriate way to estimate the human consumption in the case of food enzymes is using the so-called Budget Method (Hansen 1966; Douglass et al. 1997). This method enables to calculate a Theoretical Maximum Daily Intake (TMDI) based on conservative assumptions

regarding physiological requirements for energy from food and the energy density of food rather than on food consumption survey data.

The Budget Method was originally developed for determining food additive use limits 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

For the calculation of the TMDI, the maximum use levels are chosen. Furthermore, the calculation takes into account how much food or beverage is obtained per kg raw material (see below the table) and it is assumed that all the TOS will end up in the final product.

Applications		**Raw material (RM)**	**Suggested recommended use level (mg TOS/kg RM)**	**Final food (FF)**	**Ratio RM/FF***	**Suggested level in final food (mg TOS/kg food)**
SOLID FOODS	Baking products i.e. bread	Flour	10	Bread; baking products	0.71	7.1

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

Baking

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

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

The Total TMDI will consequently be calculated as follows:

TMDI in food (mg TOS/kg body weight/day)	TMDI in beverage (mg TOS/kg body weight/day)	Total TMDI (mg TOS/kg body weight/day)
7.1 x 0.0125 = 0.08875	0 x 0.025 = 0	**0.08875**

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

- It is assumed that ALL producers of the above-mentioned foodstuffs use the specific sterol esterase enzyme from *T. reesei* AR-777;
- It is assumed that ALL producers apply the HIGHEST use level per application;
- For the calculation of the TMDI's in food and in beverages, only THOSE foodstuffs and beverages were selected containing the highest theoretical amount of TOS.

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

The Margin of Safety (MoS) for human consumption can be calculated by dividing the NOAEL (see **Section 6**) by the Total Theoretical Maximal Daily Intake (TMDI). Total TMDI of the food enzyme is 0.08875 mg TOS/kg body weight/day. Consequently, the MoS is:

MoS = 1,000 / 0.08875 = **11,268**

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

Conclusion:

The overall conclusion is that the use of the food enzyme sterol esterase from *T. reesei* AR-777 in the production of food is safe. Considering the high safety factor – even when calculated by means of an overestimation of the intake via the Budget method – there is no need to restrict the use of the enzyme in food processing. The suggested dosage for food manufacturers is not a restrictive value and could be higher or lower depending on usage within cGMPs.

4 PART 4 §170.240 – SELF-LIMITING LEVELS OF USE

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

5 PART 5 § 170.245 – EXPERIENCE BASED ON COMMON USE IN FOOD BEFORE 1958

This part is not applicable to this notified substance.

6 PART 6 § 170.250 – GRAS NOTICE - NARRATIVE

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

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

6.1 Safety of the production strain

Production strain

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.

The original *T. reesei* isolate, QM6a is the initial parental organism of practically all currently industrially relevant food enzyme production strains, including our strain AR-777. *Trichoderma reesei* has a long history (more than 30 years) of safe use in industrial-scale enzyme production (Nevalainen et al. 1994; Olempska-Beer et al. 2006*)*. Cellulases, hemicellulases, β-glucanases, pectinases and xylanases produced by this fungus are used in food, animal feed, pharmaceutical, textile, detergent, bioethanol and pulp and paper industries.

Food enzymes deriving from *Trichoderma reesei* strains (including recombinant *T. 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.

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

Non-exhaustive list of authorized food enzymes (other than sterol esterase) 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	Schedule 18 Processing Aids
Canada	Cellulase Glucanase Pentosanase Xylanase Protease	List of Permitted Food Enzymes Health Canada
USA[6]	Pectinlyase Transglucosidase (GM)	GRAS Notice Inventory, GRN 32 GRAS Notice Inventory, GRN 315

[6] GRAS affirmations and GRAS notifications

	Glucoamylase	GRAS Notice Inventory, GRN 372
	Phospholipase A	GRAS Notice Inventory, GRN 524
	Polygalacturonase	GRAS Notice Inventory, GRN 557
	Pectin esterase	GRAS Notice Inventory, GRN 558
	Endo-1,4-beta glucanase	GRAS Notice Inventory, GRN 756
	Serine endopeptidase	GRAS Notice Inventory, GRN 813
France	Alpha-amylase (GM) Amyloglucosidase (GM) Beta-glucanase (GM) Xylanase Cellulase Lysophospholipase (GM)	Arrêté du 19 octobre 2006

As documented below, carboxylic ester hydrolyases from various micro-organisms (including genetically modified ones) are widely accepted and *Trichoderma reesei* – whether or not genetically modified[7] - is widely accepted as a safe production organism for a broad range of enzymes.

