AB Enzymes GmbH – Feldbergstrasse 78 , D-6412 Darmstadt

# GRAS Notification of a Pectin Esterase from a Genetically Modified *Aspergillus oryzae*

AB ENZYMES GmbH

November 13, 2020

# Contents

# 1 PART 1 §170.225 – SIGNED STATEMENTS AND CERTIFICATIONS

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

AB Enzymes GmbH hereby claims that Pectin esterase (IUBMB 3.1.1.11) from a Genetically Modified *Aspergillus oryzae* produced by submerged fermentation is Generally Recognized as Safe; therefore, they are exempt from statutory premarket approval requirements.

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

AB Enzymes Inc.[1]

8211 W. Broward Blvd. Suite 375

Plantation, FL 33324 USA

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

Pectin esterase (IUBMB 3.1.1.11) from a Genetically modified *Aspergillus oryzae*

**§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 Pectin esterase enzyme is to be used as a processing aid used in fruit and vegetable processing (including plant protein processing and meat analogue processing), wine production, coffee processing and flavoring production. 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 **pectin esterase from a genetically modified *Aspergillus oryzae*.**

### 2.1.1 Common name of the enzyme

Name of the enzyme protein: Pectin esterase (PE, PME)

Synonyms: pectin methylesterase; pectase; pectin demethoxylase; pectin methoxylase; pectin methyl esterase; pectinoesterase; pectin pectylhydrolase

### 2.1.2 Classification of the enzyme

| | |
|---|---|
| IUBMB # | 3.1.1.11 |
| Production Strain | *Aspergillus oryzae* AR-962 |

EC 3. is for hydrolyases;

EC 3.1. is for acting on ester bonds;

EC 3.1.1. is for carboxylic esterase hydrolases

EC3.1.1.11 is for pectin esterase

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

| | |
|---|---|
| Kingdom: | Fungi |
| Division: | *Ascomycota* |
| Class: | *Eurotiomycetes* |
| Order: | *Eurotiales* |
| Family: | *Trichocomaceae* |
| Genus: | *Aspergillus* |
| Species: | *Aspergillus oryzae (Ahlburg) Cohn* |
| Strain: | *Aspergillus oryzae* AR-962 |

## 2.2 Identity of the Source

### 2.2.1 Recipient Strain

The recipient strain used in the genetic modification for the construction of the production strain is a genetically modified spontaneous mutant of the *Aspergillus oryzae* parental strain. *Aspergillus oryzae* parental strain from South America was deposited to the RÖHM[2] strain collection in May 1984 and its taxonomy identification was reconducted in 2018 and confirmation granted that the strain is *A. oryzae* (Ahlburg) Cohn. The parental strain was identified by the Westerdijk Fungal Biodiversity Institute in the Netherlands and deposited with the accession number CBS 146745.

Therefore, the recipient can be described as followed:

| | |
|---|---|
| Kingdom: | Fungi |
| Division: | *Ascomycota* |
| Class: | *Eurotiomycetes* |
| Order: | *Eurotiales* |
| Family: | *Trichocomaceae* |

[2] RÖHM Enzymes GmbH was the previous name of AB Enzymes GmbH

Genus: *Aspergillus*

Species: *Aspergillus oryzae*

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

### 2.2.2 Donor:

The pectin esterase gene described in this application derives from *Aspergillus tubingensis Mosseray* which is a filamentous fungus belonging to *Aspergillus* section Nigri (the black aspergilli; (Samson et al. 2006). These filamentous fungi are common in causing food spoilage and biodeterioration of other materials. *A. niger*, the species having a long history of use as an industrial enzyme production organism belongs to this same *Aspergillus* section. Previously the name *A. niger* has been used for both *A. niger* and *A. tubingensis* and only the use of molecular methods has enabled division of the *A. niger* complex into two separate species.

The taxonomic lineage of *Aspergillus tubingensis* is shown below (according to http://www.uniprot.org/taxonomy/5068):

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

## 2.3 Genetic modification

*A. oryzae* AR-962 was constructed for specific pectin esterase production. The production strain differs from its recipient strain in its high pectin esterase production capacity. In the production strain the expression cassette containing *Aspergillus tubingensis* pectin methylesterase gene was

integrated into the recipient strain's genome. Besides the high pectin esterase production, no other significant changes in phenotype were made.

*A. oryzae* AR-962 secretes high amounts of pectin esterase into its culture supernatant, resulting in high pectin esterase activity in the cultivation broth. The heterologous pectin esterase is the main component of the enzyme mix produced by AR-962. In addition of the heterologous pectin esterase strain AR-962 produces endogenous *Aspergillus* enzymes in small amounts. These activities are not relevant from an application/safety point of view, due to the small amount and the fact that such activities have been approved for decades in food processing.

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

Standard transformation techniques using protoplasts were used to integrate the expression and selection cassettes into the genome of the *Aspergillus oryzae* production strain.

The production strain was constructed from the parental strain in three modification steps. The first step was the creation of the spontaneous mutant recipient strain. The second step was the co-transformation of the recipient strain with the expression cassette of the *A. tubingensis* pectin esterase gene and the nitrate reductase selection cassette. The expression cassette containing an acetamidase gene from *Aspergillus nidulans* (Hynes et al. 1983; Kelly and Hynes 1985) as a selection marker was used for the transformation of the intermediate strain in the third step.

The plasmid vectors (pUC18 and puC8) were only used in constructing the expression cassette and selection cassette respectively but were not introduced into the recipient strain in fungal transformation.

***Expression cassette:***

- ***Aspergillus tubingensis* pectin methylesterase gene:** the pectin methylesterase gene encodes a pectin methyl esterase from *Aspergillus tubingensis*.
- ***Aspergillus oryzae* promoter:** The strong promoter from *A. oryzae* is used to overexpress pectin methylesterase gene in order to obtain high yield of pectin methyl esterase. The transcription is terminated by the native terminator from the pectin methylesterase gene.
- ***Aspergillus nidulans amdS* gene:** The gene has been isolated from *Aspergillus nidulans* VH1-TRSX6 (Kelly and Hynes 1985; Hynes et al. 1983). *Aspergillus nidulans* is closely related to *Aspergillus tubigiensis*, which is used in industrial production of food enzymes. The gene codes for an acetamidase that enables the strain to grow on acetamide as a sole nitrogen source (Kelly and Hynes 1985). This characteristic has been used for selecting transformants. The product of the *amd*S gene, acetamidase, can degrade acetamide and 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.

***Selection cassette:***

- ***Aspergillus tubingensis* nitrate reductase gene:** The gene has been isolated from *Aspergillus tubingensis* Mosseray donor strain. The gene codes for a nitrate reductase that enables the strain to grow in the presence of chlorate (Campbell et al. 1989). This characteristic has been used for selecting transformants. Nitrate reductase can degrade chlorate and is not harmful or dangerous. The nitrate reductase marker gene has been widely used as a selection marker in fungal transformations without any disadvantage for more than 30 years.

### 2.3.1 Genetic stability of the production strain

When implemented, the fermentation process always starts from identical replicas of the AR-962 (production strain) seed ampoule. Production preserves from the "Working Cell Bank" are used to start the fermentation process. A Working Cell Bank is a collection of ampoules containing a pure culture. The cell line history and the production of a Cell Bank, propagation, preservation and storage is monitored and controlled. The WCB is prepared from a selected strain. A WCB ampoule is only accepted for production runs if its quality meets the required standards. This is determined by checking identity, viability, microbial purity and productivity of the WCB ampoule. The accepted WCB ampoule is used as seed material for the inoculum.

The production starts from "Working Cell Bank" preserves. A Petri dish is inoculated from the culture collection preserve in such a way that single colonies can be selected. Altogether individual colonies are picked up from plates and inoculated into shake flasks. Care is taken to select only those colonies which present the familiar picture (same phenotype). Colonies are used for inoculating 2 rounds of shake flask cultivation. Subsequently these are combined for the inoculation of the first process bioreactor.

Testimony to the stability of the strain is given by monitoring the growth behavior and by comparable levels of pectin methyl esterase activity in number of fermentation batches performed for the AR-962 strain. The activity measurements from parallel fermentations showed that the productivity of the production strain remains similar. This clearly indicates that the strain is stable. The data of the analysis of enzyme activities from preparation from different fermentation batches of the recombinant production strain is presented in Appendix # 1.

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

*Aspergillus oryzae* AR-962 strain does not harbor any vector DNA. The expression and selection cassettes were cleaved from the plasmid vectors (pUC18 and pUC8) by restriction enzyme digestions followed by isolation of the expression cassette from agarose gel.

