July 7, 2023

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 triacylglycerol lipase (IUBMB 3.1.1.3) 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 420
Plantation, FL 33324 USA

Proposed 21C.F.R. § 170.36 (c)(ii) *The common or usual name of notified substance:*
Triacylglycerol lipase (IUBMB 3.1.1.3) from a Genetically modified *Trichoderma reesei* production strain.

Proposed 21C.F.R. § 170.36 (c)(iii) *Applicable conditions of use:*
The lipase enzyme is to be used as a processing aid in the production of bakery and other cereal based products. The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements under current Good Manufacturing Practices. There are no maximal limits set, just suggested dosages.

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

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

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

§170.225(c)(8) - FOIA (Freedom of Information Act):
Parts 2 through 7 of this notification do not contain data or information that is exempt from disclosure under the FOIA (Freedom of Information Act).

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

Sincerely,

**AB Enzymes GmbH**

DocuSigned by:
452B3329EDDB45F...

**i.V. Candice Cryne**

**Senior Global Regulatory Affairs Manager**

10-Jul-2023 | 19:32 BST

DocuSigned by:
FD80C973075E42E...

**Joab Trujillo**

**Regulatory Affairs Specialist**

10-Jul-2023 | 20:48 BST

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

AB Enzymes GmbH – Feldbergstrasse 78 , D-6412 Darmstadt

# GRAS Notification of a Triacylglycerol Lipase from a Genetically Modified *Trichoderma reesei* *Strain*

AB ENZYMES GmbH

July 7, 2023

# Contents

# 1 PART 1 §170.225 – SIGNED STATEMENTS AND CERTIFICATIONS

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

In conformity with the established regulation 21 C.F.R. Section 170, subsection E, AB Enzymes GmbH hereby claims that **Triacylglycerol lipase** (IUBMB# 3.1.1.3) 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 420

Plantation, FL 33324 USA

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

**Triacylglycerol Lipase** (IUBMB 3.1.1.3) from a Genetically modified *Trichoderma reesei* production strain.

**§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 lipase is to be used as a processing aid in the production of bakery and other cereal based products. The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements under current Good Manufacturing Practices. There are no maximal limits set, just suggested dosages.

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

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

This GRAS determination is based upon scientific procedures.

**§170.225(c)(6) – Premarket approval:**

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

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

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

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

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

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

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

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

## 2.1 Identity of the notified substance

The dossier concerns a **lipase from a genetically modified *Trichoderma reesei strain*.**

### 2.1.1 Common name of the enzyme

Name of the enzyme protein: Lipase

Synonyms: Triacylglycerol acylhydrolase, triacylglycerol ester hydrolase, triacylglycerol lipase

### 2.1.2 Classification of the enzyme

| | |
|---|---|
| IUBMB # | 3.1.1.3 |
| CAS # | 9001-62-1 |

EC 3. is for hydrolyases;

EC 3.1. is for acting on ester bonds;

EC 3.1.1. is for carboxylic-ester hydrolases

EC 3.1.1.3 is for triacylglycerol lipase.

## 2.2 Strain Lineage Information

### 2.2.1 Production Strain

| | |
|---|---|
| Production strain | *Trichoderma reesei* AR-822 |

**Synopsis**

**Lipase** from *Thermomyces lanuginosus* is produced from a genetically modified *Trichoderma reesei* production strain (AR-822). The genetic modifications performed to develop the production strain are described in section 2.3 of the GRAS narrative along with confirmation on integration of expression cassettes in the *T. reesei* genome, the stability of the production strain, absence of DNA, antibiotic genes, and toxic compounds. Information on the safety of the *Trichoderma reesei* production strain is provided in section 6 of the GRAS narrative. In this notice, we provide information that the *T. reesei* production organism is **non-pathogenic** and **non-toxigenic** (for more details please refer to sections 2.3.6, 6.1.3 and 6.1.4). In short, safety of the production strain is substantiated by the safety of the genetic modifications, history of safe use for *Trichoderma reesei* as a food enzyme producer and the use of the safe strain lineage concept described in Pariza and Johnson (2001).

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

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

| **GRAS Notice** | **Description** |
|---|---|
| GRAS Notice 524[2] | Phospholipase A2 enzyme preparation from *Trichoderma reesei* |
| GRAS Notice 557[3] | Polygalacturonase produced in *Trichoderma reesei* |

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

| | |
|---|---|
| GRAS Notice 558[4] | Pectin esterase produced in *Trichoderma reesei* |
| GRAS Notice 566[5] | Mannanase produced in *Trichoderma reesei* |
| GRAS Notice 628[6] | Endo-1,4-beta-xylanase produced in *Trichoderma reesei* |
| GRAS Notice 631[7] | Triacylglycerol lipase produced in *Trichoderma reesei* |
| GRAS Notice 653[8] | Lysophospholipase produced in *Trichoderma reesei* |
| GRAS Notice 707[9] | Glucose oxidase produced in *Trichoderma reesei* |
| GRAS Notice 756[10] | Endo-1,4-beta-glucanase produced in *Trichoderma reesei* |
| GRAS Notice 817[11] | Serine endopeptidase produced in *Trichoderma reesei* |
| GRAS Notice 981[12] | Sterol esterase enzyme produced in *Trichoderma reesei* |

**The *Trichoderma reesei* production strain AR-822 is deposited in the Westerdijk Fungal Biodiversity Institute, formerly known as the "Centraalbureau voor Schimmelcultures" (CBS) in the Netherlands with the deposit number CBS 149450.**

[4] GRN No. 558
[5] GRN No. 566
[6] GRN No. 628
[7] GRN No. 631
[8] GRN No. 653
[9] GRN No. 707
[10] GRN No. 756
[11] GRN No. 817
[12] GRN No. 981

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

Kingdom: Fungi

Division: *Ascomycota*

Class: *Sordariomycetes*

Order: *Hypocreales*

Family: *Hypocreaceae*

Genus: *Trichoderma*

Species: *Trichoderma reesei*

Strain: *Trichoderma reesei* AR-822

### 2.2.2. Recipient Strain

The recipient strain *Trichoderma reesei* AR-555 used for the genetic modification is a *Trichoderma reesei* mutant strain deriving from *T. reesei* QM6a after classical mutagenesis and genetic modifications. The recipient strain used in the genetic modification for the construction of the production strain is *Trichoderma reesei* AR-555. The recipient strain was created from parental strain AR-256 through a series of native enzyme gene deletions to limit enzyme side activity expression in *Trichoderma reesei*. The recipient is negative in e.g., the genes encoding the four major cellulases (cellobiohydrolases 1 and 2 and endoglucanases 1 and 2) and the major xylanases, (xylanase I and xylanase II). The gene used in gene deletions was deriving from *T. reesei*, meaning that no heterologous marker genes were used in these gene deletions.

Therefore, the recipient can be described as followed:

Kingdom: Fungi

Division: *Ascomycota*

Class: *Sordariomycetes*

Order: *Hypocreales*

Family: *Hypocreaceae*

Genus: *Trichoderma*

Species: *Trichoderma reesei*

Strain: *Trichoderma reesei* AR-555

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

### 2.2.3 Donor:

The lipase gene described in this application derives from *Thermomyces lanuginosus* (formally known as *Humicola lanuginosa*). *Thermomyces lanuginosus* is considered to be a type of thermophilic fungus (Singh et al. 2003). In Germany, *Thermomyces lanuginosus* is a risk level 1 organism according to BAuA (German Federal Institute for Occupational Safety and Health[13]). Enzymes sourced from *Thermomyces lanuginosus* can be used in industries such as pulp and paper, fats and oils, biofuels, baking among others (Singh et al. 2003; Pedersen and Broadmeadow 2000; Fernandez-Lafuente 2010). Internationally, lipases from *Thermomyces lanuginosus* are recognized as safe, refer to the table below for non-exhaustive list of permitted enzymes from GM strains.

| **Non exhaustive list of authorized lipases sourced from *Thermomyces lanuginosus*** | | |
|---|---|---|
| **Authority** | **Source of Lipase** | **Reference** |
| **Brazil** | *Thermomyces lanuginosus* expressed in *Aspergillus oryzae*<br><br>*Thermomyces lanuginosus* and *Fusarium oxysporum* expressed in *Aspergillus oryzae* | RESOLUÇÃO - RDC Nº 728, DE 1° DE JULHO DE 2022 - RESOLUÇÃO - RDC Nº 728, DE 1° DE JULHO DE 2022 - DOU - Imprensa Nacional (in.gov.br) |

[13] **BAuA - Advanced search - Federal Institute for Occupational Safety and Health**

| | | |
|---|---|---|
| **Australia/New Zealand** | *Aspergillus oryzae,* containing the gene for Lipase, triacylglycerol isolated from *Humicola lanuginosa* | Australia New Zealand Food Standards Code – Schedule 18 – Processing aids (legislation.gov.au) |
| **Mexico** | *Aspergillus oryzae* containing the gene for a lipase isolated from *Thermomyces lanuginosus*<br><br>*Aspergillus oryzae* containing the gene for a lipase isolated from *Thermomyces lanuginosus/Fusarium oxysporum* | ANEXO_VI..pdf (www.gob.mx) |

| | |
|---|---|
| Genus: | *Thermomyces* |
| Species: | *Thermomyces lanuginosus* |
| Subspecies (if appropriate): | Not applicable |
| Commercial name: | Not applicable. The organism is not sold as such |

## 2.3 Genetic modification

The *Trichoderma reesei* AR-822 production strain was constructed for lipase production. The construction of the production strain consisted of:

- Expression cassette containing the lipase gene from *Thermomyces lanuginosus* under the control of the *T. reesei* promoter and *Aspergillus nidulans amdS* marker gene
- Introduction of expression cassette into the genome of *Trichoderma reesei* AR-555 recipient strain in one genetic modification step

The expression cassette was cleaved from the pUC19 vector plasmid by restriction enzyme digestion followed by isolation of the expression cassette from agarose gel. A Southern blot

hybridization experiment was performed on the genomic DNA of the production strain to confirm that no pUC19 vector DNA was included in the *Trichoderma reesei* AR-822 genome.

The production strain secretes high amounts of lipase into its culture supernatant, resulting in high lipase activity in the cultivation broth. In addition, the strain AR-822 produces endogenous *Trichoderma* enzymes in small amounts. These activities are not relevant from an application or safety point of view, due to the small amount and the fact that such activities are included in products which have been approved for decades in food processing.