Non-exhaustive list of authorized carboxylic -ester hydrolases from production organisms other than *Trichoderma reesei*		
Authority	**Food enzyme**	**Reference**
Australia/NZ	Rhizomucor miehei[8], Pencillium camembertii[9], (Aspergillus niger, Aspergillus oryzae, Aspergillus oryzae containing gene for Lipase isolated from Fusarium	Schedule 18 Processing Aids

[7] Overproduction of chosen enzymes and/or modification of enzyme e.g. cellulase profiles has not been observed to introduce harmful properties for the host organism or its products -animal tests Huuskonen 1990.

[8] Carboxylesterase EC 3.1.1.1

[9] Lipase, monoacylglycerol EC 3.1.1.23

	oxysporum, Aspergillus oryzae contain gene for Lipase isolated from Humicola… Candida rugosa, Mucor javanicus, Rhizopus miehei)[10], Aspergillus niger[1112], Aspergillus oryze containing gene for phospholipase A1 isolated from Fusarium venenatum[13], Streptomyces violaceoruber[14]	
France	Aspergillus oryzae (carrying a gene for lipase from Fusarium oxysporum, Thermomyces lanuginosus), Aspergillus niger (carrying a gene for lipase from Fusarium heterosporum), Rhizopus oryzae, Aspergillus niger[1516], Aspergillus oyrzae contain gene for pectin esterase from Aspergillus aculeatus, Aspergillus oryzae contain a gene for phospholipase A1 from Fusarium venenatum, Aspergillus niger[17], Streptomyces violaceoruber[18]	Arrêté du 19 octobre 2006
USA[1]	Aspergillus niger (expressing a gene for lipase from Candida antartica) Rhizopus oryzae[19]	GRAS Notice Inventory No. 158 GRAS Notice Inventory No. 216 GRAS Notice Inventory No. 462 GRAS Notice Iventory No. 145

[10] Lipase, triacylglycerol (EC 3.1.1.3)
[11] Lysophospholipase (EC 3.1.1.5)
[12] Pectin esterase (EC 3.1.1.11)
[13] Phospholipase A1 (EC 3.1.1.32)
[14] Phospholipase A2 (EC 3.1.1.4)
[15] Lysophospholipase (EC 3.1.1.5)
[16] Pectin esterase (EC 3.1.1.11)
[17] Phospholipase A2 (EC 3.1.1.4)
[18] Phospholipase A2 (EC 3.1.1.4)
[19] Lipase, triacylglycerol (EC 3.1.1.3)

	Pseudomonas fluorescens Biovar I [20] Streptomyces violaceoruber[21] Talaromyces leycettanus produced in Aspergillus niger[22] Aspergillus oryzae[23] Aspergillus niger[24]	GRAS Notice Inventory No. 651 GRAS Notice Inventory No. 811 GRAS Notice Inventory No. 857
Canada	(Aspergillus niger, Aspergillus oryzae, Rhizomucor miehei…)[25], (Streptomyces violaceoruber, Aspergillus oryzae, Aspergillus niger)[26]	List of Permitted Food Enzymes Health Canada
JECFA	Fusarium venenatum expressed in Aspergillus Oryzae[27] Aspergillus oryzae[28]	WHO Food Additives Series 56 NMRS 54/TRS 557-JECFA 18/20

T. reesei strains have been cultivated in the production plant of Alko Oy/Roal Oy starting from year 1987 and the parental strain, a classical mutant originating from QM6a, has been used from year 1995 on.

[20] Lipase, triacylglycerol (EC 3.1.1.3)
[21] Phospholipase A2 (EC 3.1.1.4)
[22] Phospholipase A1 (EC 3.1.1.32)
[23] Phospholipase A1 (EC 3.1.1.32)
[24] Phospholipase A1 (EC 3.1.1.32)
[25] Lipase
[26] Phospholipase
[27] Phospholipase A1 (EC 3.1.1.32)
[28] Lipase, triacylglycerol (EC 3.1.1.3)

6.1.1 Pathogenicity and toxigenicity

Trichoderma reesei strains are non-pathogenic for healthy humans and animals (*Nevalainen et al. 1994)*. *Trichoderma reesei* is not present on the list of pathogens in the EU (Directive Council Directive2000/54/EC) and is present in major culture collections worldwide.