A Southern blot hybridization experiment using plasmid with the pUC18 vector backbone as a labeled probe and genomic DNA of the production host AR-962 was performed to confirm no vector DNA is included in the genome of AR-962. The results of the experiment were negative (no hybridization), demonstrating that the plasmid vector was completely removed to generate the linear transforming DNA fragments introduced into the *Aspergillus* 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 production strain is recovered from the fermentation broth by a widely used process that results in a cell-free enzyme concentrate. The absence of the production strain is confirmed for every production batch, using an internal Roal method. This method has been validated in-house. The sensitivity of the method is 1 cfu/20 ml in liquid and 1 cfu/0.2 gram in dried semifinals.

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

The AR-962 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 recombinant DNA

The pectin esterase enzyme preparation is produced by an aerobic submerged microbial fermentation using a genetically modified *Aspergillus oryzae* strain. All viable cells of the production strain, AR-962, are removed during the down-stream processing: the fermentation broth is filtered with pressure filters and subsequent sheet filters, concentrated with ultra-filtration, and optionally followed by sheet filtration(s).

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

### 2.3.6 Absence of Antibiotic Genes and Toxic Compounds

As 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[3] (Food and Agriculture Organization of the United Nations 2006) has been also tested from the fermentation product of the *Aspergillus oryzae* strain AR-962. The Food Chemicals Codex ("FCC", 12th edition 2020), states the following: "Although limits have not been established for mycotoxins, appropriate measures should be taken to ensure that the products do not contain such contaminants." Adherence to specifications of microbial counts is routinely analyzed. The absence of antibiotic activities, according to the specifications recommended by JECFA (Food and Agriculture Organization of the United Nations 2006), was also confirmed from three AR-962 enzyme production batches in Appendix #1 and no antibiotic or toxic compounds were detected.

[3] 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.

## 2.4 ENZYME PRODUCTION PROCESS

### 2.4.1 Overview

The food enzyme is produced by ROAL Oy[4] by submerged fermentation of *Aspergillus oryzae* AR-962 in accordance with current Good Manufacturing Practices for Food (GMP) and the principles of Hazard Analysis of Critical Control Points (HACCP). As it is run in the EU, it is also subject to the Food Hygiene Regulation (852/2004).

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

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

### 2.4.2 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

[4] See footnote 1

in the Food Chemical Codex, 12th edition, 2020 or The Council Regulation 93/315/EEC, setting the basic principles of EU legislation on contaminants and food, and Commission Regulation (EC) No 1881/2006 setting maximum limits for certain contaminants in food. The maximum use levels of antifoam are ≤0.15% respectively.

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

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

### 2.4.5 Inoculum

A suspension of a pure culture of AR-962 is aseptically transferred to shake flasks containing fermentation medium.

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

### 2.4.6 Seed fermentation

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

### 2.4.7 Main Fermentation

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

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

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

### 2.4.8 Recovery

The purpose of the recovery process is:

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

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

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

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

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

### 2.4.9 Materials

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

- Flocculants
- Filter aids

- pH adjustment agents

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

### 2.4.10 Pre-Treatment

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

### 2.4.11 Primary solid/liquid separation

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

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

### 2.4.12 Concentration

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

### 2.4.13 Polish and germ filtration

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

### 2.4.14 General Production Controls and Specifications

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

*Identity and purity of the producing microorganism:*

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

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

*Microbiological hygiene:*

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

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

- Hygienic design of equipment:

- all equipment is designed, constructed and used to prevent contamination by foreign micro-organisms

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

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

- Training of staff:
  - all the procedures are executed by trained staff according to documented procedures complying with the requirements of the quality system.
- Procedures for the control of personal hygiene
- Pest control
- Inspection and release by independent quality organization according to version-controlled specifications

- Procedures for cleaning of equipment including procedures for check of cleaning efficiency (inspections, flush water samples etc.) and master cleaning schedules for the areas where production take place
- Procedures for identification and implementation of applicable legal requirements
- Control of labelling
- Requirements to storage and transportation

*Chemical contaminants:*

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

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

These in-process controls comprise:

*Microbial controls:*

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

*Monitoring of fermentation parameters may include:*

- pH
- Temperature

- Aeration conditions

The measured values of these parameters are constantly monitored during the fermentation process. The values indicate whether sufficient biomass or enzyme protein has been developed and the fermentation process evolves according to plan.
Deviations from the pre-defined values lead to adjustment, ensuring an optimal and consistent process.

*Enzyme activity and other relevant analyses (like dry matter, refraction index or viscosity):*
This is monitored at regular intervals and at critical steps during the whole food enzyme production process.

### 2.4.15 Formulation and Packaging

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

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

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

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

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

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

## 2.5 Composition and specifications

### 2.5.1 Characteristics of the enzyme preparation

The characteristics of the enzyme preparation are:

| **Property** | **Requirement** | |
|---|---|---|
| Activity | min. | 10900 PE/g |
| Appearance | Brown liquid | |
| Density | 1.1 g/ml | |

### 2.5.2 Formulation of a typical enzyme preparation

| **Composition** | |
|---|---|
| **Constituent** | **%** |
| Pectinase concentrate | 17 |
| Glycerol | 30 |
| Sodium chloride | 10 |
| Water | Remainder |

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

The pectin esterase protein subject for this dossier consists of 314 amino acid residues with a calculated molecular mass of 34.0 kDa (or 34,000 Da).

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

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

| | |
|---|---|
| Lead: | Not more than 5 mg/kg |
| *Salmonella* sp.: | Absent in 25 g sample |
| Total coliforms: | Not more than 30 per gram |
| *Escherichia coli*: | Absent in 25 g of sample |
| Antimicrobial activity: | Not detected |
| Mycotoxins: | No significant levels[5] |

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

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

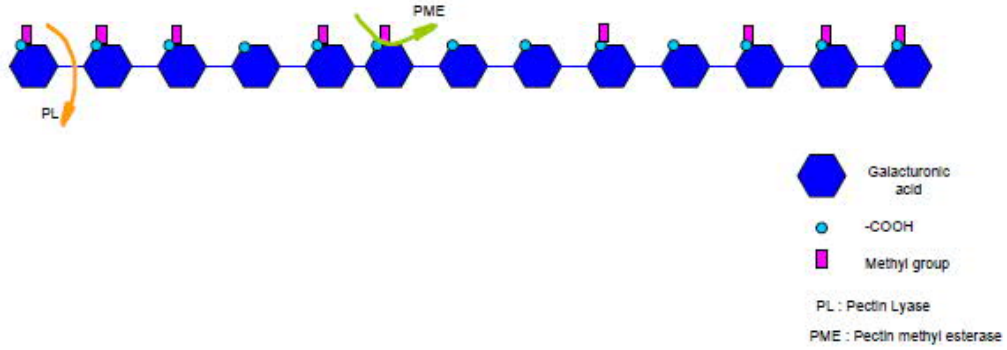
Therefore, there are no relevant side activities from an application and/or safety point of view.

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

## 2.6 Enzymatic Activity

The main activity of AR-962 enzyme preparation is pectin esterase (IUBMB 3.1.1.11). Pectin esterase is a hydrolase enzyme that catalyzes the de-esterification of methyl ester linkages of galacturonan backbone of pectic substances to release acidic pectins and methanol. Pectin is the substrate as pectin esterase acts on pectin. Pectin esterase is naturally present in plants (tomato, papaya, and grapes), plant pathogenic bacteria and fungi (Jayani et al. 2005). Pectin is a structural polysaccharide found in primary cell wall and middle lamina of fruit and vegetables. The breakdown of pectin (pectolysis) is an important process for plants, as it assists in cell elongation, growth, and fruit ripening. Microbial pectolysis is important in plant pathogenesis, symbiosis and decomposition of plant deposits. Pectic enzymes have two classes namely pectin esterases and pectin depolymerases. Pectin esterases have the ability to de-esterify pectin by the removal of methoxy residues. Pectin depolymerases readily split the main chain and have been further classified as polygalacturonases (PG) and pectin lyases (PL).

The figure below demonstrates the catalytic reaction of the de-esterification of "smooth" region-pectin converting the pectin into a partially demethylated version or pectic acid




The method to analyze the activity of the enzyme is company specific and is capable of quantifying pectin esterase activity as defined by its IUBMB classification. The enzyme activity is usually reported in PE/g. Pectin esterase activity is determined using in-house validated methods. The method is based on titration of COOH-groups which are released on pectin degradation.

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

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

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

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

Enzymes is not aware of any adverse effects from the side activities present in the pectin esterase enzyme preparation.