**Expression Cassette Components Table**

| Component | Description |
|---|---|
| *Thermomyces lanuginosus* **lipase gene** | The synthetic lipase gene encoding the *Thermomyces lanuginosus* protein engineered lipase enzyme. |
| **Synthetic *amdS* gene and promoter** | The marker gene has originally been isolated from *A. nidulans* VH1-TRSX6 (Kelly and Hynes 1985; Hynes et al. 1983) which is closely related to *Aspergillus tubingensis*, which is used in industrial production of food enzymes. The gene codes for an acetamidase that enables the strain to grow on acetamide as a sole nitrogen source (Kelly and Hynes 1985). This characteristic has been used for selecting transformations without any disadvantage for more than 30 years. In the expression cassette, a synthetic *amdS* gene was included which |

| | |
|---|---|
| | encodes the amino acid sequence of the native *amdS*. |
| ***Trichoderma reesei* promoter and terminator** | The lipase gene is fused to *T. reesei* native promoter. This promoter is strong and used to overexpress lipase gene transcription, to obtain high yields of lipase. The transcription is terminated by the native terminator from *T. reesei*. |

**Information relating to the genetic modification**

Standard DNA techniques were used in the construction of the plasmid and transformation of the expression cassette. The expression cassette was constructed using synthetic gene encoding the lipase enzyme. The transformation of the *T. reesei* recipient strain with the expression cassette containing the lipase gene was performed as described in (Penttilä et al. 1987) with modifications. No vector DNA was expected to be included in the DNA preparation used for transformation. The transformants were selected according to their ability to grow on acetamide selection plates (*amdS* marker gene).

**Genes of concern**

The production organism does not contain any genes of concern. No antibiotic resistance marker genes were used for the construction of the production strain. The inserted sequences are well described and do not contain any sequences of concern.

**Confirmation of Insertion**

Confirmation of expression cassette insertion was done via Whole Genome Sequencing.

### 2.3.1 Genetic stability of the production strain

The fermentation process always starts from identical replicas of the AR-822 (production strain) seed ampoule. Production preserves from the "Working Cell Bank" are used to start the fermentation process. A Working Cell Bank is a collection of ampoules containing a pure culture. The cell line history and the production of a Cell Bank, propagation, preservation and storage is monitored and controlled. Ampoules in a WCB ampoule Bank are only accepted for production runs if their quality meets the required standards. This is determined by checking identity, viability, microbial purity and productivity of the WCB ampoules. The accepted WCB ampoules are used as seed material for the inoculum.

Testimony to the stability of the strain is given by monitoring the growth behavior and by production of comparable levels of lipase activity in number of fermentation batches performed for the AR-822 strain. The activity measurements from parallel fermentations show that the productivity of the AR-822 strain remains similar. This clearly indicates that the strain is stable. In addition, to confirm the genetic stability of AR-822, a Southern blot was prepared using the genomic DNAs isolated from the mycelia collected from the end of three independent fermentations. Three different restriction digestions were performed, and the expression cassette transformed was used as a probe in the hybridization. The hybridization patterns were identical in all samples. The data of the analysis of enzyme activities from preparations deriving from different fermentation batches of the recombinant AR-822 strain is presented in Appendix #1.

Another conformational method was used to determine genetic stability of the production strain. The production strain was cultivated in liquid medium for three subsequent cultivation cycles. The cultivation started from the original strain deposit at -80 ºC being cultivated on solid agar. The spores were collected and inoculated to liquid medium. After finalizing the cultivation, a sample from the liquid culture was transferred to fresh cultivation medium and liquid cultivation was repeated. Still another transfer to fresh cultivation medium was done similarly, leading to three subsequent cultivation cycles in liquid medium. After the cultivations the lipase activities were analyzed from the culture supernatants and samples were run on SDS-PAGE gels. The genomic DNAs were isolated from mycelia (representing different cultivation cycles) and were analyzed by

using Southern blot. The results from the analysis showed that the production strain remained stable: the enzyme activities and protein patterns remained comparable in all culture supernatants and there were no changes in the strain genome. It is expected that in case the strain is unstable, the enzyme activities would decrease considerably from cultivation cycle to the next and, also, differences in the banding patterns (in SDS-PAGE and Southern blot) would be detected.

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

No vector sequences were integrated.

A Southern blot hybridization experiment using pUC19 vector backbone as a labelled probe and genomic DNA of the production host AR-822 was performed to confirm that no vector DNA is included in the genome of AR-822. The results of the experiment were negative (no hybridization), demonstrating that no vector DNA is inserted into the genome of *Trichoderma reesei* 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 lipase enzyme production strain is recovered from the fermentation broth by a widely used process that results in a cell-free enzyme concentrate. The absence of the production strain is confirmed for every production batch, using an internal Roal[14] 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.

[14] Roal Oy is the sole manufacturer of AB Enzymes' enzyme preparations.

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

The AR-822 enzyme preparation is free from detectable, viable production organism as demonstrated in Appendix #1. As the absence of the production strain is confirmed for every production batch, no additional information regarding the inactivation of the GMM cells is required.

### 2.3.5 Information on the possible presence of DNA

The lipase enzyme preparation is produced by an aerobic submerged microbial fermentation using a genetically modified *Trichoderma reesei* strain. All viable cells of the production strain, AR-822, are removed during the down-stream processing: the fermentation broth is filtered with pressure filters and subsequent sheet filters, concentrated with ultra-filtration, and optionally followed by sheet filtration(s).

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

### 2.3.6 Absence of Antibiotic Genes and Toxic Compounds

As noted above, the transformed DNA does not contain any antibiotic resistance genes. Further, the production of known mycotoxins according to the specifications elaborated by the General Specifications for Enzyme Preparations Used in Food Processing Joint FAO/WHO Expert Committee on Food Additives, Compendium of Food Additive Specifications, FAO Food and Nutrition Paper[15] (FAO/WHO 2006) has been also tested from the fermentation product of the *Trichoderma reesei* strain AR-822. The Food Chemicals Codex ("FCC", 13th edition 2022), states

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

the following: "Although limits have not been established for mycotoxins, appropriate measures should be taken to ensure that the products do not contain such contaminants." Adherence to specifications of microbial counts is routinely analyzed. The absence of antibiotic activities, according to the specifications recommended by JECFA (FAO/WHO 2006), was also confirmed from three AR-822 enzyme production batches in Appendix #1 and no antibiotic or toxic compounds were detected.

We confirm that the *T. reesei* AR-822 production strain is non-toxigenic. Regarding mycotoxins produced by *Trichoderma reesei*, the composition report provided as Appendix #1 demonstrates mycotoxin values below the levels of quantification (LoQs) for the enzyme concentrate batches tested.

## 2.4 ENZYME PRODUCTION PROCESS

### 2.4.1 Overview

The food enzyme is produced by ROAL Oy[16] by submerged fermentation of *Trichoderma reesei* AR-822 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,

[16] See footnote 14

the enzyme protein in question is only partially separated from the other organic material present in the food enzyme.

### 2.4.2 Fermentation

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

- Inoculum
- Seed fermentation
- Main fermentation

### 2.4.3 Raw Materials

The raw materials used in the fermentation and recovery processes are standard ingredients that meet predefined quality standards controlled by Quality Assurance for Roal Oy. The safety is further confirmed by toxicology studies. The raw materials conform to either specifications set out in the Food Chemical Codex, 13$^{th}$ edition, 2022 or The Council Regulation 93/315/EEC, setting the basic principles of EU legislation on contaminants and food, and Commission Regulation (EC) No 1881/2006 setting maximum limits for certain contaminants in food. The maximum use levels of antifoam and flocculant are ≤0.15% and ≤1.5% respectively.

Regarding the use of raw materials that contain known food allergens, the enzyme preparation is formulated with a wheat-based ingredients (as indicated in section 2.5.2) which contains gluten.

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

- Potable water
- A carbon source
- A nitrogen source

- Salts and minerals
- pH adjustment agents
- Foam control agents

### 2.4.5 Inoculum

A suspension of a pure culture of AR-822 is aseptically transferred to shake flasks containing fermentation medium.

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

### 2.4.6 Seed fermentation

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

### 2.4.7 Main Fermentation

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

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

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

### 2.4.8 Recovery

The purpose of the recovery process is:

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

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

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

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

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

### 2.4.9 Materials

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

- Flocculants
- Filter aids
- pH adjustment agents

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

### 2.4.10 Pre-Treatment

Flocculants and/or filter aids are added to the fermentation broth, in order to get clear filtrates, and to facilitate the primary solid/liquid separation. Typical amount of filter aids is 2.5 %.

### 2.4.11 Primary solid/liquid separation

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

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

### 2.4.12 Concentration

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

### 2.4.13 Polish and germ filtration

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

### 2.4.14 General Production Controls and Specifications

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

*Identity and purity of the producing microorganism:*

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

Production of the required enzyme protein is based on a well-defined Master (MCB) and Working Cell Bank (WCB). The MCB contains the original deposit of the production strain. The WCB is a collection of ampoules containing a pure culture prepared from an isolate of the production strain in MCB. The cell line history, propagation, preservation and the production of a Working Cell Bank is monitored and controlled. A WCB is only accepted for production runs if its quality meets the required standards. This is determined by checking identity, viability, microbial purity and productivity of the WCB. The accepted WCB is used as seed material for the inoculum.

*Microbiological hygiene:*

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

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

- Hygienic design of equipment:
    - all equipment is designed, constructed and used to prevent contamination by foreign micro-organisms
- Cleaning and sterilization:
    - Validated standard cleaning and sterilization procedures of the production area and equipment: all fermentor, vessels and pipelines are washed after use with a CIP-system (Cleaning in Place). After cleaning, the vessels are inspected manually;

all valves and connections not in use for the fermentation are sealed by steam at more than 120°C; critical parts of down-stream equipment are sanitized with disinfectants approved for food industry

- Sterilization of all fermentation media:
    - all the media are sterilized with steam injection in fermentors or media tanks
- Use of sterile air for aeration of the fermentors:
    - Air and ammonia water are sterilized with filtration (by passing a sterile filter).
- Hygienic processing:
    - Aseptical transfer of the content of the WCB ampoule, inoculum flask or seed fermentor
    - Maintaining a positive pressure in the fermentor
- Germ filtration

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

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

*Chemical contaminants:*

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

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

These in-process controls comprise:

*Microbial controls:*

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

*Monitoring of fermentation parameters may include:*

- pH
- Temperature
- Aeration conditions

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

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

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

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

### 2.4.15 Formulation and Packaging

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

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

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

### 2.4.16 Stability of the enzyme during storage and prior to use

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

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

## 2.5 Composition and specifications

### 2.5.1 Characteristics of the enzyme preparation

The characteristics of the formulated enzyme preparation are:

| Property | Requirement | |
|---|---|---|
| Activity | min. | 10,000 ALU/g |
| Appearance | Light beige to light brown powder | |
| Particle size distribution | Max 1 % 250 µm | |

### 2.5.2 Formulation of a typical enzyme preparation

| Composition | |
|---|---|
| **Constituent** | **%** |
| Enzyme concentrate | 5.0 – 10.0 |
| Sunflower oil | 0.4 |
| Wheat flour | Remainder |

### 2.5.3 Molecular mass and amino acid sequence of the enzyme

The approximate molecular mass of the protein engineered lipase enzyme is about 30 kDa. It is 94.53% identical to the native *Thermomyces lanuginosus* lipase.