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 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, *Trichoderma reesei* can be used under the lowest containment level at large scale, GILSP, as defined by *(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

[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

[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;

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 (Food and Agriculture Organization of the United Nations 2006), was also confirmed for AR-777. 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.

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

http://amfep.drupalgardens.com/sites/amfep.drupalgardens.com/files/Amfep-List-of-Commercial-Enzymes.pdf

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.

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 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 (Nevalainen et al. 1994; Blumenthal 2004). The safety of this organism as an enzyme producer has been reviewed by Nevalainen et al. (1994), Blumenthal (2004), and (Olempska-Beer et al. 2006) and it is concluded 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 the heterologous sterol esterase produced by *Trichoderma reesei* AR-777 originates from *Melanocarpus albomyces*. The donor microorganism is a thermophilic fungus classified as a biosafety level 1 microbe. AB Enzymes limits the possibilities of mutations through the inoculation of the seed culture for the fermentation with controlled spore stocks that have been stored at -80°C.

As the sterol esterase protein is not toxic, 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 20 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-777 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-777 can be regarded as safe as the recipient or the parental organism to be used for production of enzymes for food processing.

6.2 DATA FOR RISK ASSESSMENT

6.2.1 Toxicological testing

The sterol esterase is produced with the help of the genetically modified *Trichoderma reesei* AR-777. The AR-777 production strain was constructed by inserting the sterol esterase gene into the RF10310 Trichoderma reesei host. This host was similarly used for the expression of a glucose oxidase, i.e. *Trichoderma reesei* RF11400 (GRAS Notice No. 703). The safety of *Trichoderma reesei* RF11400 was substantiated based upon a complete package of toxicological studies:

- *In vitro* bacterial reverse mutation test
- *In vitro* mammalian chromosome aberration test
- 13-week oral toxicity study in rats

As mentioned above both the AR-777 and RF11400 have been developed from the same host (RF10310). Expression constructs are very similar, only differing by the expression cassette / enzyme gene of interest. No presence of vector DNA is included in either of the production strains as stated in section 2.3.2 of this application and section 2.4.1. of the GRAS No. 707.

As the 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-777 and RF11400 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-777 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-777.

Based on the rationale provided above, as well as on the review of both strains meeting the requirements of Pariza and Johnson Decision Tree (Appendix #3 & Appendix #1 for GRAS No. 707), AB Enzymes concludes sterol esterase from *Trichoderma reesei* AR-777 to be safe and does not pose a significant risk to human health.

7 PART 7 §170.255 – LIST OF SUPPORTING DATA AND INFORMATION

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

Appendices

1. AR-777 Composition Report
2. Manufacturing Flow Chart
3. Pariza and Johnson Decision Tree AR-777

Publication bibliography

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DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

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

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

FDA USE ONLY

GRN NUMBER	DATE OF RECEIPT
GRN 000981	Nov 17, 2020
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: Joab Trujillo		Position or Title: Junior Regulatory Affairs Specialist	
Organization *(if applicable)*: AB Enzymes Inc.			
Mailing Address *(number and street)*: 8211 W. Broward Blvd.			
City: Plantation	State or Province: Florida	Zip Code/Postal Code: 33324	Country: United States of America
Telephone Number: +1 954 800 8606	Fax Number:	E-Mail Address: joab.trujillo@abenzymes.com	

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

Name of Contact Person:		Position or Title:	
Organization *(if applicable)*:			
Mailing Address *(number and street)*:			
City:	State or Province:	Zip Code/Postal Code:	Country:
Telephone Number:	Fax Number:	E-Mail Address:	

SECTION C – GENERAL ADMINISTRATIVE INFORMATION

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

Sterol Esterase (IUBMB 3.1.1.13) from a Genetically Modified 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 707

☐ b) GRAS Affirmation Petition No. GRP _____

☐ c) Food Additive Petition No. FAP _____

☐ d) Food Master File No. FMF _____

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

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

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

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

☐ Yes *(Proceed to Item 8*

☒ No *(Proceed to Section D)*

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

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

☐ No

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

☐ Yes, a redacted copy of the complete submission

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

☐ No

SECTION D – INTENDED USE

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

The sterol esterase is to be used as a processing aid for partial or extensive hydrolysis of lipids from plant sources and in bread making. The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements under current Good Manufacturing Practices.