## 2.7 Allergenicity

There have been reports of enzymes manufactured for use in food to cause inhalation allergy in sensitive workers exposed to the enzyme dust in manufacturing facilities. In the case of pectin esterase, 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 pectin esterase residues in food seems remote. To address allergenicity by ingestion of the enzyme, the following may be considered:

- The allergenic potential of enzymes was studied by (Bindslev-Jensen et al. 2006) and reported in the publication: "*Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry*". The investigation conducted involved enzymes produced by wild-type and genetically modified strains as well as wild-type enzymes and protein engineered variants. To add on, the investigation comprised 400 patients with a diagnosed allergy to inhalation allergens, food allergens, bee or wasp. The conclusion from the study was that ingestion of food enzymes in general is not likely to be a concern regarding food allergy.
- Previously, the AMFEP Working Group on Consumer Allergy Risk from Enzyme Residues in Food performed an in-depth analysis of the allergenicity of enzyme food products (Dauvrin et al. 1998). The overall conclusion is that exposure to enzyme proteins by ingestion, as opposed to exposure by inhalation, are not potent allergens and that sensitization to ingested enzymes is rare.
- Enzymes when used as digestive (Abad et al. 2010) aids are ingested daily, over many years, at much higher amounts when compared to enzymes present in food (up to 1 million times more).

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

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

- The majority of proteins are not food allergens and based on previous experience, the enzyme industry is not aware of any enzyme proteins used in food that are homologous to known food allergens[6].
- Only a small amount of the food enzyme is used during food processing, which leads to very small amount of enzyme protein present in the final food. A high concentration generally equals a higher risk of sensitization, whereas a low level in final food equals a lower risk (Goodman et al. 2008).
- For cases where the proteins are denatured which is the case for this enzyme due to the food process conditions, the tertiary conformation of the enzyme molecule is destroyed. These types of alterations to the conformation in general, are associated with a decrease in the antigenic reactivity in humans. In the clear majority of investigated human cases, denatured proteins are much less immunogenic than the corresponding native proteins (Valenta and Kraft 2002; Valenta 2002; Takai et al. 2000; Nakazawa et al. 2005; Kikuchi et al. 2006).
- To add on, residual enzyme still present in the final food will be subjected to digestion in the gastro-intestinal system, that reduces further the risk of enzyme allergenicity. While stability to digestion is considered as a potential risk factor of allergenicity, it is believed 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)

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

are naturally present in food. This is in contrast with most known food allergens, which are naturally present in a narrow range of foods.

### 2.7.1 Allergenicity Search

To specifically evaluate the risk of the pectin esterase enzyme cross reacting with known allergens and induce a reaction, the sequence homology testing to known allergens was performed. The testing involved using an 80-amino acid (aa) sliding window search and conventional FASTA alignment (overall homology), with the threshold of 35% homology as recommended in the most recent literature (Food and Agriculture Organization of the United Nations January/2001; Ladics et al. 2007; Goodman et al. 2008).

For the results of the allergenicity search, the following allergen databases were used, "AllergenOnline" database also known as FARRP and the Structural Database of Allergenic Proteins (SDAP). AB Enzymes followed the recommendations for bioinformatics searches proposed in EFSA (2010). Two databases out of the 13 databases listed in the above publication were used in the searches, since other databases are no longer maintained; of these one has been updated this year (2020) and also contains risk assessment tools, namely AllergenOnline (FARRP). The comparison of query sequence with sequences of known allergens using the sliding 80-mer window was recommended by the FAO/WHO Expert panel in 2001 (Food and Agriculture Organization of the United Nations January/2001) and by the Codex Alimentarius Commission in 2003 (Codex Alimentarius Commission 2003) as a method to evaluate the extent of which a protein is similar in structure to known allergens. The alignments methods used in the searches are as following, alignment of the entire amino acid sequence to sequences in allergen databases and alignment of sliding 80-amino acid windows of the query protein to known protein allergens. The results of the two allergenicity searches conducted are summarized below.

Four of the five hits in the SDAP database were included in the 6 hits of FARRP database. FARRP gave the highest identity of 31.5 % to Sal k 1, and SDAP 30.9 % to Act d. The identity percentages

of all the hits were below the set 35 % identity limit. Aalberse suggested that "cross-reactivity is rare below 50% amino acid identity and, in most situations requires more than 70% identity" (Aalberse 2000), making it unlikely that the pectin esterase in question can be presumed to be allergenic based on full-length sequence relatedness to known allergens.

In the 80-mer sliding window analysis the pectin esterase protein sequence did show degrees of identity from 36.3 % to 38.8 % with pollen allergens of a common weed *Salsola kali* and olive tree *Olea europaea*. As recommendation by the FAO/WHO, a possible cross-reactivity has to be considered, when there is more than 35% identity in the amino acid sequence of the expressed protein using an 80 amino acids window and a suitable gap penalty (Food and Agriculture Organization of the United Nations January/2001). This recommendation has been however challenged. According to Ladics et al. (2007) comparing the predictive value of a full-length (conventional) FASTA search to the 80-mer analysis, "a conventional FASTA search provides more relevant identity to the query protein and better reflects the functional similarities between proteins. It is recommended that the conventional FASTA analysis be conducted to compare identities of proteins to allergens". This judgement on the predictive inferiority of the 80-mer (35% threshold) approach was supported recently by Goodman and Tetteh (2011) who suggested: "Because the purpose of the bioinformatics search is to identify matches that may require further evaluation by IgE binding, full-length sequence evaluation or an increase in the threshold from 35% identity toward 50% for the 80 amino acid alignment should be considered" (Goodman and Tetteh 2011). Using the latter recommendation, the pectin esterase in question would be below threshold even using the 80-mer sliding window.

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 pectin esterase produced by *Aspergillus oryzae* is of no concern.

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

The pectin esterase from *Aspergillus oryzae* AR-962 object of this dossier is specifically intended to be used in fruit and vegetable processing (including plant protein processing and meat analogue processing). Furthermore, pectin esterase AR-962 is intended to be used in wine production, coffee processing and flavoring production.

Pectin esterase (PE, Pectin pectylhydrolase, E.C.3.1.1.11), often referred to as pectinmethylesterase, pectase, pectin methoxylase, pectin demethoxylase and pectolipase, is a carboxylic acid esterase and belongs to the hydrolase group of enzymes. It catalyzes the deesterification of methyl ester linkages of galacturonan backbone of pectic substances to release acidic pectins and methanol (Jayani et al. 2005). The resulting pectin is then acted upon by polygalacturonases and lyases.

Like any other enzyme, pectin esterase 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.

Pears, apples, guavas, quince, plums, gooseberries, oranges and other citrus fruits, contain large amounts of pectin. The daily intake of pectin from fruits and vegetables can be estimated to be around 5 g (assuming consumption of approximately 500 g fruits and vegetables per day). Pectin is a high-molecular weight, biocompatible, non-toxic and anionic natural polysaccharide extracted from cell walls of higher plants and make up about one third of the cell wall dry substance of higher plants. The primary roles of cell walls are to give physical strength to the plant and to provide a barrier against the outside environment. The main role of pectin is to participate in these two functions together with the other polymers. The highest concentrations of pectin are found in the middle lamella of the plant cell wall, with a gradual decrease as one passes through the primary wall toward the plasma membrane (Chen et al. 2014). Pectic polysaccharides exist in the

cell wall as either "smooth" regions of a linear copolymer of α-(1–4)-linked GalUA (galacturonic acid units) or "hairy" regions that have attached α-(1–2)-linked rhamnosyl residues that may be substituted with araban and Gal-rich side chains. The pectin structure is further elaborated by divalent cation cross-linkages and possible esterification to other cell wall polymers. See figure #1 below for pectin schematic:

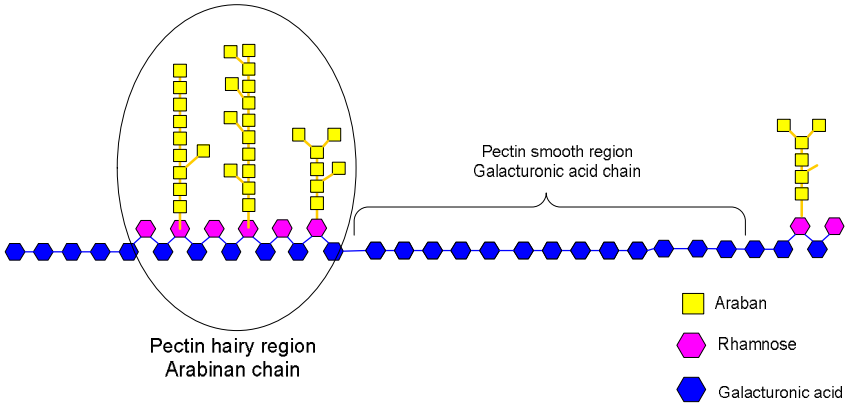



*Figure #1: Pectin schematic*

Pectin is present not only throughout primary cell walls but also in the middle lamella between plant cells, where it helps to bind cells together (Sharma *et al*. 2013). Pectin is a natural part of the human diet. The daily intake of pectin from fruits and vegetables can be estimated to be around 5 g (assuming consumption of approximately 500 g fruits and vegetables per day). Consequently, the substrate for polygalacturonase occurs naturally in vegetable based foods.