### 2.5.4 Purity and identity specifications of the enzyme preparation

It is proposed that the food enzyme lipase should comply with the internationally accepted JECFA specifications for chemical and microbiological purity of food enzymes (FAO/WHO 2006):

| | |
|---|---|
| Lead: | Not more than 5 mg/kg[17] |
| *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[18] |

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

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

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

Enzyme concentrate batches and the commercial enzyme preparation

The samples tested in the composition report found in Appendix #1 are liquid enzyme concentrates. As described in section 2.5.2, the commercial enzyme preparation is a dry powder product. The liquid concentrate batches are representative to the commercial enzyme preparation even though the enzyme activity levels of the liquid batches are greater than the minimum activity level stated in section 2.5.2. The liquid enzyme concentrates were obtained after the downstream processing/recovery step in the manufacturing of lipase produced by AR-822 (see section 2.4. for more details), prior to the addition of the formulation ingredients. During the formulation and

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

[18] See JECFA specifications, Food safety and quality: enzymes (fao.org)

packaging step in the manufacture of the commercial enzyme preparation (see section 2.4.16 for more details), we adjust the amount of dry concentrate in the preparation to reach the desired activity by either adding more or less enzyme concentrate.

## 2.6 Enzymatic Activity

The main activity of the *Trichoderma reesei* AR-822 enzyme preparation is lipase (IUBMB 3.1.1.3). The **function** of lipase to catalyze the hydrolysis of ester bonds of triacylglycerols (at the position 1 and 3 of the glycerol molecule), resulting in the formation of mono- and diacylglycerols, free fatty acids and, in some cases, also glycerol.

**The substrates** for the lipase are non-polar lipids such as triglycerides or triacylglycerol. Triglycerides are formed by combining glycerol with three fatty acids molecules. The glycerol molecule has three hydroxyl (OH-) groups. Each fatty acid has a carboxyl group (COOH-). In triglycerides, the hydroxyl groups of the glycerol join the carboxyl groups of the fatty acid to form esters bonds.

Triglycerides are found in plants and animals: they are the main constituents of vegetable oils and animal fats. Triglycerides and triacylglycerols are also found for instance in wheat flour: wheat flour contains approximately 2.0–2.5% lipids; wheat lipids can be divided into glycolipids, phospholipids and non-polar lipids (triacylglycerides, mono-glycerides) as shown in Figure 1 below.

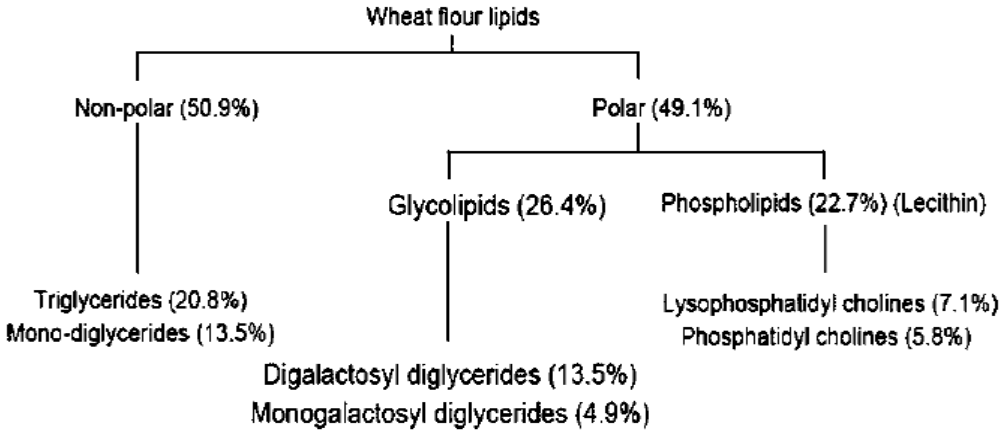


*Figure 1. Classification of wheat flour lipids*

**The reaction products** of the hydrolysis of non-polar lipids with the help of lipase are mainly mono- and diacylglycerols and free fatty acids. As the non-polar lipids containing organisms themselves produce lipases, these reaction products are naturally present in these organisms. Consequently, also the reaction products occur naturally in foods.

Lipase activity is widely present in nature and in food ingredients. The substrates and the reaction products are themselves present in food ingredients. No reaction products which could not be considered normal constituents of the diet are formed during the production or storage of the enzyme treated food. Consequently, no adverse effect on nutrients is expected.

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

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

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

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

Furthermore, the presence of such enzyme activities and the potential reaction products in food is not new and should not be of any safety concern. During the production of food enzymes, the main enzyme activity contains several other enzymes excreted by the microbial cells. As in the case of the enzyme for this application, the lipase is clearly the main enzyme in the product and the minor amounts of side activities are native to the production strain. It is generally accepted that the enzyme proteins themselves do not pose any safety concern and are recognized to be generally considered as safe. AB Enzymes is not aware of any adverse effects from the side activities present in the lipase enzyme preparation.

Adverse effects from side activities are not expected from the lipase enzyme preparation. *Trichoderma reesei* has a long history of safe use in the food industry can described in the dosser (section 6.1.). The side activities in the enzyme preparation derive from the production microorganism *Trichoderma reesei*. This microorganism is known to produce enzymatic side activities, e. g. beta-glucanase and cellulase in low amounts. These side activities have no adverse consequence to human health as they are considered globally as safe food enzymes and are part of the human diet.

## 2.7 Allergenicity

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

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

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

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

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

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

- The majority of proteins are not food allergens and based on previous experience, the enzyme industry is not aware of any enzyme proteins used in food that are homologous to known food allergens[19].
- Only a small amount of the food enzyme is used during food processing, which leads to very small amount of enzyme protein present in the final food. A high concentration generally equals a higher risk of sensitization, whereas a low level in final food equals a lower risk (Goodman et al. 2008).
- For cases where the proteins are denatured which is the case for this enzyme due to the food process conditions, the tertiary conformation of the enzyme molecule is destroyed. These types of alterations to the conformation in general, are associated with a decrease in the antigenic reactivity in humans. In the clear majority of investigated human cases, denatured proteins are much less immunogenic than the corresponding native proteins (Valenta and Kraft 2002; Valenta 2002; Takai et al. 2000; Nakazawa et al. 2005; Kikuchi et al. 2006).
- To add on, residual enzyme still present in the final food will be subjected to digestion in the gastro-intestinal system, that reduces further the risk of enzyme allergenicity. While

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

stability to digestion is considered as a potential risk factor of allergenicity, it is believed that small protein fragments resulting from digestion are less likely to be allergenic (Food and Agriculture Organization of the United Nations January/2001; Goodman et al. 2008).

- Lastly, enzymes have a long history of safe use in food processing, with no indication of adverse effects or reactions. Moreover, a wide variety of enzyme classes (and structures) are naturally present in food. This is in contrast with most known food allergens, which are naturally present in a narrow range of foods.

### 2.7.1 Allergenicity Search

Alignments of the lipase mature amino acid sequence to the sequences in the allergen database were performed and results obtained were used to estimate the level of potential allergenicity of this enzyme. Similarity searches were performed to the sequences available in the public Allergen Online (FARRP) allergen database version 21 (last updated on February 14, 2021). The allergenicity searches were conducted on February 28, 2023.

The alignment methods used in the searches are:

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

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

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

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

**Results of Allergenicity searches:**

| Type of Search | Outcome |
|---|---|
| **Alignment of the lipase mature amino acid sequence to sequences in allergen online databases** | One hit was identified over the 35 % identity using the full-length search from the AllergenOnline database. *One hit was identified over the 35 % identity using the full-length search from the AllergenOnline database*, with high E-value (0.99) indicating a non/less significant match – the proteins are not likely to be related in evolution or structure. |
| **Alignment of sliding 80-amino acid window of the query protein to known protein allergens** | No matches having greater than 35% identity were found from the AllergenOnline database using the 80-mer sliding window search. |
| **8-mer sequence search** | Zero sequences with at least one 8-mer match were detected |

To summarize, the bioinformatics approach to estimate potential allergenicity and cross-reactivity based on relatedness to known allergens and taking into account the most recent scientific recommendations on the interpretation of such data leads us to conclude that the **lipase** produced by *Trichoderma reesei* AR-822 is of no concern.

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

Like any other enzyme, the lipase acts as a biocatalyst: with the help of the enzyme, a certain substrate is converted into a certain reaction product. It is not the food enzyme itself, but the result of this conversion that determines the effect in the food or food ingredient. After the conversion has taken place, the enzyme no longer performs a technological function. The main activity of the *T. reesei* AR-822 enzyme preparation is lipase (IUBMB 3.1.1.3).

As mentioned in section 2.6:

| | |
|---|---|
| **Enzymatic function** | catalyze the hydrolysis of ester bonds of triacylglycerols (at the position 1 and 3 of the glycerol molecule) |
| **Substrates** | non-polar lipids such as triglycerides or triacylglycerol |
| **Reaction Products** | mono- and diacylglycerols and free fatty acids |

Like most of the enzymes, lipase performs its technological function during food processing. The lipase from *Trichoderma reesei* AR-822, object of this dossier, acts as processing aid in the manufacture of bakery products (e.g. bread, biscuits, tortillas, cakes, steamed bread and croissants) and other cereal based products (pasta, noodles and snacks). Lipases have been used in baking for the last 30 years and their use in the bakery industry is continuously increasing. This application has been specifically approved for a number of years in Canada, Denmark and France (including the "Pain de tradition Française"), USA, Mexico, Canada, Australia/New Zealand which together with the extensive use for decades demonstrates the technological need of lipases in

these food processes. Much of the information presented in this section can also be found in GRN 631.

**Baking processes**

In baking, lipase performs its technological function during dough or batter handling in order to contribute to an improved and consistent baking process. During mixing, wheat flour free lipids become bound or trapped within the gluten fraction. Limited hydrolysis of the triglycerides with the help of lipase results 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 destabilise gas cells in dough and therefore limit bread volume. The use of lipase helps removing this negative effect.

In addition, the degradation of the substrate triglycerides with the help of lipase leads to the creation of monoacyl-glycerol, that interacts with gelatinizing starch, in particular with amylose to form irreversible monoacyl-glycerol-amylose complexes.