Recommended use levels: 10 mg TOS/kg Raw Material

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 Sterol Esterase (IUBMB 3.1.1.13) from a Genetically Modified 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
(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: 2020.11.16 16:34:11 -05'00'	Joab Trujillo Junior Regulatory Affairs Specialist	11/16/2020

SECTION G – LIST OF ATTACHMENTS

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

Attachment Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)
	Form3667 AB Enzymes Sterol Esterase.pdf	Administrative
	Cover Letter for Sterol Esterase.pdf	Administrative
	GRAS Notice Sterol Esterase.pdf	Submission
	1_AR-777 Composition Report.pdf	Submission
	2_Flow Chart of the manufacturing process with control steps. pdf	Submission
	3_Pariza and Johnson Decision Tree AR-777.pdf	Submission
	References for Sterol Esterase GRAS Notice.zip	Submission

Viebrock, Lauren

From: Trujillo, Joab <Joab.Trujillo@abenzymes.com>
Sent: Friday, March 11, 2022 1:38 PM
To: Viebrock, Lauren
Subject: RE: [External] GRN 00981 Questions
Attachments: Lason et al_2010.pdf; Pariza and Johnson 2001.pdf; ABE Response to Questions for Notifier of GRN 981.pdf; AR-777 Composition Report Update 2022.pdf; Barriuso et al 2016.pdf

Dear Lauren,

Thank you for providing an extension for the submission of the responses. Please see the attached documents to contain our responses. Along with the questions and answers document I provide an updated composition report containing the batch analysis for a third enzyme lot, values for the heavy metals and method requested. The literature references cited in the questions and answers document are also attached above. If you have any further questions please do not hesitate to contact me, thank you.

Best Regards,

Joab Trujillo
Regulatory Affairs Specialist - Americas
T: +1 954 800 8606 M: +1 954 439 4632
8211 W. Broward Blvd., Suite 375 | Plantation, FL 33324 | USA
Joab.Trujillo@abenzymes.com
www.abenzymes.com

From: Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
Sent: Wednesday, February 23, 2022 8:55 AM
To: Trujillo, Joab <Joab.Trujillo@abenzymes.com>
Subject: RE: [External] GRN 00981 Questions

Dear Jaob,

March 14th would be no problem for submission of your responses to our questions for GRN 981. Thank you.

Best,
Lauren

From: Trujillo, Joab <Joab.Trujillo@abenzymes.com>
Sent: Tuesday, February 22, 2022 2:09 PM

To: Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
Subject: RE: [External] GRN 00981 Questions

Dear Lauren,

I wanted to follow up on my request for an extension for the questions provided last week. Can you please let me know if the March 14, 2022 extension acceptable?

Looking forward to your feedback at your earliest convenience, thank you.

Best Regards,
Joab

From: Trujillo, Joab
Sent: Friday, February 18, 2022 9:17 AM
To: Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
Subject: RE: [External] GRN 00981 Questions

Dear Lauren,

Thank you for your follow up email with question #10. I am contacting you to see if we can get an additional time to respond to the questions. Can we please have 10 additional business days to respond? The new deadline would be **March 14, 2022**.

Please let me know if this new deadline is acceptable if not please let know what other options are available, thank you.

Best Regards,
Joab

From: Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
Sent: Wednesday, February 16, 2022 3:44 PM
To: Trujillo, Joab <Joab.Trujillo@abenzymes.com>
Subject: RE: [External] GRN 00981 Questions

Dear Mr. Trujillo,

Please find attached an additional question (#10) to be addressed for GRN 981. This is an addition to the questions #1-9 sent on 2/14/22. I apologize for omitting this question in my last email.

Please let me know if you have any questions.

Best,
Lauren

From: Trujillo, Joab <Joab.Trujillo@abenzymes.com>
Sent: Monday, February 14, 2022 5:04 PM
To: Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
Subject: RE: [External] GRN 00981 Questions

Dear Dr. Viebrock,

Thank you for your email and questions on GRAS Notice No. 000981. I will email you in the next few days on any questions or if we need more time to respond.

Best Regards,

Joab Trujillo
Regulatory Affairs Specialist - Americas
T: +1 954 800 8606 M: +1 954 439 4632
8211 W. Broward Blvd., Suite 375 | Plantation, FL 33324 | USA
Joab.Trujillo@abenzymes.com
www.abenzymes.com

From: Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
Sent: Monday, February 14, 2022 2:00 PM
To: Trujillo, Joab <Joab.Trujillo@abenzymes.com>
Subject: [External] GRN 00981 Questions

Dear Mr. Trujillo,

During our review of GRAS Notice No. 000981, we noted questions that need to be addressed. Please find the questions attached to this email.