Pectin esterase is a pectinolytic enzyme (i.e. acts on pectin, which is the major component of middle lamella in plant cell walls), which has been identified in many sources, including plants, microorganisms and animals. Pectin esterase catalyzes the de-esterification of "smooth" region-pectin, i.e. to remove the methyl-group from the pectin backbone, converting the pectin into a partially demethylated version or pectic acid (also known as pectinic acid or polygalacturonic acid), as shown in the figure below:

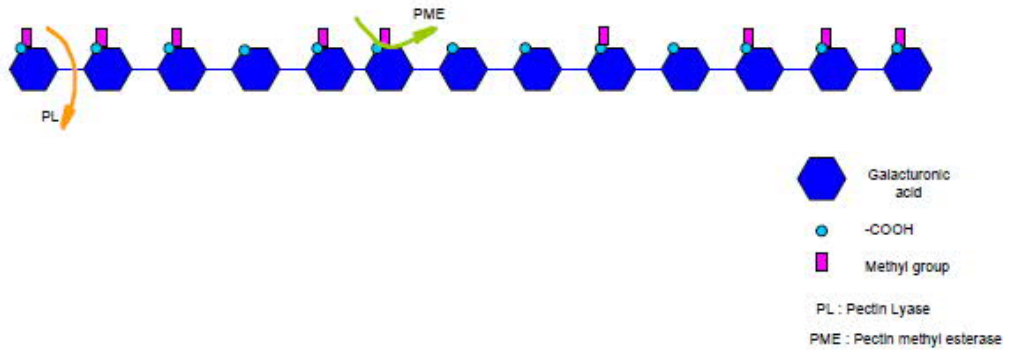



*Figure #2 Pectin esterase schematic*

The complexity of pectin sometimes hampers enzymatic degradation. As a consequence, a lot of substitutions and structural organizations require treatment with several enzymes simultaneously, and several pectin-degrading enzymes have been demonstrated to act synergistically. Since pectin esterase is specific for the "smooth region" of the pectin molecule, it does not provide complete pectin enzymatic hydrolysis and is most often used with other enzymes. Thus, to achieve complete pectin degradation, pectin (methyl) esterase is commonly combined in an enzyme preparation with pectin esterase, as it removes the methyl-group from the pectin backbone, converting the pectin to a partially demethylated version (pectinic acid) or pectic acid.

In principle, the hydrolysis of pectin with the help of pectin esterase can be of benefit in the processing of all fruits and vegetables based foods and food ingredients which naturally contain pectin.

In general, the technological need of the enzymatic conversion of pectin with the help of pectin esterase can be described as: degradation of a component (the substrate pectin) which causes technical difficulties due to its high viscosity and gelling properties in processing of raw materials containing this component.

As described above, pectin esterase is naturally present in fruit and vegetable raw materials. The natural enzymatic conversion of pectin in such materials is of technological benefit in several industrial food manufacturing processes, like fruits and vegetables processing, wine production, oil extraction, etc. However, the levels of endogenous pectin esterase are often inadequate and vary from batch to batch of raw material, and the specificity of the enzyme may not be optimal to give desired process advantages. Therefore, industrial pectin esterase is used during food processing.

**This dossier is specifically submitted for the use of polygalacturonase in fruit and vegetable processing (including plant protein processing and meat analogue processing), wine production, coffee processing and flavoring production.** Below, the benefits of the use of industrial polygalacturonase in those processes are described. The beneficial effects are of value to the food chain because they lead to better and/or more consistent product quality. Moreover, the applications lead to more effective production processes, resulting in better production economy and environmental benefits such as the use of less raw materials and the production of less waste. The use of pectinases, including polygalacturonase, has been specifically approved for a number of years, which together with the extensive use since the 1930s (Godfrey and West; Sharma et al. 2013) in a number of countries including the EU[7] and USA[8] and in the rest of the world demonstrates the technological need of such food enzymes in food processes.

**Fruit and vegetable processing:**

Enzymes are useful in the processing of fruit and vegetable juice to help break down the cell walls within the fruits and vegetables to release the liquids and sugars. Pectinases, amylases and

[7] 1 The use of pectinolytic enzymes is allowed in fruit juices processing and wine making, according to the Council Directive 2001/112/EC relating to fruit juices and certain similar products intended for human consumption and the Regulation (EC) No 606/2009 laying down certain detailed rules for implementing Council Regulation (EC) No 479/2008 as regards the categories of grapevine products, oenological practices and the applicable restrictions
[8] GRN 89: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=89

cellulases all break down different structures of the plant cell walls and effect the extraction process in various ways. Pectin esterase is a pectinolytic enzyme and will assist in degradation of pectin in the processing of juice. Raw fruit and vegetables contain a naturally varied concentration of pectin esterase, which has been shown to be involved in cell wall metabolism including cell growth, fruit ripening, abscission, senescence and pathogenesis (Jayani et al. 2005). In industrial processing of fruit and vegetables, it is technological advantageous to employ the use of exogenous pectinase to degrade plant pectin, as pectin causes technical difficulties during processing due to its high viscosity and gelling properties. When the plant tissue is crushed mechanically, the pectin will be found in the liquid phase (soluble pectin), which causes an increase in viscosity and pulp particles. Whereas, other pectin molecules will still remain bound to cellulose fibrils of side chains hemicelluloses and facilitate water retention (Kashyap et al. 2001). This causes the fruit juice to remain bound to the pulp in a jelly-like mass. With the addition of pectinases, like pectin esterase, the viscosity of the juice drops, pressability improves, the jelly structure disintegrates, and the fruit juice can be easily obtained with higher yields. In addition, when the gelling behavior of pectin needs to be changed (e.g. to maintain the original texture and appearance of fruits and vegetables for canned or frozen fruits, and jams) the pectin smooth region needs to be demethylated in order to transform the high-methylated pectin into low-methylated pectin. The flow charts are presented below as figures 3, 4, and 5.

The benefits of the depolymerization of pectin with the help of pectin esterase[9] in fruits and vegetable processing/purees are:

- Better peels removals
- Faster viscosity reduction, increased press/centrifugation capacity and filtration efficiency
- Increased concentrate ability
- Higher juice extract yield, due to efficient solubilization of pectin
- Increased cloud stability (reduced turbidity) of the clear concentrate

[9] In most industrial processing of fruit and vegetable juice, pectin esterase is combined with other enzymes in order to complete the full pectin degradation.

Please refer to figure #3 below.

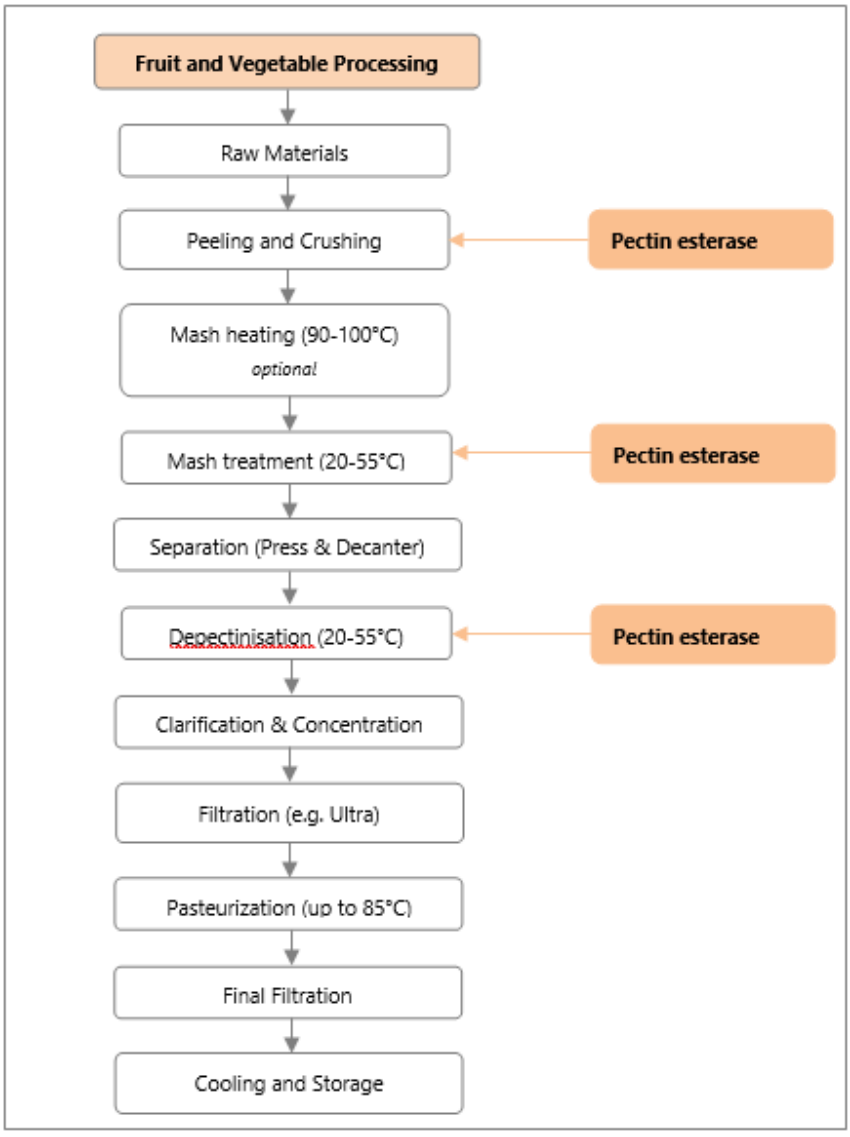



*Figure #3 Enzymatic Fruit and Vegetable Processing*

The benefits of the pectin backbone demethylation using pectin esterase (in presence of calcium[10]) to produce fruit pieces products and purees are:

- Improved product structure and texture
- Maintained fruit integrity
- Increased puree viscosity

Consequently, the enzymatic conversion of the pectin results in an improved process economy and improved product owing to the increased integrity and firmness of the fruits and vegetables during processing. See below for schematic of fruit puree and fruit firming.