The use of lipase 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 triglycerides (non-polar lipids) with the help of lipase 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 behaviour during the baking steps
- Regulate batter viscosity, beneficial in the production process for e.g., waffles, pancakes and biscuits

The process flow of lipase used in the baking process is presented below:

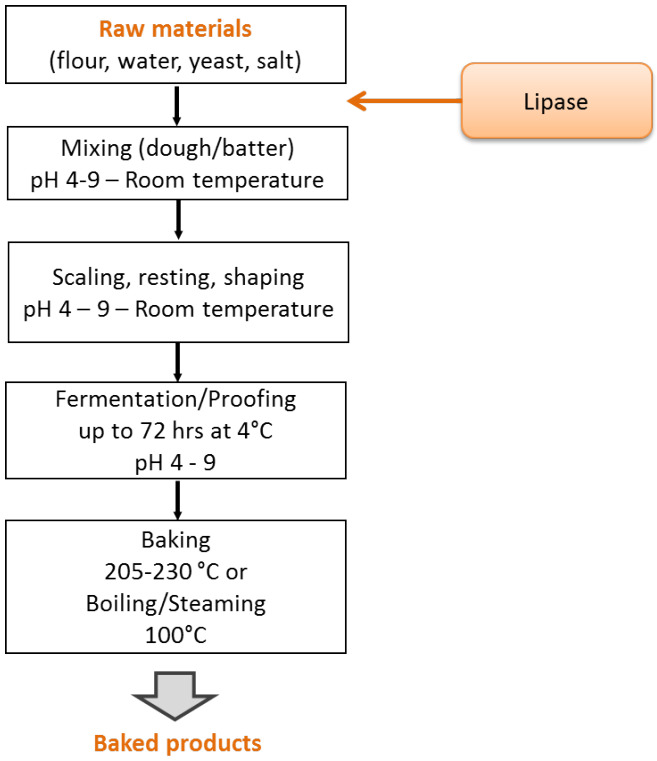


Figure #1: Lipase used in baking products

**Other cereal based processes:**

Lipids provide functional properties during pasta, noodle and snack making - due to their ability to interact with gluten and the water phase. Limited hydrolysis of lipids with the help of lipase improves the functional properties of the flour endogenous lipids, as explained below.

Dried pasta has, among cereal derived foodstuffs, a very distinct microscopic structure. It has a continuous protein mixture phase (the gluten or the protein network) wherein the starch granules are dispersed. While cooking in hot water, the starch granules gelatinize, i.e. absorb water, swell and turn into starch paste. The gluten (the protein network) is denatured through cooking and if it is not sufficiently resistant, the starch granules, when swelling, can tear the meshes of its continuous phase, thereby giving rise, at the periphery of the pasta, to a viscous layer of starch paste.

The state of the protein network after cooking can also affect the elasticity of the pasta. The main problem which has to be solved to obtain elastic and non-sticky pasta thus consists in increasing the resistance of the protein network to cooking[20].

Pasta treated with lipase show higher amylose-lipid melting enthalpies (increase of around 75% more melting enthalpy in the cooked pasta treated by lipase), indicating that hydrolysis products of lipase do form complexes with amylose during cooking. These complexes inhibit the swelling of starch and the leakage of amylose during cooking, resulting in a firmer texture and smoother surface. Further, the complex-building capability of the lipase hydrolysis products with amylose reduces leaching of amylose, resulting in less stickiness of products[21].

Because gluten has a predominant role in the structure, the use of lipase, by increasing the gluten protein network resistance to cooking also plays a role in reducing the porosity and oil uptake during (noodles) frying (Gulia et al. 2014).

Therefore, the benefits of the conversion of the triglycerides (non-polar lipids) with the help of lipase in other cereal based processes can be summarized as follows:

- Facilitate the handling of the dough
- Improve dough stability and strength which results in processing tolerance
- Improved texture after boiling/steaming

[20] USA (1970) - US Patent, US 3520702 A "*Method of making dried pasta having a protein network that withstands cooking*")1 available online: http://www.google.com/patents/US3520702

[21] VTT Biotechnology and TNO Nutrition and Food Research Institute (1999) – Second European Symposium on Enzymes in Grain processing - VTT Symposium 207- ESEGP-2 . p. 167 Available online: http://www2.vtt.fi/inf/pdf/symposiums/2000/S207.pdf

The process flow of lipase used in other cereal based processes is presented below:

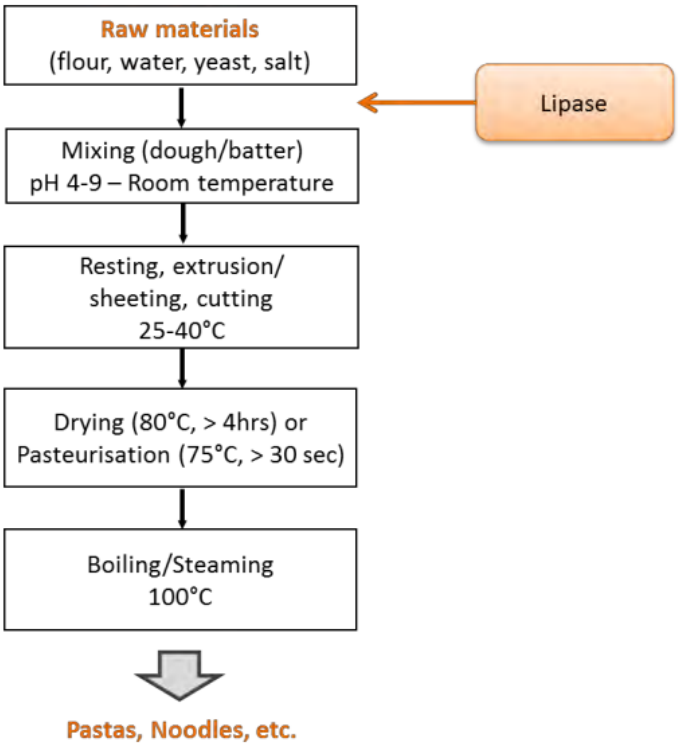


Figure #2: Lipase use in other cereal based products

## 2.9 Use Levels

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

Therefore, the enzyme manufacturer can only issue a recommended enzyme dosage range. Such a dosage range is the starting point for the individual food producer to fine-tune this process and determine the amount of enzyme that will provide the desired effect and nothing more. Consequently, from a technological point of view, there are no 'normal or maximal use levels' and **lipase** is used according to the QS principle. A food producer who would add much higher doses

than the needed ones would experience untenable costs as well as negative technological consequences.

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

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

The table below shows the range of recommended use levels for each application where the **lipase** from *Trichoderma reesei* AR-822 may be used:

| Food Application | Raw material (RM) | Maximal recommended use levels (mg TOS/kg RM) |
|---|---|---|
| **Baking and other cereal based products** | Cereals | 4 |

## 2.10 Fate in food

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

**Lipase** performs its technological function during food processing. In some cases, the enzyme may no longer be present in the final food. In other cases, where the enzyme protein is still present

in the final food, it does not perform any technological function in the final food, just like the endogenous lipase present in the fruit and vegetable raw materials and ingredients. In order to be able to perform a technological function in the final food, a number of conditions have to be fulfilled at the same time:

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

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

— the lipase is denatured by heat during the baking or steaming steps of the baking process and during the drying, boiling or steaming steps of processes of other cereal-based products: it is inactivated during regular baking processes, where temperatures inside the dough reach between 95° and 100°C for a period of at least 10-15 minutes.

— the remaining water content (water activity) within baked goods is much too low to support any hydrolytic enzymatic activity in the baking matrix.

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

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

# 3 Part 3 § 170.325- Dietary Exposure

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

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

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

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

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

| Applications | | Raw material (RM) | Maximal recommended use level (mg TOS/kg RM) | Final food (FF) | Ratio RM/FF* | Maximal level in final food (mg TOS/kg food) |
|---|---|---|---|---|---|---|
| Solid foods | Baking and other cereal based products | Cereals | 4 | Baked products | 0.71 | 2.84 |

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

<u>Baking:</u>

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

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

| TMDI in food<br><br>(mg TOS/kg body weight/day) | TDMI in beverage<br><br>(mg TOS/kg body weight/day) | Total TMDI<br><br>(mg TOS/kg body weight/day) |
|---|---|---|
| 2.84 x 0.0125 = 0.036 | 0 | **0.036** |

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 specific lipase from *Trichoderma reesei* AR-822;
- It is assumed that ALL producers apply the HIGHEST use level per application;
- For the calculation of the TMDI's in food, only the above foodstuffs were selected containing the highest theoretical amount of TOS. Therefore, foodstuffs containing lower theoretical amounts were not included;
- It is assumed that the final food containing the calculated theoretical amount of TOS is consumed DAILY over the course of a lifetime;
- Assumptions regarding food and beverage intake of the general population are overestimates of the actual average levels (Douglass et al. 1997).

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

The Margin of Exposure (MoE)[22] for human consumption can be calculated through the division of the NOAEL[23] (no-observed adverse effect) value by the TMDI (Total Theoretical Maximal Daily Intake). Total TMDI of the food enzyme is 0.036 mg TOS/kg body weight/day.

As a result, the MoE is:

MoE =1000/0.036 =**28,169**.

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

**Conclusion**:

To conclude, the use of the food lipase from *Trichoderma reesei* AR-822 in the production of food is safe. Considering the high safety value determined by the MoE, even when calculating using means of overestimation of intake via the Budget method, there is no need to restrict the use of the enzyme in food. The suggested dosage for food manufacturers is not a restrictive value and could be higher or lower depending on usage within cGMPs.

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

[23] The NOAEL value is taken from the 90-Day Repeated Dose Oral Toxicity Study in Wister Rats study of the reference production strain AR-852 (see section 6.1.3 for more details).

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

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

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

This part is not applicable to this notified substance.

# 6 Part 6 § 170.250- GRAS Notice- Narrative

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

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

## 6.1 Safety Risk Assessment for Production Strain

### 6.1.1 Safety of *Trichoderma reesei*

The safety of *Trichoderma reesei* as an enzyme producer has been reviewed by (Frisvad et al. 2018; Nevalainen et al. 1994; Olempska-Beer et al. 2006; Blumenthal 2004) . *T. reesei* is regarded as a safe organism for production of industrial enzymes.

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

At AB Enzymes, *Trichoderma reesei* strains have been used as enzyme producer for many years without any safety problems.