We respectfully request a response within **10 business days**. If you are unable to complete the response within that time frame, please contact me to discuss further options.

If you have questions or need further clarification, please feel free to contact me. Thank you in advance for your attention to our comments.

Regards,
Lauren VieBrock

Lauren VieBrock, Ph.D.
Regulatory Review Scientist/Microbiology Reviewer

Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration
Tel: 301-796-7454
lauren.viebrock@fda.hhs.gov

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Three pages have been removed in accordance with copyright laws. The removed reference citation is:

E.Lason, and J. Ogonowski, “Lipase – Chracterization, applications and methods of immobilization”, CHEMIK, vol. 64, no. 2, pp. 97-102, 2010.

To: Lauren Viebrock

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

Regulatory Affairs
E-Mail info@abenzymes.com
Date: 2022-03-11

RE: Questions for Notifier of GRN 981

1. Please provide additional information on the production of the Trichoderma reesei recipient strain and/or differences from its parent strain.

AB Enzymes' Response:

The recipient strain used in construction of the sterol esterase production strain is a *Trichoderma reesei* strain which compared to its original parental strain produces lower amounts of native proteases and from which the genes encoding the major *T. reesei* cellulases and xylanases have been genetically deleted. The gene deletions have been made using *pyr4* selection – counter selection method. The *pyr4* gene used is a native *T. reesei* gene. No heterologous marker genes or antibiotic marker genes were used in the gene deletions.

2. Please provide additional information on how the sequence integrity of the integrated DNA and genetic stability of the production strain were confirmed.

AB Enzymes' Response:

The sequence integrity of the integrated DNA was confirmed using Southern blot analysis in which the genomic DNA isolated from the production strain was digested with several restriction enzymes and labelled expression cassette was used as a probe. Basing on the analysis of the Southern blot results (the lengths of the hybridizing bands and overall hybridization patterns) it could be concluded that the production strain contained the transformed expression cassette integrated into its genome. The genetic stability of the production strain was confirmed by liquid

cultivations of spores from several generations and analysis of the production yields as follows:

- The production strain was first cultivated on PD slants for five consecutive generations (inoculating of spores from one PD slant to the next one).
- The spores from the PD slants representing each five generations were then collected and inoculated into liquid medium (parallel flasks for each generation spores).
- At the end of the cultivations, the culture supernatants were collected, and the enzyme activities were analyzed.
- The enzyme activities were shown to remain similar. The activities in the later generation cultivations were not decreasing compared to the earlier generation cultivations. In case the production strain would be unstable (would have expression cassette), the yield of the enzyme activity would be lower in the later generation cultivations.

3. Please provide more detail of the "carrier protein" in your expression cassette, and why the presence/expression of this carrier protein in your construct is not a safety concern.

AB Enzymes' Response:

The carrier protein encoded by the expression cassette is the CBM (carbohydrate binding domain) of the native *Trichoderma reesei* cellobiohydrolase 2 (CBHII). There are no safety concerns in using this carrier protein as the CBMs have no enzymatic activity but are only acting in binding of carbohydrase to its substrates. Also, CBHII is one of the four major cellulases secreted by *T. reesei* strains and this protein (including the CBM) is included in the cellulase products deriving from industrial *T. reesei* mutant strains. The carrier protein is used as it has been shown that use of a carrier very often improves the production of heterologous proteins in filamentous fungi. The carrier proteins are generally deriving from the native, well produced proteins, for *Trichoderma* e. g. CBHI (cellobiohydrolase I) enzyme core and CBHII CBM can be used and for *Aspergillus* the glucoamylase enzyme core is used.

4. Please confirm that there are no functional or transmissible antibiotic resistance genes present in the production strain.

AB Enzymes' Response:

Trichoderma reesei does not contain any functional or transmissible antibiotic resistance genes and no antibiotic resistance genes have been included in any deletion or expression cassette used in generation of the recipient or production strain. The expression cassette used in the transformation of the recipient strain was isolated from the plasmid vector prior to transformation (using agarose gel isolation). It was also confirmed by Southern blot that there is no plasmid vector integrated into the genome of the production strain.

5. Please state whether the enzyme is secreted into the culture medium.

AB Enzymes' Response:

Yes, this enzyme is secreted into the culture medium.