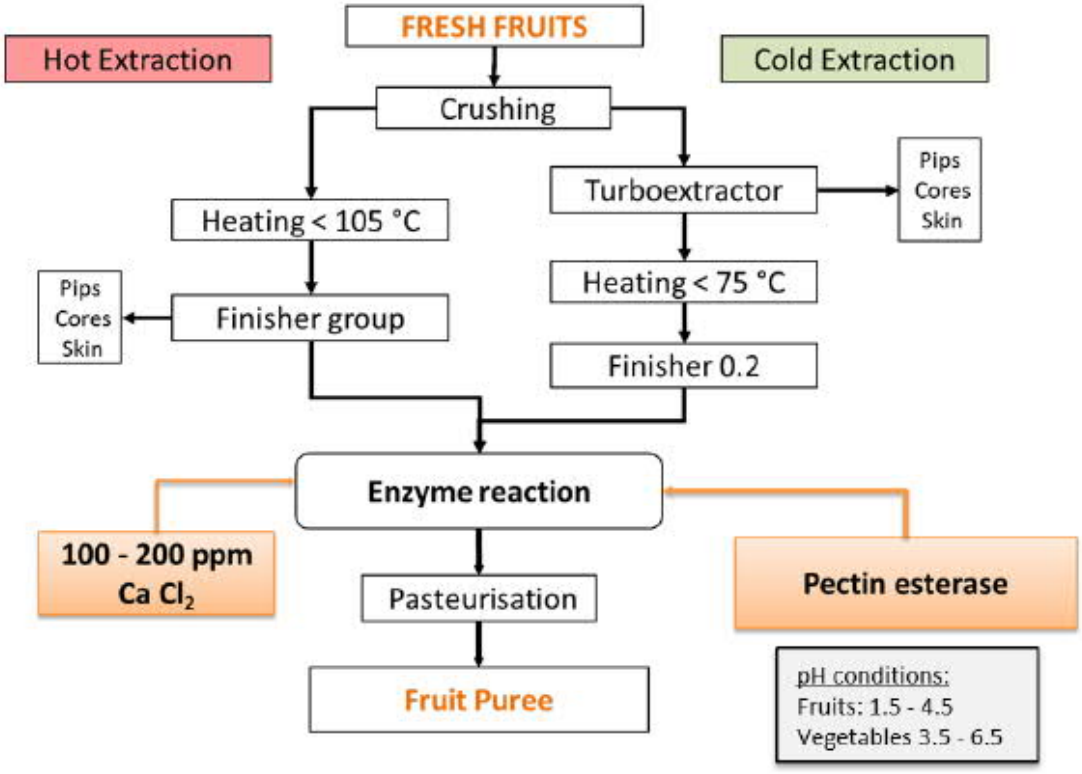



*Figure #4 Enzymatic Fruit Puree processing*

[10] The calcium ions form intermolecular ionic bonds with the Low Methyated pectin leading to a strong gel structure. The pectin is then trapped into a tri-dimensional network.

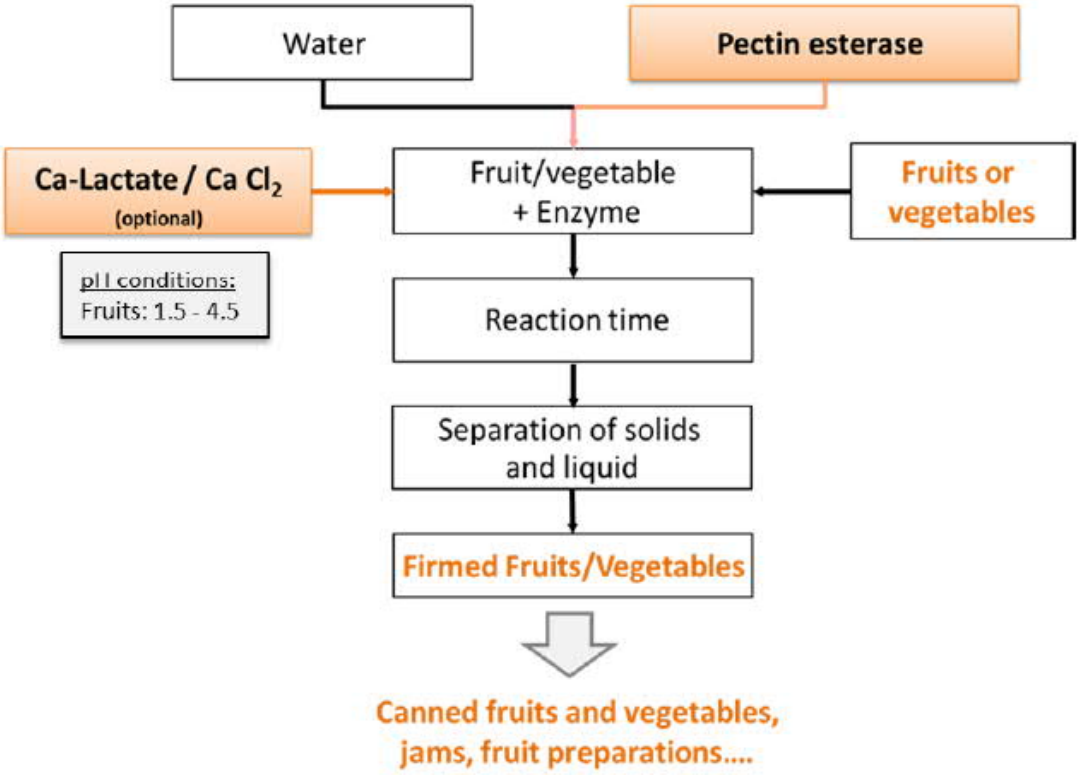



*Figure #5 Enzymatic Fruit firming*

**Meat analogue and Plant Protein Processing:**

As mentioned under Fruit and Vegetable processing, the role of pectin esterase is in the breakdown of the elements of the cell wall. The function of pectin esterase in fruit firming applications is very similar to the enzyme's use in meat analogue and plant protein processing. The removal of pectin is a necessary process in the development of meat analogue and or plant protein food products as much of the ingredients are vegetable derived. Pectin esterase acts on its substrate, pectin leading to the removal of pectin from the cell wall through the process of degradation. Pectin esterase aids in the removal of the pectin from plant protein in order to initiate the process of protein hydrolysis conducted by protease enzymes.

Texture, an important quality attribute of fresh and processed fruits and vegetables, is closely related with the structural integrity of the primary cell walls and middle lamella, which are mainly

composed of pectic substances. In industry, most of the processes such as blanching, freezing, dehydration, pasteurization and sterilization applied to preserve fruits and vegetables cause irreversible physical damages on cellular tissues. However, the negative effects of processing on texture can be overcome by applying different processes, which involve the use of pectin esterase enzyme. The mechanism of firming in this method is based on the demethylation of naturally occurring pectin in plant tissues by the action of PME and the chelation of the added or natural calcium with the free carboxyl groups generated in pectin molecules. Chelation would cause an event of networks forming among pectin molecules, which results in pectin stabilization plus increase in the firmness of plant tissues (Kohli et al., 2015).

The example provided by (Kashyap et al. 2001) on fruit and vegetable processing with pectin esterase can be applied to meat analogue and plant protein processing. When the plant tissue is crushed mechanically, the pectin will be found in the liquid phase (soluble pectin), which causes an increase in viscosity and pulp particles. Whereas, other pectin molecules will still remain bound to cellulose fibrils of side chains hemicelluloses and facilitate water retention (Kashyap et al. 2001). Furthermore, a jelly like mass in the pulp is created which disintegrates with the addition of pectin esterase. Some examples of meat analogue products where the use of pectin esterase is applicable are soy protein, tofu, chickpea protein and pea protein.