Non-exhaustive list of authorized food enzymes (other than lipase) used *Trichoderma reesei*:

| **Non exhaustive list of authorized food enzymes (other than lipases) produced by *Trichoderma reesei*** | | |
|---|---|---|
| **Authority** | **Food Enzyme** | **Reference** |
| **JECFA** | Cellulase<br>Beta-glucanase<br>Glucoamylase | FAS 30-JECFA 39/15 and FAS 22-JECFA 31/31<br>FAS 22-JECFA 31/25, JECFA monograph gluco amylase |
| **Australia/New Zealand** | Cellulase<br>Glucan 1-3 beta-glucosidase<br>Beta-glucanase<br>Hemicellulase complex<br>Gluco-amylase<br>Endo 1,4-beta- xylanase<br>Pectinases | Australia New Zealand Food Standards Code – Schedule 18 – Processing aids (legislation.gov.au) |
| **Canada** | Cellulase<br>Glucanase<br>Pentosanase<br>Xylanase<br>Protease<br>Pectinase | 5. List of Permitted Food Enzymes (Lists of Permitted Food Additives) |
| **USA**[24] | Pectin lyase<br>Transglucosidase (GM)<br>Glucoamylase<br>Phospholipase A<br>Polygalacturonase<br>Pectin esterase<br>Mannanase | GRAS Notice Inventory, GRN 32<br>GRAS Notice Inventory, GRN 315<br>GRAS Notice Inventory, GRN 372<br>GRAS Notice Inventory, GRN 524<br>GRAS Notice Inventory, GRN 557<br>GRAS Notice Inventory, GRN 558<br>GRAS Notice Inventory, GRN 566 |

[24] GRAS affirmations and GRAS notifications

| | | |
|---|---|---|
| | Endo-1,4-beta-xylanase | GRAS Notice Inventory, GRN 628 |
| | Lipase | GRAS Notice Inventory, GRN 631 |
| | Lysophospholipase | GRAS Notice Inventory, GRN 653 |
| | Glucose oxidase | GRAS Notice Inventory, GRN 707 |
| | Serine endopeptidase | GRAS Notice Inventory, GRN 817 |
| **France** | Alpha-amylase (GM)<br>Amyloglucosidase (GM)<br>Beta-glucanase (GM)<br>Xylanase<br>Cellulase<br>Lysophospholipase (GM) | Arrêté du 19 octobre 2006 |

### 6.1.2 Safety of the genetic modification

The recipient strain used for the genetic modification is a *Trichoderma reesei* mutant strain, which derives from a wild-type *T. reesei* QM6a after several mutagenesis and genetic engineering steps. This mutant strain has been shown to be genetically stable for industrial production. The production strain only differs from its recipient strain (AR-555) by the production of lipase from *Thermomyces lanuginosus*. As explained in section 2.2.3, the lipases from *Thermomyces lanuginosus* has an established history of safe use in the food industry. AB Enzymes limits the possibilities of mutations in the product enzyme, as well as in the production strain, through the inoculation of the seed culture for the fermentation with controlled spore stocks that have been stored at -80º C. AB Enzymes has conducted an internal risk assessment to verify the strain is stable and safe, for more information please refer to the sections discussing safety, toxicity and stability of the production strain found in this dossier.

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

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

No additional mutagenesis cycles have been performed after the AR-822 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-822 can be regarded as safe as the recipient or the parental organism to be used for production of enzymes for food processing.

**Enzyme Safety Narrative:**

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

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

- Fate of the enzyme in food

Lipases are safe, and they have a long history of use in food (Pariza M.W. and Foster E.M. 1983; Pariza and Johnson 2001). Lipases have been evaluated worldwide, apart from US, with multiple national (Canada, Denmark, France, Australia & New Zealand) and international (JECFA) bodies concluding that these food enzymes do not constitute a toxicological hazard. As indicated in sections 2.8, lipases have been used in the baking industry for many years.

Non-exhaustive list of authorized lipases from production organisms other than *Trichoderma reesei*:

| **Non exhaustive list of authorized lipases from production organisms other than *Trichoderma reesei*** | | |
|---|---|---|
| **Authority** | **Production Organism** | **Reference** |
| **Australia/NZ** | *Aspergillus niger*<br><br>*Aspergillus oryzae*<br><br>*Aspergillus oryzae*, containing the gene for Lipase, triacylglycerol isolated from *Fusarium oxysporum*<br><br>*Aspergillus oryzae*, containing the gene for Lipase, triacylglycerol isolated from *Humicola lanuginose*<br><br>*Candida rugosa* | Australia New Zealand Food Standards Code – Schedule 18 – Processing aids (legislation.gov.au) |
| **France** | Genetically modified *Aspergillus oryzae* containing a gene for a lipase from *Fusarium oxysporum*<br><br>*Candida rugosa* | Arrêté du 19 octobre 2006 |

| | | |
|---|---|---|
| | Genetically modified *Aspergillus niger* containing a gene coding a lipase from *Fusarium culmorum* | |
| **USA**[25] | *Penicillium camemberti*<br><br>*Rhizopus oryzae* produced in *Aspergillus niger* | GRAS Notice Inventory, GRN No. 908<br><br>GRAS Notice Inventory, GRN No. 783 |
| **Canada** | *Aspergillus niger var.*<br><br>*Aspergillus oryzae var.*<br><br>*Rhizopus oryzae var.*<br><br>*Rhizomucor miehei*<br><br>*Rhizopus niveus* | 5. List of Permitted Food Enzymes (Lists of Permitted Food Additives) |
| **JECFA** | *Aspergillus oryzae* | JECFA Evaluations-LIPASE FROM ASPERGILLUS ORYZAE, VAR. |

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

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

Furthermore, the allergenic potential of the enzyme is not of significant concern. As explained in the next section the allergen searches detected only one matches over 35% homology. Based on the allergenicity searches we conclude that there is an unlikely chance of the enzyme acting as an allergen.

The notified lipase is sourced from *Thermomyces lanuginosus* which is recognized to be a safe microbial source for lipases (see section 2.2.3). As the notified lipase is protein engineered a similarity analysis to other lipases was conducted. For the purpose of the similarity analysis, NCBI Identical Protein Groups (IPG) database (https://www.ncbi.nlm.nih.gov/ipg/) was searched using the word "lipase". The IPG database contains a single entry for each protein translation found in several sources at NCBI, including annotated coding regions in GenBank and RefSeq, as well as records in SwissProt and PDB. From IPG 250,243 entries were retrieved. The database was retrieved on June 20th, 2023. A protein-protein BLAST search was used to see which lipases (E-value threshold 0.05) are most similar to the notified lipase. The most similar lipases (similarity % calculated with Blosum 75, threshold 0) to the protein engineered lipase subject to this GRAS notice are from *T. lanuginosus* (~98 % and ~91 %) and *T. dupontii* (~95 %). The next similar lipase is from another thermophilic fungus *Rasamsonia emersonii* (~81 %). In section 6.1.3 we include a summary of a toxin search conducted via bioinformatics confirming the notified enzyme is not a toxin.

Lastly, the fate of the enzyme in the final food is relevant to consider regarding the safety of the enzyme. Sections 2.8 and 2.10 demonstrate that lipase does not perform any technical function in the final product. Based on detailed assessment, including the high safety factor calculated by means of an overestimation of the intake, the overall conclusion is that the use of this enzyme in the production of food is safe.

### 6.1.3 Toxicological testing

The safety of the lipase produced by the genetically modified *Trichoderma reesei* AR-822 from a toxicological perspective is supported by the historical safety of strain lineage and a bioinformatics toxin search. From the safe strain lineage perspective, toxicological studies were performed on a *Trichoderma reesei* strain (AR-852) which derives from the same recipient strain as *Trichoderma reesei* AR-822.

The following studies were performed for strain AR-852:

- Reverse Mutation Assay using Bacteria (*Salmonella typhimurium*) with Cellulase produced with *Trichoderma reesei*
- *In vitro* Mammalian Micronucleus Assay in Human Lymphocytes with Cellulase produced with *Trichoderma reesei*
- 90-Day Repeated Dose Oral Toxicity Study in Wister Rats with Cellulase produced with *Trichoderma reesei*

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

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

As mentioned above both the AR-822 and AR-852 have been developed from the same recipient strain. Expression constructs are very similar, differing by the expression cassette/enzyme gene of interest. As both production strains are free of any harmful sequences or any potential hazards, the expression cassettes are very similar and are stably integrated into the genome of the strains without any additional growth/mutagenesis cycles thereafter, differences in the genetic modification of AR-822 and AR-852 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-822 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-822.

**Safety of the Production strain (SSL):**

For more details on the safety of the *Trichoderma reesei* AR-822 production strain, we refer to the following appendices:

- **Pariza and Johnson Decision Tree** (Appendix #3)
- **JECFA Safe Progeny Strain statement** (Appendix #4)
- **Differences between tox tested AR-852 strain and AR-822 production strain** (Appendix #4)
- **Diagram on Strain Lineage** (Appendix #4)

**Toxin Search**

A prediction of toxicity of the notified lipase using bioinformatics tools was conducted to rule out the possibility of the enzyme being a toxin. A homology search was performed from the NCBI Identical Protein Groups (IPG) database using the BLAST-P. For the searches, a set of control sequences were created (using five different shuffled version of the lipase amino acid sequence). BLAST-P search using the five shuffled (control) sequences returned 25-272 hits when a query from the NCBI Identical Protein Groups (IPG) database limited with word "toxin" was done. The bit-scores ranged from 26.1 to 34.2 and e-values from 99.5 to 0.5. Based on these results, it was concluded that bit-score or below 34, and E-values larger than about 0.5 are considered random,

and do not represent proteins of possibly significant amino acid homology. Using the amino acid sequence of the notified lipase, a BLAST-P search was conducted which returned one match with Bit-score above 34 and the E-value below 0.5.

Furthermore, the match detected in the BLAST-P of the notified lipase's amino acid sequence was a benign lipase from *Vibrio* species that were sequenced as part of a toxin related project and is not considered a toxin for this analysis for the following reasons.

- While almost all of the over 200 serogroups of V. cholerae can cause disease, cholera epidemics and pandemics are attributed to serogroups O1 and O139 that harbor cholera toxin genes, encoded on a prophage. Serogroup O1 can be further divided into two biotypes, Classical and El Tor, and two serotypes, Inaba and Ogawa. Vibrio cholerae RC27 is an O1 Classical isolate from Indonesia, 1991." Comment in the database report for EEY40808.1, a lipase family protein from Vibrio cholerae RC27.
- The query coverage is also below 25% and is considered not relevant as a true hit.

Based on the results of the searches, AB Enzymes concludes the notified lipase subject to this GRAS notice does not show significant homology to any toxin sequence.

### 6.1.4 Pathogenicity and Toxigenicity

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

*Trichoderma reesei* is globally regarded as a safe microorganism:

- In the USA, *Trichoderma reesei* is not listed as a Class 2 or higher Containment Agent under the National Institute of Health (NIH, 1998) Guidelines for Recombinant DNA Molecules. Data submitted in Generally Recognized as Safe (GRAS) petitions to the Food and Drug

Administration (FDA) for numerous enzyme preparations from *T. reesei* for human and animal consumption demonstrate that the enzymes are nontoxic. The Environmental Protection Institute (EPA) completed a risk assessment on *T. reesei* in 2011 resulting in a Proposed Rule in 2012, concluding that it is appropriate to consider *T. reesei* as a recipient microorganism eligible for exemptions from full reporting requirements[26], 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**[27] as part of the 40 Code of Federal Regulations Part 725 where *Trichoderma reesei* is classified as a Tier I organism.

As a result, AR-822 can be used under the lowest containment level at large scale, GILSP, as defined by OECD (OECD 1992).

The genus *Trichoderma* contains filamentous fungi which are frequently found on decaying wood and in soil. Industrial *T. reesei* strains have a long history of safe use and several of the *Trichoderma* based products have been approved for food and feed applications[28]. *T. reesei* is listed as a "Risk Group 1" organism according to German TRBA classification (Federal Institute for Occupational Safety and Health, www.baua.de) and as "Biosafety Level 1" organism by the American Type Culture Collection (www.atcc.org). *Trichoderma reesei* strains are non-pathogenic for healthy humans and animals. The DNA based identification methods have shown that *T. reesei* is taxonomically different from the other *Trichoderma* species of the section *Longibrachiatum* (Druzhinina et al. 2005).