6. Please provide an updated Food Chemicals Codex reference.

AB Enzymes' Response:

We confirm that the raw materials used in the fermentation and recovery processes during the manufacture of the commercial enzyme preparation conform to the specifications of the current Food Chemicals Codex, edition 13th, released in March 2022.

7. Please provide a CAS registry number for the sterol esterase enzyme

AB Enzymes' Response:

The CAS registry number for the sterol esterase is 9026-00-0.

8. The method for elemental analysis stated in the Attachment 1 (ISO 17294-2) is validated for water samples.

a. Please provide an appropriate method for analysis that has been validated for food or biological matrices such as FDA WAM 4.7, AOAC 2015.01.

b. Please also provide results for other heavy metal such as As, Cd and Hg.

c. FDA noted that the specification for lead is 5 mg/kg. If the batch analysis results support a lower specification, please consider reducing the specification to reflect the batch analysis.

AB Enzymes' Response:

As part of our response to question #8 we provide an updated composition report covering the requests made for parts a & b. For part a, in the updated composition report we list the method EN 13805:2014 in conjunction with ISO 17294-2:2016. We want to clarify why the use of both methods meets the request for part a. Method EN 13805:2014 has been validated for food matrix, it is a European standard focusing on the use of pressure digestion for determining the presence of trace elements (i.e., heavy metals) in foodstuffs. This method works in collaboration with methods containing the protocol for use of ICP-MS (Inductively Coupled Plasma/ Mass Spectrometry). Method ISO 17294-2:2016 contains the protocol for use of ICP-MS which is needed to measure the concentration of heavy metals in the digested sample and thus referenced in the updated composition report.

For part b, please refer to the updated composition report containing the results for the metals, arsenic, cadmium, and mercury. For part c, the specification for lead remains the same.

9. Batch analysis data was provided for two lots of the enzyme. If additional batches have been produced since the notice has been submitted, please provide the results of batch analysis for an additional lot.

AB Enzymes' Response:

Please refer to the updated composition report provided in this response for the batch analysis data of the third lot of enzyme.

10. Part 6 of the notice mainly discusses safety related information on the production strain but not on information that specifically speaks to the safety of the enzyme, the article of commerce. Please provide a short and concise safety narrative on the enzyme itself.

AB Enzymes' Response:

We focused on the production strain for Part 6 of the notice based on the following consideration. 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 sterol esterase subject to this GRAS notice can also be considered safe for use in food processing based on:

- Similarity in enzymatic activity with other known food enzymes
- 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

Sterol esterase (IUBMB 3.1.1.13) is a member of the lipase family of enzymes within the IUBMB 3.1.1. category for carboxylic ester hydrolases, featuring triacylglycerol lipase (IUBMB 3.1.1.3), lysophospholipase (IUBMB 3.1.1.5), phospholipase A1 (IUBMB 3.1.1.32), phospholipase A2 (IUBMB 3.1.1.4), and other fatty acid ester hydrolases. Within the lipase family, sterol esterase is in the *Candida rugosa* like lipase group (Barriuso et al 2016). This group of lipases, including the sterol esterase of this GRAS

notice, hydrolyzes fatty acid esters of sterol and of glycerol, including triacylglycerol esters, which are also hydrolyzed by triacylglycerol lipase. The enzyme reaction products of sterol esterase are similar to other known lipases used in food processing (mentioned above). These enzyme reaction products are hydrolyzed lipids and fatty acids, and are naturally present in food, and also in foods that are processed by other lipases that are GRAS. Lipases are commonly occurring enzymes with various industrial applications (Lason and Ogonowski 2010).

To add on, the manufacturing conditions of the enzyme are relevant to consider in regard to safety. The sterol esterase enzyme preparation (commercial) 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 sterol esterase. 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. Sterol esterase is used in baking in order to improve the baking process and Sections 2.8 and 2.10 demonstrate that sterol esterase is denatured by the end of the baking process and therefore does not perform any technical function in the final product. The fate of sterol esterase is not different to other (lipase) enzymes already safely applied in similar applications. 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.

AB Enzymes, Inc.
8211 W. Broward Blvd. Suite# 375
Plantation, Florida 33324

Publication bibliography

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.

Barriuso, J.; Vaquero, M.E.; Prieto A. and Martínez, M.J. (2016): Structural traits and catalytic versatility of the lipases from the Candida rugosa-like family: A review. In *Biotechnology Advances* 34 pp. 874–885

Lason, Elwira; Ogonowski, Jan (2010): Lipase - Characterization, applications and methods of immobilization. In *Chemik* 64 (2), pp. 97–102.