**Wine Production:**

Enzymes are used at various stages of winemaking, depending on the variety of grape and processing technology. Enzyme preparations may be used to facilitate wine clarification, decoloration, dealcoholization, enhance flavor development, or augment anthocyanin liberation. Pectinases have been used since the 1960's in wine production (Kashyap et al. 2001) and FDA had no objection to their use in foods in GRAS GRN#000089. Pectinases preparations may be added before or after pressing to improve quality, juice clarity and filterability. See figure #6 below.

Grapes have high pectin content (5-10 g $1^{-1}$) and are difficult to crush and press. They are de-stemmed, crushed, and heated to 60°C or 80°C to release color (red grapes) from the skins and to destroy endogenous polyphenoloxidase (Kashyap et al. 2001). Pectin esterase together with other pectinases (polygalacturonase), cellulases, and hemicellulases are used to reduce haze or gelling of the grape juice at any one of three stages in the process. At the first stage, when the grapes are crushed; at the second stage, which involves the must (free-run juice) before its fermentation or after; and/or at the final stage, once the fermentation is complete, when the wine is ready for transfer or bottling (Kashyap et al. 2001).

The advantages of the addition of pectinases during winemaking are:

- First stage: increases volume of free-run juice and reduces pressing time
- Second stage (before or during fermentation): settles out suspended particles and other undesirable microorganisms.
- Final stage: increase filtration rate and clarity
- Release of anthocyanins into the juice
- Better extraction yield and quality
- Increased amount of free run must
- Easier pressing

When added to the macerated grapes before the addition of wine yeast in the process of producing red wines, pectin esterase (in combination with polygalacturonase) improves visual characteristics (color stability and turbidity) as compared to untreated wines.

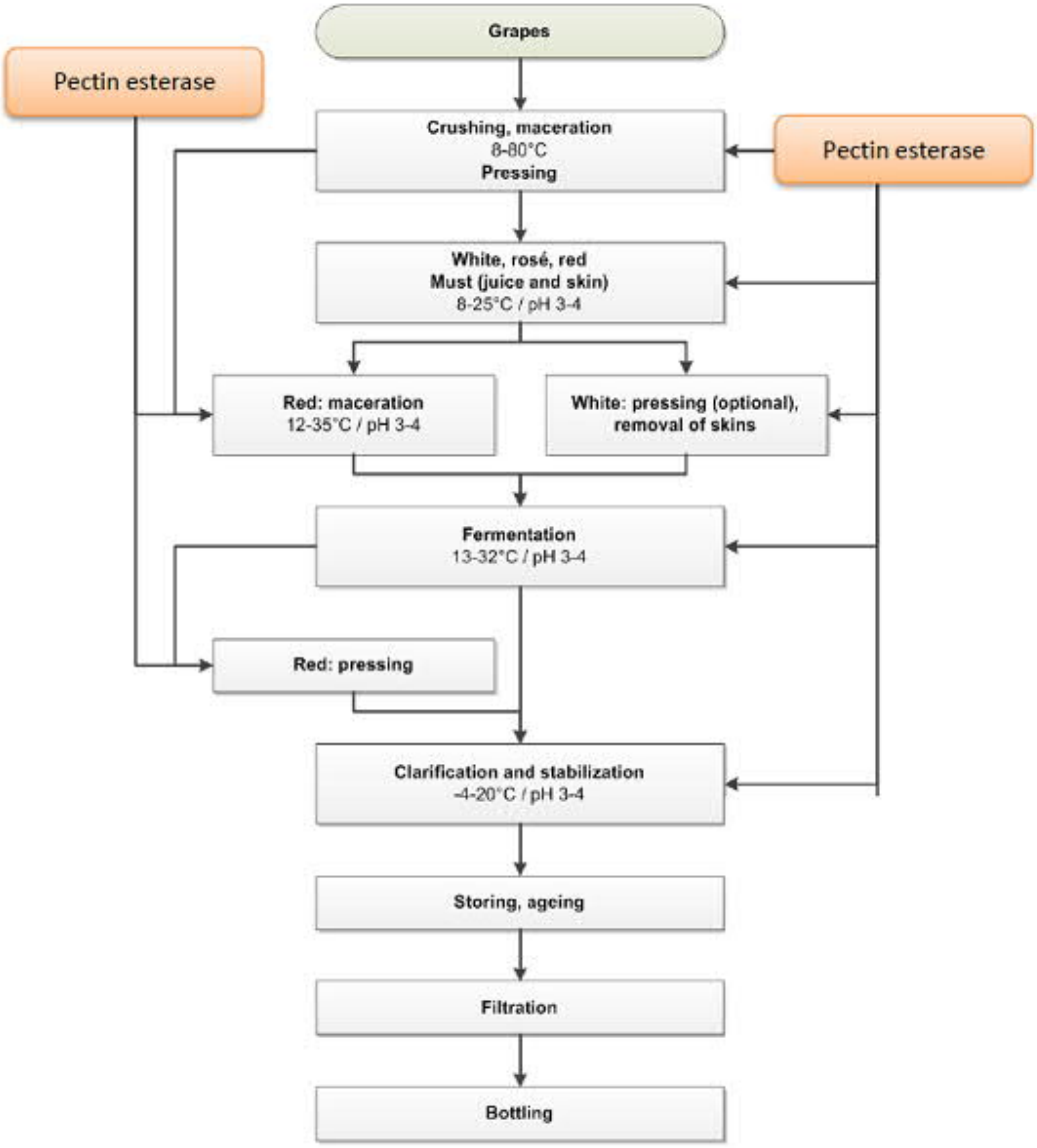
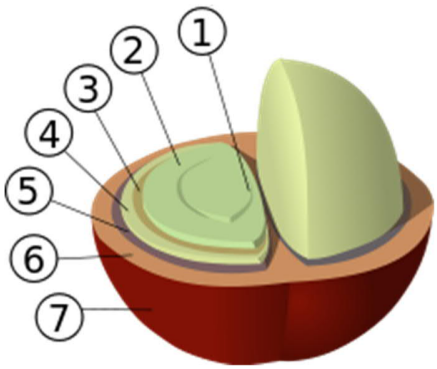



*Figure #6: Enzymatic Wine Processing*

## Coffee Processing

A Coffee bean is a seed of the coffee plant, and the pit inside the red/purple fruit is commonly referred to as a cherry. During green coffee production from harvested coffee cherries, the fruit

covering the coffee beans need to be removed before the coffee beans can be dried. The following diagram details the structure of coffee berries.




*Structure of coffee berry and beans: 1: center cut 2: bean (endosperm) 3: silver skin (testa, epidermis), 4: parchment (hull, endocarp) 5: pectin layer 6: pulp (mesocarp) 7: outer skin (pericarp, exocarp)*

There are two methods for processing coffee cherries – the wet and dry methods. During the wet method the flesh and some of the pulp of the berries is separated from the seed by pressing the fruit mechanically in water through a screen. At that stage, the bean will still have a significant amount of the pulp clinging to it that needs to be removed. Pectins are the major structural polysaccharide of the mesocarp (commonly called mucilage) of the coffee cherries. This mucilage is removed by microbial fermentation (therefore also called demucilation step). When the fermentation is complete, the coffee is thoroughly washed with clean water in tanks or in special washing machines and the beans are dried in the sun or by machine.

Pectin esterase (often together with other pectinases, such as pectin lyase and/or polygalacturonase) is added during the first steps of the coffee processing – mainly during fermentation/demucilation step - (see process flow below) which helps to:

- Improve demucilation of the pulp coffee cherries in a faster, consistent and complete way.

- Improve the green coffee characteristics and provide consistent quality: shorter fermentation and drying times reduce bean defection, formation of acids and negative aroma components. It is also reported that after storage of the green coffee beans the enzyme treated batches has a better quality with less "old" flavor.
- Improve environmental impact and sustainability of the entire milling process: No water is added during fermentation and less washing during post fermentation. Simultaneously less polluted wastewater is achieved.
    - No water is added during fermentation and less washing during post fermentation. Simultaneously less polluted wastewater is achieved. Furthermore, the processing of the wastewater is much easier, as the polysaccharides causing the problems in wastewater processing are degraded and the separation of clean water and waste sediments are easier to manage
    - As the complete removal of the mucilage layer reduces the drying time, a significant saving of energy is achieved.