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

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

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

Some species belonging to *Trichoderma* genus are able to secrete various types of antibiotics in laboratory cultures. However, strains of *T. reesei* used in industrial applications are proven to be devoid of antibiotic activities (Coenen et al. 1995; Hjortkjaer et al. 1986). The absence of antibiotic activities, according to the specifications recommended by JECFA (FAO/WHO 2006), was also confirmed for AR-822. The analyzed data are presented in Appendix #1.

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

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

- Results of the toxicological studies provided in the narrative (Appendix #3);
- Safety and history of use of the production organism *Trichoderma reesei;*
- Mycotoxin testing results presented in the composition report (Appendix #1).

With the use of safe strain lineage, we have substantiated the safety of the AR-822 *Trichoderma reesei* production strain via three toxicological studies on the *Trichoderma reesei* AR-852 production strain to demonstrate non-toxigenicity of the strain lineage. The toxicological studies conducted include, a reverse mutation assay using bacteria, a Micronucleus Assay in Bone Marrow Cells of the Rat, and a 90-day repeated dose oral toxicity study in Wister rats. All three toxicological studies showed negative findings demonstrating the AR-852 production strain to be non-mutagenic, to not induce structural and/or numerical chromosomal damage, and to not cause toxigenic effects on the Wister rats tested in the 90-day oral toxicity study. For more details on the results of the toxicological studies conducted on the production strain, please refer to Appendix #3.

To add on, as mentioned in this section of the dossier, the *Trichoderma reesei* as a production organism has a long history of use for the production of industrial food enzymes. Food enzymes, including those derived from recombinant *Trichoderma reesei* strains, have been evaluated by JECFA and many countries which regulate the use of food enzymes such as France Denmark, Australia, and Canada, apart from the USA. Also, AB Enzymes has used *Trichoderma reesei* strains

for food enzyme production for many years without any safety problems. Lastly, we have demonstrated the low presence of the mycotoxins produced by the *Trichoderma reesei* microorganism. The composition report provided as an appendix to this GRAS notification demonstrates mycotoxin values below the levels of quantification (LoQs) for the enzyme concentrate batches tested.

**Conclusion**: Based on the above-mentioned available data, it is concluded that the organism *T. reesei*, has a long history of safe use in industrial-scale enzyme production and can be considered as a safe production organism for enzymes for food as well as feed processing and numerous other industrial applications. As an example, *T. reesei* strains have been cultivated in the production plant of Alko Oy/Roal Oy since 1987. During this time, genetic engineering techniques have been used to improve the industrial production strains of *Trichoderma reesei* and considerable experience on the safe use of recombinant *Trichoderma reesei* strains at industrial scale has accumulated. Thus, *Trichoderma reesei* and its derivatives can be considered generally safe not only as a production organism of its natural enzymes, but also as a safe host for heterologous gene products. In section 6.1.2 we also provided a short narrative on the safety of the lipase enzyme.

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

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

## Appendices

1. AR-822 Composition Report
2. Flow Chart of the manufacturing process with control steps
3. Summary of Toxicological Studies and Decision Tree Lipase
4. Safe Strain Lineage Narrative AR-822

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

Analytical report
12.4.2023

**Objective:** **Chemical Composition Analysis of Lipase from *Trichoderma reesei*, Strain AR-822**

**Samples:**

| Description | Batch | LIMS ID |
|---|---|---|
| **1. Liquid enzyme concentrate** | **P220022** | **1-22-02265-002** |
| **2. Liquid enzyme concentrate** | **B220096** | **1-22-01407-002** |
| **3. Liquid enzyme concentrate** | **B220095** | **1-22-01406-002** |

Table 1. Main activity

| | Batch | | |
|---|---|---|---|
| | **P220022** | **B220096** | **B220095** |
| ALU/g | 24700 | 30400 | 31500 |

ALU: Assay of lipase activity, Roal internal method B079

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

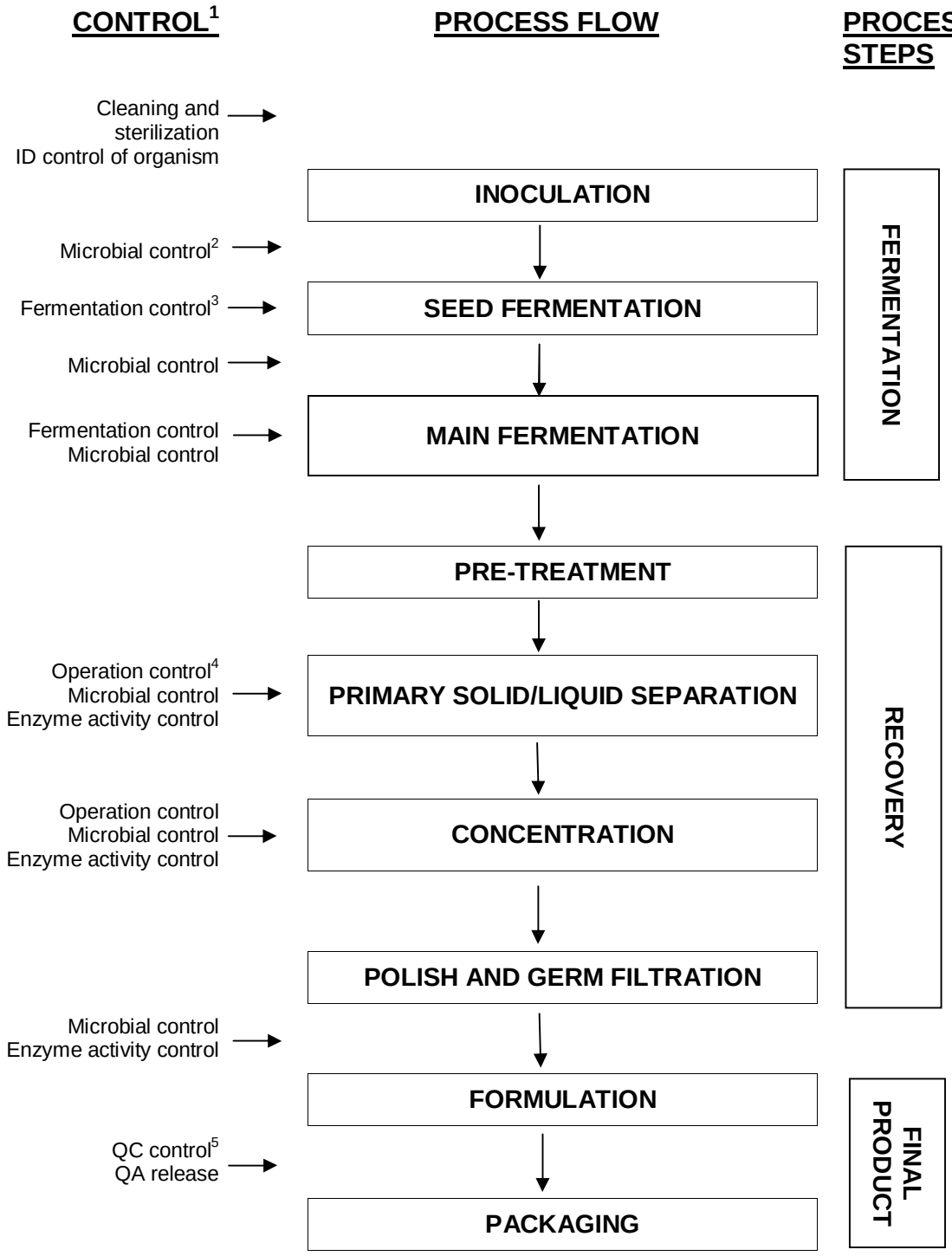
| | Batch | | |
|---|---|---|---|
| | **P220022** | **B220096** | **B220095** |
| Antimicrobial activity | Not detected | Not detected | Not detected |
| Presence of production strain | Not detected | Not detected | Not detected |
| Escherichia coli (/25 g) | Not detected | Not detected | Not detected |
| Salmonella (/25 g) | Not detected | Not detected | Not detected |
| Total coliforms (cfu*/g) | <30 | <30 | <30 |
| Lead (mg/kg) | <0,05 | <0,05 | <0,05 |
| T2-Toxin (µg/g) | <10 | <10 | <10 |
| HT-2-Toxin (µg/g) | <10 | <10 | <10 |

Antimicrobial 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)*, 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
Lead: ISO 17294-2
T2-Toxin and HT-2 toxin: Eurofins internal method, LC-MS/MS

Rajamäki 12.4.2023

Anna He
Quality Information Specialist
Roal Oy

# Production Process of Food Enzymes from Fermentation




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

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

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

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

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

**TOXICOLOGICAL STUDIES SUMMARY**

**Reverse Mutation Assay using Bacteria (*Salmonella typhimurium*)**

In order to investigate the potential of Cellulase produced with *Trichoderma reesei* for its ability to induce gene mutations the plate incorporation test (experiment I) and the pre-incubation test (experiment II) were performed with the *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and TA102.

In two independent experiments several concentrations of the test item were used. Each assay was conducted **with** and **without** metabolic activation. The concentrations, including the controls, were tested in triplicate. The following concentrations of the test item were prepared and used in the experiments:

31.6, 100, 316, 1000, 2500 and 5000 µg/plate

No precipitation of the test item was observed in any tester strain used in experiment I and II (**with** and **without** metabolic activation).

No toxic effects of the test item were noted in any of the five tester strains used up to the highest dose group evaluated (**with** and **without** metabolic activation) in experiment I and II.

No biologically relevant increases in revertant colony numbers of any of the five tester strains were observed following treatment with cellulase produced with *Trichoderma reesei* at any concentration level, neither in the presence nor absence of metabolic activation in experiment I and II.

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

***In vitro* Mammalian Micronucleus Assay in Human Lymphocytes with a cellulase produced with *Trichoderma reesei***

In order to investigate a possible potential of a cellulase produced with *Trichoderma reesei* to induce micronuclei in human lymphocytes an *in vitro* micronucleus assay was carried out. The selection of the concentrations was based on data from the pre-experiment. In the first main experiment **without** and **with** metabolic activation 2500 µg/mL and 4000 µg/mL test item, respectively, and in experiment II 200 µg/mL test item was selected as the highest concentration for microscopic evaluation.

The following concentrations were evaluated for micronuclei frequencies:
**Experiment I** with short-term exposure (4 h):
**without** metabolic activation: 250, 500 and 2500 µg/mL
**with** metabolic activation: 500, 1000, 2000 and 4000 µg/mL

**Experiment II** with long-term exposure (44 h):
**without** metabolic activation: 125, 175 and 200 µg/mL

No precipitate of the test item was noted in the cultures at the end of treatment in any concentration group evaluated in experiment I and II.