Joab Trujillo
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www.abenzymes.com

Analytical report
AR-777
2.3.2022

Objective: **Chemical Composition Analysis of Sterol esterase from *Trichoderma reesei***

Sample:
1. Liquid enzyme concentrate batch P190023B, lims ID 19-2049-2
2. Liquid enzyme concentrate batch P200008B, lims ID 20-612-4
3. Liquid enzyme concentrate batch 212131789, lims ID 21-1294-1

Table 1. Enzyme activity

	Batch		
	P190023B	**P200008B**	**212131789**
Sterol esterase activity (SEU/g)	22000	29000	30800

SEU: Assay of Sterol esterase activity, Roal internal method B082

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

	Batch		
	P190023B	**P200008B**	**212131789**
Antibiotic activity	not detected	not detected	not detected
Presence of production strain	not detected	not detected	not detected
Escherichia coli (/25 g)	not detected	not detected	not detected
Salmonella (/25 g)	not detected	not detected	not detected
Total coliforms (cfu*/g)	<30	<30	<30

Antibiotic activity: Specifications for Identity and Purity of Certain food Additives, FAO Food and Nutrition Paper 65 (2006), Rome, Vol.4, p. 122.
Production strain: Detection of production strain (Trichoderma, Aspergillus), Roal internal method M001
E. coli: ISO 16649-3:2015, mod.
Salmonella: NMKL 71:1999, mod.
Total coliforms: ISO 4832:2006, mod.
*cfu: colony forming units

Table 3. Heavy metals (mg/kg)

	Batch		
	P190023B	**P200008B**	**212131789**
Arsenic, As	<0.5	<0.5	<0.5
Mercury, Hg	<0.05	<0.05	<0.05
Cadmium, Cd	<0.05	<0.05	<0.05
Lead, Pb	<0.05	<0.05	<0.05

Heavy metals: EN 13805:2014 and ISO 17294-2:2016

Table 4. Mycotoxins (µg/kg)

	Batch		
	P190023B	**P200008B**	**212131789**
T2-Toxin	<10	<10	<10
HT-2-Toxin	<10	<10	<10

T2- and HT-2-Toxin: Eurofins international method, LC-MS/MS

Milla Frantzi
Quality Information Specialist
Roal Oy

Twelve pages have been removed in accordance with copyright laws. The removed reference citation is:

J. Barriuso, M. E. Vaquero, et al., “Structural traits and catalytic versatility of the lipases from the Candida rugosa-like family: A review”, Biotechnology Advances, vol. 34, pp. 874-885, 2016.

Viebrock, Lauren

From: Trujillo, Joab <Joab.Trujillo@abenzymes.com>
Sent: Thursday, April 14, 2022 5:34 PM
To: Viebrock, Lauren
Subject: RE: [External] GRN 00981 Questions

Dear Lauren,

I confirm that the reference used regarding allergenicity guidelines from FAO/WHO was the 2001 version as referenced in the notice, linked to the FAO website: Evaluation of Allergenicity of Genetically Modified Foods (fao.org).

Please let me know if you have further questions.

Best Regards,
Joab

From: Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
Sent: Thursday, April 14, 2022 12:12 PM
To: Trujillo, Joab <Joab.Trujillo@abenzymes.com>
Subject: RE: [External] GRN 00981 Questions

Dear Joab,

Thank you for your response and the additional information regarding GRN 981. We have one additional question:

Please confirm that the reference used for Codex Alimentarius Commission regarding allergenicity guidelines was the 2009 version, and not the 2001 version as referenced in the notice.

Thank you,
Lauren

From: Trujillo, Joab <Joab.Trujillo@abenzymes.com>
Sent: Friday, March 11, 2022 1:38 PM
To: Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
Subject: RE: [External] GRN 00981 Questions

Dear Lauren,

Thank you for providing an extension for the submission of the responses. Please see the attached documents to contain our responses. Along with the questions and answers document I provide an updated composition report containing the batch analysis for a third enzyme lot, values for the heavy

metals and method requested. The literature references cited in the questions and answers document are also attached above. If you have any further questions please do not hesitate to contact me, thank you.