The process flow is presented on the next page in figure #8:

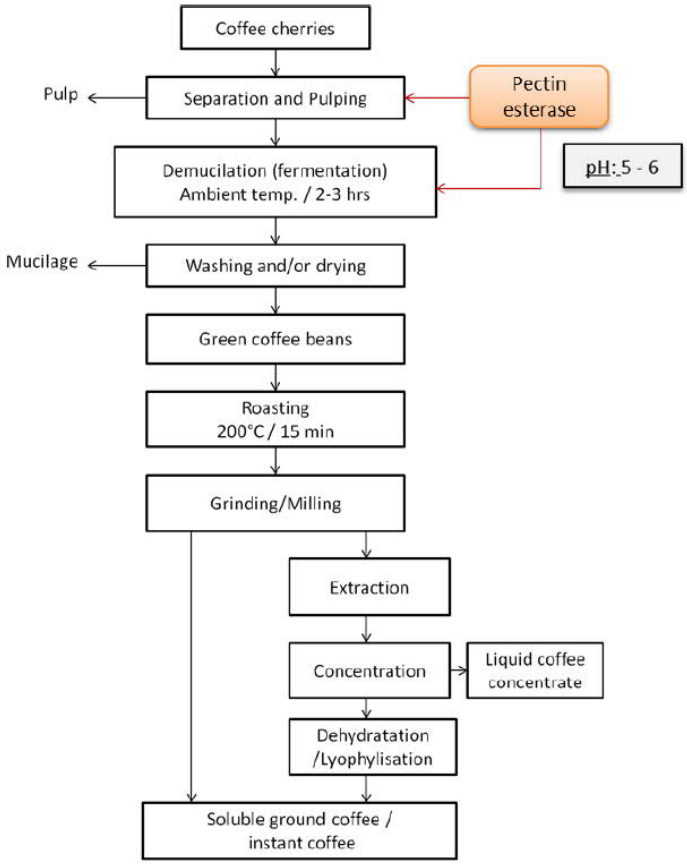



*Figure #8 Coffee Production Flow*

**Flavoring Production**

Pectin esterase may be used in the production of flavoring substances and/or preparations. Flavoring substances and preparations are used as ingredient in a wide variety of final foods (including soups, sauces, bouillons, dressings, condiments, processed foods, snack foods, meat-derived foods, breads/crackers, etc.).

Recent studies have shown that enzymatic pre-treatment for the extraction of flavor components from various plant materials have shown enhancement in aroma recovery. Enzymes such as cellulases, hemicellulases, and pectinases, and a combination of these have been used for the pre-treatment of plant materials (Sowbhagya and Chitra 2010).

## 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 endoglucanase 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 pectin esterase from *Aspergillus oryzae* may be used:

| Food Application | | Raw material (RM) | Suggested recommended use levels (mg TOS/kg RM) |
|---|---|---|---|
| **Coffee processing** | | Coffee cherries | 0.26 |
| **Flavoring production** | | Fruits/Vegetables | 2 |
| **Fruit and vegetable processing** | Fruit juices | Fruits/Vegetables | 2 |
| | Fruit purees | Fruits/Vegetables | 13 |
| | Fruit firming / meat analogue | Fruits/Vegetables | 26 |
| **Wine production** | | Grapes | 0.52 |

## 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.

Pectin esterase 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 polygalacturonase 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 pectin esterase does not exert any (unintentional) enzymatic activity in the final food are:

Pectin esterase (fruit-own and microbial) are inactivated or removed during processing of fruit and vegetable juices. Fruits and vegetables products are mostly all pasteurized.
Inactivation conditions in pasteurized products:

- Fruit-own pectin esterase: >80°C / >2min
- Pectin esterase: >75°C / >2min

Removal of pectin esterase in non-pasteurized products:

- Precipitation by bentonite (which is added prior to filtration to absorb and therefore remove proteins for wine stabilization)
- Removal by filtration processes (removal of proteins in general); inactivation by some natural wine ingredients like alcohol, polyphenols, metals, sulfur in form of $SO_2$ (forming the so called tannin-protein cloudiness), etc.

In wine production, the pectin esterase is added during maceration (essentially for red wines), fermentation, and/or before clarification- filtration steps. At the end of the wine production, one or more of the following unit operations may be used which will lead to removal or denaturation of the enzyme protein:

- White and rosés wines need to be stabilized by removing specifically thermo-labile proteins. Therefore, bentonite is added prior filtration (and bottling), leading to adsorption and therefore removal of proteins in general, including the enzyme proteins;
- Wine is filtrated on membranes that remove proteins in general (the cut-off of the membrane – usually 20 kDa- is smaller than the molecular size of enzyme proteins);

- In certain rare cases, wines may be even be heat treated, leading to denaturation of the enzyme protein;
- Natural wine ingredients such as alcohol, polyphenols, metals, sulfur in form of SO2, inhibit and naturally precipitate the enzyme (forming the so-called tannin-protein cloudiness).

Due to the above-mentioned reasons, it can be concluded that the enzyme has no technological function in the wine anymore.

During fruit firming and puree production, because calcium is added, the formation of calcium pectate depletes the substrate rendering the enzyme non-functional. In addition, it should be noted that the polygalacturonic acid backbone is completed demethylated in the process, therefore there is indeed no substrate available for further enzyme action. The depletion of the substrate also occurs in meat analogue and plant protein processing.

With respect to green coffee beans, they are typically roasted at 240–275 °C for a period of time ranging from 3 to 30 minutes. From this, it can be concluded that the enzyme will be denatured and has no technological function in the final coffee anymore.

In terms of flavoring production, the enzyme protein is denatured during a heating step (at temperatures at around 100°C) in the final food manufacturing. The denatured protein is subsequently separated from the targeted final food (i.e. flavoring substance) during a purification step (distillation for example).

Due to the above-mentioned reasons, it can be concluded that pectin esterase enzyme from *Aspergillus oryzae* AR-962 has no technological function in the final food products.

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

| Applications | | Raw material (RM) | Suggested recommended use level (mg TOS/kg RM) | Final food (FF) | Ratio RM/FF* | Suggested level in final food (mg TOS/kg food) |
|---|---|---|---|---|---|---|
| Liquid foods | Fruit and vegetable processing | Fruit/vegetable | 2 | Juices | 1.3 | 2.6 |
| | Coffee processing | Coffee cherries | 0.26 | Coffee | 0.4 | 0.1 |
| | Flavoring production | Various | 2 | Various beverages | 0.01 | 0.02 |
| | Wine production | Grape | 0.52 | Wine | 1.6 | 0.83 |
| Solid foods | Fruit and vegetable processing | Fruit/vegetable | 26 | Processed fruits (like canned fruits, jams,) + meat analogue applications | 1 | 26 |
| | | Fruit/vegetable | 13 | Purees | 1 | 13 |

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

- *For fruit juices, we assume that a RM/FF ratio of 1.3 kg fruit per L of fruit juice will be used (typically 0.75-0.9 l juice is produced per kg of fruit thus the range for RM/FF will be 1.1-1.3 kg fruit per L of fruit juice).*
- *Flavorings are generally used in small amounts in final foods. Depending on the composition of the flavoring and the final food application, the typical use levels / dosages range from 0.1 to 1%. Therefore, the corresponding RM/FF ratio is 0.01 kg flavoring per kg of final food.*
- *For fruit purees, we assume a RR/FF of 1 (1 kg of fruits / kg of puree).*
- *For coffee processing, we assume that a RM/FF of 0.4 will be used (1kg de-pulped coffee cherries lead to 330 g green coffee (ratio: 3) and 1kg green coffee leads to the production of 380 g ground coffee (ratio 2.6), typically 50g ground coffee makes 1 L coffee beverage (ratio 0.05)).*
- *For wine production, we assume that a RM/FF ratio of 1.60 kg grapes per litre of wine will be used (corresponding to a yield of 100 L of wine per 160 kg of grapes).*

The Total 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) | TMDI in beverage<br><br>(mg TOS/kg body weight/day) | Total TMDI<br><br>(mg TOS/kg body weight/day) |
|---|---|---|
| 26 x 0.125 = 0.325 | 2.6 x 0.025 = 0.065 | **0.390** |

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

- It is assumed that ALL producers of the above-mentioned foodstuffs use the specific pectin esterase enzyme from *Aspergillus oryzae* AR-962;
- It is assumed that ALL producers apply the HIGHEST use level per application;
- For the calculation of the TMDI's in food and in beverages, only THOSE foodstuffs and beverages were selected containing the highest theoretical amount of TOS.
- Thus, foodstuffs and beverages containing lower theoretical amounts were not taken into account;
- It is assumed that the amount of TOS does not decrease because of the food production process;
- It is assumed that the final food containing the calculated theoretical amount of TOS is consumed DAILY over the course of a lifetime;
- Assumptions regarding food and beverage intake of the general population are overestimates of the actual average levels (Douglass et al. 1997).

The margin of Safety (MoS) for human consumption can be calculated through the division of the NOAEL (no-observed adverse effect) value by the TMDI (Total Theoretical Maximal Daily Intake). Total TMDI of the food enzyme 0.390 mg TOS/kg body weight/day.