If cytotoxicity is observed the highest concentration evaluated should not exceed the limit of 55% ± 5% cytotoxicity according to the OECD Guideline 487 [4]. Higher levels of cytotoxicity may induce chromosome damage as a secondary effect of cytotoxicity. The other concentrations evaluated should exhibit intermediate and little or no toxicity. However, OECD 487 does not define the limit for discriminating between cytotoxic and non-cytotoxic effects. According to laboratory experience this limit is a value of the relative cell growth of 70% compared to the negative/solvent control which corresponds to 30% of cytostasis.

In experiment I **without** metabolic activation no increase of the cytostasis above 30% was noted up to 500 µg/mL. At 2500 µg/mL a cytostasis of 54% was observed. In experiment I **with** metabolic activation no increase of the cytostasis above 30% was noted up to 500 µg/mL. At

1000 µg/mL a cytostasis of 39%, at 2000 µg/mL a cytostasis of 49% and at 4000 µg/mL a cytostasis of 59% was observed. In experiment II **without** metabolic activation no increase of the cytostasis above 30% was noted up to 175 µg/mL. At 200 µg/mL a cytostasis of 53% was observed.

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

The nonparametric $\chi^2$ Test was performed to verify the results in both experiments. No statistically significant enhancement ($p<0.05$) of cells with micronuclei was noted in the dose groups of the test item evaluated in experiment I and II **with** and **without** metabolic activation. The $\chi^2$ Test for trend was performed to test whether there is a concentration-related increase in the micronucleated cells frequency in the experimental conditions. No statistically significant increase in the frequency of micronucleated cells under the experimental conditions of the study was observed in experiment I and II.

Methylmethanesulfonate (MMS, 25 and 65 µg/mL) and cyclophosphamide (CPA, 15 µg/mL) were used as clastogenic controls. Colchicine (Colc, 0.01 and 0.4 µg/mL) was used as aneugenic control. All induced distinct and statistically significant increases of the micronucleus frequency. This demonstrates the validity of the assay.

In conclusion, it can be stated that during the study described and under the experimental conditions reported, the test item cellulase produced with *Trichoderma reesei* did not induce structural and/or numerical chromosomal damage in human lymphocytes. Therefore, a cellulase produced with *Trichoderma reesei* is considered to be non-mutagenic with respect to clastogenicity and/or aneugenicity in the *in vitro* Mammalian Cell Micronucleus Test.

**90-Day Repeated Dose Oral Toxicity Study in Wistar Rats with a cellulase produced with *Trichoderma reesei***

The aim of this study was to assess the possible health hazards which could arise from

repeated exposure of a cellulase produced with *Trichoderma reesei* via oral administration to rats over a period of 90 days.

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

Control: 0 mg TOS/kg body weight

Low Dose: 100 mg TOS/kg body weight

Medium Dose: 300 mg TOS/kg body weight

High Dose: 1000 mg TOS/kg body weight

The test item formulation was prepared at least every 7 days. The test item was dissolved in *aqua ad injectionem* and administered daily during a 90-day treatment period to male and female animals. Dose volumes were adjusted individually based on weekly body weight measurements.

During the period of administration, the animals were observed precisely each day for signs of toxicity. At the conclusion of the test, surviving animals were sacrificed and observed macroscopically.

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

A full histopathological evaluation of the tissues was performed on high dose and control animals. Any gross lesion macroscopically identified was examined microscopically in all animals.

There were no test item-related clinical signs of systemic toxicity observed during the treatment period in any of the animals. In addition, detailed clinical examinations, functional observation battery (FOB) and ophthalmoscopy examination did not reveal any test item related effects in any of the treatment groups.

In males and females, there was no test item-related effect on body weight during the treatment period.

There was no effect of toxicological relevance on food consumption in any of the dose groups during the treatment period.

No toxicologically relevant effects on parameters of haematology, blood coagulation, clinical biochemistry, and hormone analysis were observed in test item-treated animals.

Macroscopic examination as well as organ weight parameters revealed no toxicologically relevant findings at the end of the treatment period.

No test item related alterations were reported during histopathological examination.

Under the condition of this study all animals survived their scheduled period. There were no gross lesions or histology findings that could be related to treatment with the test item.

At dose formulation analysis nominal concentrations were confirmed for all dose groups, as measured concentrations were within acceptance criterion of 15%.

On the basis of the present study, the 90-Day Repeated Dose Oral Toxicity study with a cellulase produced with *Trichoderma reesei* in male and female Wistar rats, with dose levels of 100, 300, and 1000 mg/kg body weight day the following conclusions can be made:

There was no test item-related effect observed on mortality, clinical signs, body weight development, food consumption, functional observation battery, weekly detailed clinical

observations, haematology and blood coagulation, hormone analysis, clinical biochemistry, urinalysis, gross pathological findings, organ weight and histopathology in males and females sacrificed at the end of treatment period. Therefore, the histopathological NOAEL (no observed adverse effect level) could be established at 1000 mg/kg body weight.

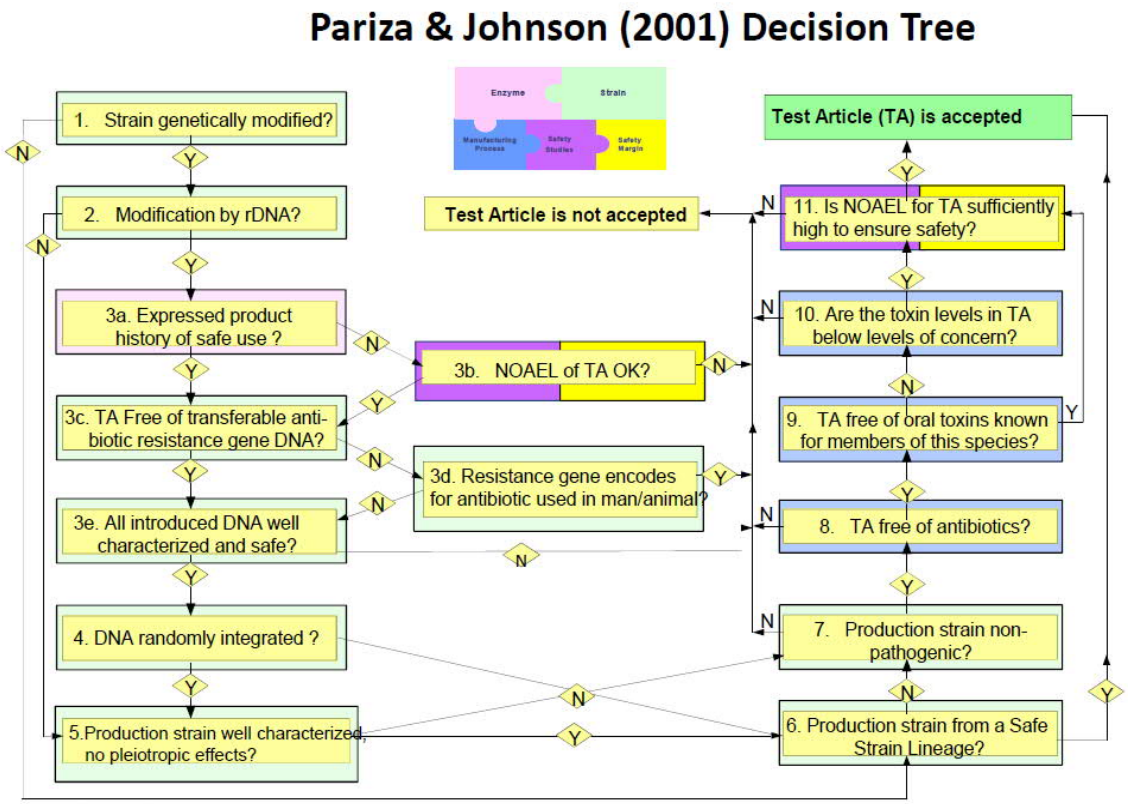
## ANALYSIS OF SAFETY BASED ON PARIZA AND JOHNSON DECISION TREE

Pariza and Johnson have published updated guidelines for the safety assessment of microbial enzyme preparations (2001)[1] from the IFBC Decision Tree[2]. The safety assessment of a given enzyme preparation is based upon an evaluation of the toxigenic potential of the production organism. The responses below follow the pathway indicated in the decision tree as outlined in Pariza and Johnson, 2001. The outcome of this inquiry is that *Trichoderma reesei* AR-822 production strain producing a lipase is "ACCEPTED" as safe for its intended use.

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

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

**Decision Tree:**




1. **Strain genetically modified? (Yes, AR-822 strain is genetically modified, see section 2.3 for genetic modification description).** Go to #2

2. **Modification by rDNA? (Yes, AR-822 strain is modified by rDNA)**. Go to #3a

3. **Does the expressed enzyme product which is encoded by the introduced DNA have a history of safe use in food[3]?**

   3a. Expressed product history of safe use: Yes, Lipase (IUBMB 3.1.1.3) has been widely and safely used in food applications for many years. The safety of the lipase is further supported by whole genome sequencing, allergenicity analysis and lack of sequence similarity with known oral toxins. The protein engineered variant is derived from a safe lineage of previously tested enzymes expressed in the same

[3] Engineered enzymes are considered not to have a history of safe use in food, unless they are derived from a safe lineage of previously tested engineered enzymes expressed in the same host using the same modification system.

host using a similar modification system (refer to section 6.1 on the safety of the production strain and section 2.8 technological purpose for evidence). Go to #3c

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

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

4. **DNA randomly integrated?** (No, targeted integration into the chromosome) Go to #6

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

Thus, AB Enzymes concludes that the decision tree shows that the *Trichoderma reesei* production strain AR-822 is **ACCEPTED.**

# Safety of Lipase from *Trichoderma reesei* AR-822

The lipase under evaluation is produced by a genetically modified strain of *Trichoderma reesei* (*T. reesei*). *T. reesei* has a long history (more than 30 years) of safe use in industrial-scale enzyme production. Food enzymes produced by *T. reesei* strains have been evaluated by the EFSA, JECFA and many countries which regulate the use of food enzymes, such as Brazil, the USA, France, Denmark, Australia and Canada, resulting in the approval of the use of food enzymes produced by *T. reesei* in the production of various foods, such as baking, brewing, juice production, wine production and the production of dairy products.

*T. reesei* is a hypercellulolytic fungus which was first found during the Second World War on deteriorating military fabrics such as tents and clothing. This original isolate, designated as QM6a, was initially named *Trichoderma viride*. Approximately 20 years later, QM6a was re-classified as *T. reesei*. In the 1980s, it was suggested that *T. reesei* should be placed into synonymy with *Trichoderma longibrachiatum*. Later however, evidence appeared that the two species were not identical, and it was decided to go back to the *T. reesei* name. It is of relevance to note that many enzymes which are produced by *T. reesei* have been approved under the name of *Trichoderma longibrachiatum*.

Taxonomic studies have shown that the strains of the species *T. reesei* used in industry originate only from this single isolate QM6a and its derivatives. The American Type Culture Collection (ATCC) designation for this original strain of *T. reesei* QM6a is ATCC 13631.