Best Regards,

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Joab.Trujillo@abenzymes.com
www.abenzymes.com

From: Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
Sent: Wednesday, February 23, 2022 8:55 AM
To: Trujillo, Joab <Joab.Trujillo@abenzymes.com>
Subject: RE: [External] GRN 00981 Questions

Dear Jaob,

March 14th would be no problem for submission of your responses to our questions for GRN 981. Thank you.

Best,
Lauren

From: Trujillo, Joab <Joab.Trujillo@abenzymes.com>
Sent: Tuesday, February 22, 2022 2:09 PM
To: Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
Subject: RE: [External] GRN 00981 Questions

Dear Lauren,

I wanted to follow up on my request for an extension for the questions provided last week. Can you please let me know if the March 14, 2022 extension acceptable?

Looking forward to your feedback at your earliest convenience, thank you.

Best Regards,
Joab

From: Trujillo, Joab
Sent: Friday, February 18, 2022 9:17 AM

To: Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
Subject: RE: [External] GRN 00981 Questions

Dear Lauren,

Thank you for your follow up email with question #10. I am contacting you to see if we can get an additional time to respond to the questions. Can we please have 10 additional business days to respond? The new deadline would be **March 14, 2022**.

Please let me know if this new deadline is acceptable if not please let know what other options are available, thank you.

Best Regards,
Joab

From: Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
Sent: Wednesday, February 16, 2022 3:44 PM
To: Trujillo, Joab <Joab.Trujillo@abenzymes.com>
Subject: RE: [External] GRN 00981 Questions

Dear Mr. Trujillo,

Please find attached an additional question (#10) to be addressed for GRN 981. This is an addition to the questions #1-9 sent on 2/14/22. I apologize for omitting this question in my last email.

Please let me know if you have any questions.

Best,
Lauren

From: Trujillo, Joab <Joab.Trujillo@abenzymes.com>
Sent: Monday, February 14, 2022 5:04 PM
To: Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
Subject: RE: [External] GRN 00981 Questions

Dear Dr. Viebrock,

Thank you for your email and questions on GRAS Notice No. 000981. I will email you in the next few days on any questions or if we need more time to respond.

Best Regards,

Joab Trujillo
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www.abenzymes.com

From: Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
Sent: Monday, February 14, 2022 2:00 PM
To: Trujillo, Joab <Joab.Trujillo@abenzymes.com>
Subject: [External] GRN 00981 Questions

Dear Mr. Trujillo,

During our review of GRAS Notice No. 000981, we noted questions that need to be addressed. Please find the questions attached to this email.

We respectfully request a response within **10 business days**. If you are unable to complete the response within that time frame, please contact me to discuss further options.

If you have questions or need further clarification, please feel free to contact me. Thank you in advance for your attention to our comments.

Regards,
Lauren VieBrock

Lauren VieBrock, Ph.D.
Regulatory Review Scientist/Microbiology Reviewer

Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration
Tel: 301-796-7454
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Viebrock, Lauren

From: Trujillo, Joab <Joab.Trujillo@abenzymes.com>
Sent: Friday, May 6, 2022 9:09 AM
To: Viebrock, Lauren
Subject: RE: [External] GRN 00981 Questions

Dear Lauren,

I confirm that the allergenicity bioinformatic analysis using 8 amino acid sliding window is based on the Codex Alimentarius (2009) reference listed in your email sent on May 4, 2022.

If you have any additional questions please let me know.

Best Regards,
Joab

From: Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
Sent: Wednesday, May 4, 2022 1:44 PM
To: Trujillo, Joab <Joab.Trujillo@abenzymes.com>
Subject: RE: [External] GRN 00981 Questions

Dear Joab,

Please confirm that the allergenicity bioinformatic analysis using 8 amino acid sliding window is based on the following reference:
Codex Alimentarius (2009). Foods Derived from Modern Biotechnology, 2nd edition. Geneva, Switzerland: World Health Organization (WHO) / Rome, Italy: Food and Agriculture Organization of the United Nations (FAO), Codex Alimentarius Commission. Available at: http://www.fao.org/docrep/011/a1554e/a1554e00.htm.

Regards,
Lauren

From: Trujillo, Joab <Joab.Trujillo@abenzymes.com>
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Subject: RE: [External] GRN 00981 Questions

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If you have questions or need further clarification, please feel free to contact me. Thank you in advance for your attention to our comments.

Regards,
Lauren VieBrock

Lauren VieBrock, Ph.D.
Regulatory Review Scientist/Microbiology Reviewer

Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
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Tel: 301-796-7454
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