**Safe Strain Lineage:**

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

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

As of 2020, JECFA has evaluated over 80 food enzyme preparations from a variety of microorganisms and has never recorded a positive result in any toxicity study, suggesting either that toxins were not present or that toxins were present at levels that were below the limit of detection of the bioassays.

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

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

JECFA concluded provided that the genetic modification (either recombinant DNA or chemical mutagenesis) is well characterized, additional toxicological testing would not be required.

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

## Production organism *T. reesei* AR-822 (Lipase) in relation to safe *T. reesei* strain *T. reesei* AR-852 (Cellulase)

The lipase is produced by *Trichoderma reesei* AR-822 production strain. The transformation of the recipient strain AR-555 *Trichoderma reesei* with the expression cassette results in the recombinant strain AR-822 (see Table 1 below).

The production organism *Trichoderma reesei* has been genetically engineered by deleting genes from the genome and by transformation of the strain with the expression cassette to promote lipase production. All genetic modifications are well characterized and as such the safe strain lineage concept was employed as the *Trichoderma reesei* recipient strain, AR-555, was similarly used for the AR-852 strain which has been assessed in several toxicological studies as presented below in **Table 1 and Table 2**. The differences between the two strains (AR-822 and AR-852) are the inserted expression cassettes containing the enzyme genes of interest.

**Table 1: Comparison of the AR-822 and Toxicological tested strain AR-852 Expression Cassettes**

| Production Strain | Promoter[3] | Signal Sequence | Enzyme | Terminator[4] | Selection marker |
|---|---|---|---|---|---|
| AR-822 *T. reesei* | *T. reesei* promoter | *T. reesei* signal sequence | Lipase | *T. reesei* terminator | *A. nidulans amdS* |
| AR-852 *T. reesei* | *T. reesei* promoter | Native cellulase signal sequence | Cellulase | *T. reesei* terminator | *A. nidulans amdS* |

[3] Expression cassettes for AR-822 and AR-852 use the same *T. reesei* promoter
[4] Expression cassettes for AR-822 and AR-852 use different *T. reesei* terminators

**Table 2: Toxicological Test Summaries**

| Production Strain | Enzyme | Toxicology Test | Result |
|---|---|---|---|
| AR-852 *T. reesei* | Cellulase | 90-Day Repeated Dose Oral Toxicity Study in Wistar Rats | No adverse effects |
| | | Reverse Mutation Assay using Bacteria (*Salmonella typhimurium and E. coli*) | Non-mutagenic |
| | | *In vitro* Mammalian Micronucleus Assay in Human Lymphocytes | Non-clastogenic |

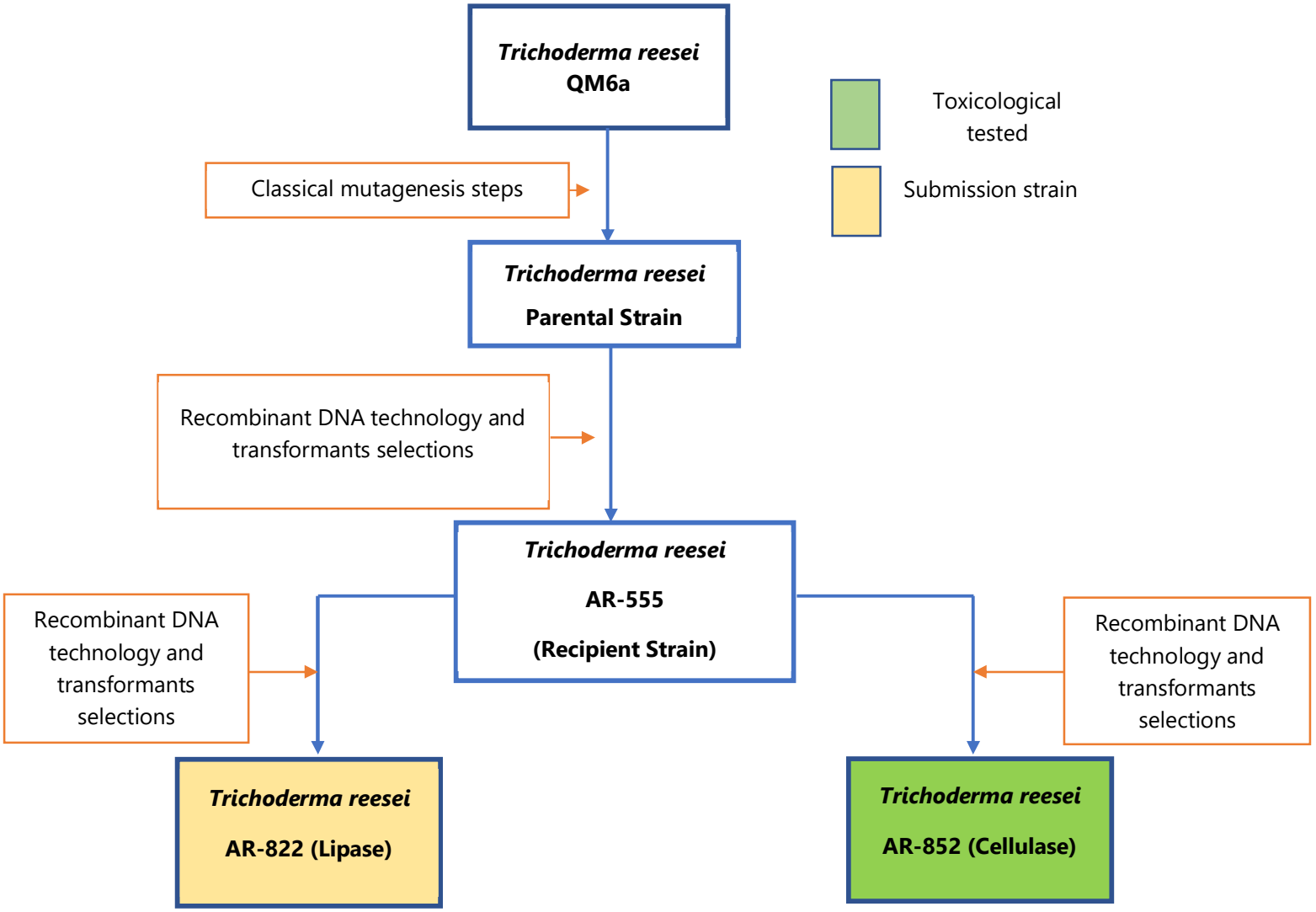



**Figure 1: *Trichoderma reesei* Safe Strain Lineage of AR-822**

**Safety Narrative:**

As described above the AR-822 and the safe strain AR-852 come from the same parental strain and recipient strain **(Fig. 1)**. Both strains are very similar. This is also true for the expression cassette, which differs by the enzyme of interest as described in Table 1.

As the production strains (including the expression cassettes) are free of any harmful sequences or any potential hazards, differences in the genetic modification of AR-822 and AR-852 are not a safety concern.

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

To add on, enzyme product from AR-822 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-822.

Based on the rationale provided above as per JECFA, 2020, as well as on the review of the strains meeting the requirements of Pariza and Johnson Decision Tree (Appendix #3 of GRAS narrative), AB Enzymes concludes lipase produced by *Trichoderma reesei* AR-822 to be safe and does not pose a significant risk to human health.

**Margin of Exposure:**

According to the Safe Strain Lineage concept, the Margin of Exposure (MoE) for human consumption can be calculated by dividing the NOAEL by the Total Theoretical Maximal Daily Intake (TMDI). In the case of the safe strain lineage concept for AR-822, there is no NOAEL. However, the NOAEL from the 90-day toxicological study from the closely related toxicological production strain *Trichoderma reesei* AR-852 is used to calculate the MoE and support the safety of the *Trichoderma reesei* AR-822.

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 |
|---|---|
| 001150 | |
| ESTIMATED DAILY INTAKE | INTENDED USE FOR INTERNET |
| NAME FOR INTERNET | |
| KEYWORDS | |

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

## SECTION A – INTRODUCTORY INFORMATION ABOUT THE SUBMISSION

1. Type of Submission *(Check one)*

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

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

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

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

## SECTION B – INFORMATION ABOUT THE NOTIFIER

**1a. Notifier**

| Name of Contact Person | Position or Title |
|---|---|
| Joab Trujillo | Regilatory Affairs Specialist |
| Organization *(if applicable)* | |
| AB Enzymes Inc. | |
| Mailing Address *(number and street)* | |
| 8211 W. Broward Blvd Suite 420 | |

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

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

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

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

| City | State or Province | Zip Code/Postal Code | Country |
|---|---|---|---|
| | | | |

| Telephone Number | Fax Number | E-Mail Address |
|---|---|---|
| | | |

## SECTION C – GENERAL ADMINISTRATIVE INFORMATION

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

Triacylglycerol Lipase from 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 631

☐ 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 lipase is to be used as a processing aid in the production of bakery and other cereal based products. The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements under current Good Manufacturing Practices. No maximal limits are set, just suggested dosages.

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

*(Check one)*

☐ Yes ☒ No

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

(Check one)

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

## SECTION E – PARTS 2 -7 OF YOUR GRAS NOTICE

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

☒ PART 2 of a GRAS notice: Identity, method of manufacture, specifications, and physical or technical effect (170.230).

☒ PART 3 of a GRAS notice: Dietary exposure (170.235).

☒ PART 4 of a GRAS notice: Self-limiting levels of use (170.240).

☒ PART 5 of a GRAS notice: Experience based on common use in foods before 1958 (170.245).

☒ PART 6 of a GRAS notice: Narrative (170.250).

☒ PART 7 of a GRAS notice: List of supporting data and information in your GRAS notice (170.255)

**Other Information**

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

☒ Yes ☐ No

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

☒ Yes ☐ No

## SECTION F – SIGNATURE AND CERTIFICATION STATEMENTS

1. The undersigned is informing FDA that AB Enzymes Inc.
*(name of notifier)*

has concluded that the intended use*(s)* of Triacylglycerol Lipase from 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 420 Plantation, Florida 33324 USA
*(address of notifier or other location)*

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

| 3. Signature of Responsible Official, Agent, or Attorney | Printed Name and Title | Date (mm/dd/yyyy) |
|---|---|---|
| Joab Trujillo Digitally signed by Joab Trujillo Date: 2023.07.10 15:45:34 -04'00' | Joab Trujillo Regulatory Affairs Specialist | 07/10/2023 |

## 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 Triacylglycerol lipase GRAS AB Enzymes 2023 | Administrative |
| | Cover Letter for Triacylglycerol lipase GRAS Notice AB Enzymes | Administrative |
| | GRAS Notice Dossier Triacylglycerol lipase AB Enzymes | Submission |
| | 1_AR-822 Composition Report | Submission |
| | 2_Flow Chart of the manufacturing process with control steps | Submission |
| | 3_Summary of Toxicological Studies and Decision Tree Lipase | Submission |
| | 4_Safe Strain Lineage Narrative AR-822 | Submission |
| | References for AB Enzymes' Triacylglycerol lipase GRAS Notice. zip | Submission |
| | | |