As a result, the MoS is:

MoS= 1,000/0.390 = **2564**

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 Safety in practice will be some magnitudes higher. Consequently, there are no safety reasons for laying down maximum levels of use.

**Conclusion:**

The overall conclusion is that the use of the food enzyme pectin esterase from *Aspergillus oryzae* AR-962 in the production of food is safe. Considering the high safety factor-even when calculated by means of an overestimation of the intake via the Budget method- there is no need to restrict the use of the enzyme in food processing. The suggested dosage for food manufacturers is not a restrictive value and could be higher or lower depending on usage within cGMPs.

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

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

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

This part is not applicable to this notified substance.

# 6 Part 6 § 170.250- GRAS Notice- Narrative

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

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

## 6.1 Safety of the Production Strain

Production strain

*Aspergillus oryzae* is not present on the list of pathogens in the EU (Council Directive 2000/54/EC). This means that it is unlikely to cause human disease.

The safety of *Aspergillus oryzae* as an enzyme producer has been reviewed by Barbesgaard et al. 1992. *A. oryzae* is regarded as a safe organism for production of industrial enzymes.

*Aspergillus oryzae* has a long history of use in the food industry. It has been used in Chinese and other East Asian cuisines to ferment soybeans for making soy sauce and fermented bean paste, and also to saccharify rice, other grains, and potatoes in the making of alcoholic beverages such as *huangjiu*, *sake*, *makgeolli*, and *shōchū*. *A. oryzae* is also used for the production of rice vinegars.

Food enzymes, including those derived from recombinant *Aspergillus oryzae* 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

*Aspergillus oryzae* in the production of various foods, such as baking, brewing, juice production, wine production and the production of dairy products.

At AB Enzymes, *Aspergillus* strains have been used as enzyme producer for many years without any safety problems. The first genetically modified *Aspergillus* production strain developed by AB Enzymes (formerly Röhm GmbH) was made by transforming an *A. foetidus* lysophospholipase gene into *Aspergillus* strains such as *A. foetidus* strain RH3046 and *A. sojae* strain RH3782 (Löffler et al. 1999).

Pectinases[11] from various micro-organisms (including genetically modified ones) are widely accepted and *A. oryzae* – whether or not genetically modified - is widely accepted as safe production organism for a broad range of enzymes. Similar food enzymes and/or food enzymes from similar production organisms have been evaluated by EFSA and internationally, food enzymes similar to the one described in this dossier have already been evaluated. A non-exhaustive list of authorized food enzymes produced by the same production organisms as well as a non-exhaustive list of pectinases is provided below.

Non-exhaustive list of authorized food enzymes (other than pectin esterase) used *Aspergillus oryzae*:

| Authority | Food enzyme | Reference |
|---|---|---|
| **JECFA** | Alpha amylase, lipase, laccase, phospholipase A1, glucoamylase, proteinase, aspergillopepsin I, aspergillopepsin II | WHO Food Additives Series 22 , WHO Food Additives Series 52 , WHO Food Additives Series 56 , TRS 759-JECFA 31/17 , NMRS 54/TRS 557-JECFA 18/20 , TRS 759-JECFA 31/17 |

[11] The name "pectinase" covers several pectinolytic enzymes (enzymes acting on pectin), mostly pectin lyase – IUBMB 4.2.2.10 -, polygalacturonase – IUBMB 3.2.1.15 - and polygalacturonase - IUBMB 3.1.1.11). Those names may be used alternatively in the current positive listings of authorized food enzymes.

| | | |
|---|---|---|
| **Australia/NZ** | Aminopeptidase, alpha amylase, asparaginase, aspergillopepsin I, xylanase, beta galactosidase, beta glucanase, glucoamylase, glucose oxidase, alpha glucosidase, lipase, metalloproteinase, mucorpepsin, pectin esterase, phospholipase A1, serine proteinase | Schedule 18 Processing Aids |
| **Canada** | Amylase, asparaginase, glucoamylase, glucoase oxidase, lactase, lipase, phospholipase, protease | List of Permitted Food Enzymes Health Canada |
| **France** | Alpha amylase, Aminopeptidase, Amyloglucosidase, beta galactosidase, asparaginase, glucose oxidase, lactase, lipase, polygalacturonase, phospholipase A1, protease, xylanase | Arrete 2006 Enzymes |
| **USA**[12] | lipase, carbohydrase and protease, glucose oxidase, laccase, asparaginase, phospholipase A1 | GRAS Notice Inventory No. 43 & GRAS Notice Inventory No. 75 , GRAS Notice Inventory No. 90 , GRAS Notice Inventory No. 106 , GRAS Notice Inventory No. 122 , GRAS Notice Inventory No. 201 , GRAS Notice Inventory No. 811 |

[12] GRAS affirmations and GRAS notifications

| Non-exhaustive list of authorized pectinases from production organisms other than *Aspergillus oryzae* | | |
|---|---|---|
| **Authority** | **Production Organism** | **Reference** |
| **Australia/NZ** | *Aspergillus niger* (pectin lyase, polygalacturonase, pectin esterase), pectin esterase from *A. aculeatus* expressed in *A. niger*, pectinase / polygalacturonase from *Trichoderma reesei* | Schedule 18 Processing Aids |
| **France** | *Aspergillus niger* (pectinase, pectinmethylesterase, pectinmetylesterase from *A. aculeatus* in *A. niger*, or from self-cloned *A. niger*, polygalacturonase from *A. niger*), *Aspergillus wentii* (pectinase), *Trichoderma reesei* (pectin methylesterase and polygalacturonase from *A. tubingensis* in *Trichoderma reesei*) | Arrêté du 19 octobre 2006 |
| **USA**[13] | *Aspergillus niger, Trichoderma reesei* | GRAS Notice Inventory No. 89, GRAS Notice Inventory No. 557 & GRAS Notice Inventory No. 558 |

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

| | | |
|---|---|---|
| | | |
| **Canada** | *Aspergillus niger, Bacillus amyloliquefaciens, Bacillus subtilis, Trichoderma reesei* | List of Permitted Food Enzymes Health Canada |
| **JECFA** | *Aspergillus alliaceus*<br><br>*Aspergillus niger* | FAS 22-JECFA 31 and TRS 789-JECFA 35/18.pdf<br><br>FAS 22-JECFA 31/21 and JECFA Monograph 305 |

### 6.1.1 Pathogenicity and Toxigenicity

*Aspergillus oryzae* strains are non-pathogenic for healthy humans and animals. As mentioned above, *Aspergillus oryzae* 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:

*Aspergillus oryzae* is globally regarded as a safe microorganism:

- In the USA, has exempted *Aspergillus oryzae* from review by the Agency, due to its extensive history of safe use (EPA 1997);
- In Europe, *Aspergillus oryzae* is classified as a low-risk-class microorganism, as exemplified in the listing as Risk Group 1 in the microorganism classification lists of the German Federal Institute for Occupational Safety and Health (BAuA) (BauA, 2002) and the Federal Office of Consumer Protection and Food Safety (BVL) (BVL, 2010). It is not mentioned on the list of pathogens in Belgium (Belgian Biosafety Server, 2010[14]).

[14] https://www.biosafety.be/content/contained-use-definitions-classes-biological-risk

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

**Secondary Metabolites:**

Metabolites of human toxicological concern are usually produced by microorganisms for their own protection. Microbes in natural environments are affected by several and highly variable abiotic (e.g. availability of nutrients, temperature and moisture) and biotic factors (e.g. competitors and predators). Their everchanging environments put a constant pressure on microbes as they are prompted by various environmental signals of different amplitude over time. In nature, this results in continuous adaptation of the microbes through inducing different biochemical systems; e.g. adjusting metabolic activity to current availability of nutrients and carbon source(s), or activation of stress or defense mechanisms to produce secondary metabolites as 'counter stimuli' to external signals (Earl et al. 2008; Klein and Paschke 2004). On the contrary, culture conditions of microbial production strains during industrial scale fermentation have been optimized and 'customized' to the biological requirements of the strain in question (see e.g. review by Parekh et al. 2000). Thus, the metabolic activity and growth of a particular microbial production strain during the fermentation process (primarily the 'exponential growth phase') will focus on efficiently building cell biomass which in turn produces the molecule of interest. Industrial fermentations are run as monocultures (i.e. no external competitors or predators) with optimal abiotic conditions; and the fermentation process is terminated before or when the production strain enters the 'stationary growth phase'. Hence, there are no strong environmental signals that would induce stress (e.g. lack of nutrient or low/high temperature) or defense mechanisms (e.g. production of antibiotic, antiviral or neurotoxic molecules). Biosynthesis of stress and/or defense secondary metabolites of toxicological relevance by industrial microbial production organisms during the fermentation process is thus highly unexpected (Sanchez and Demain 2002) and is furthermore avoided from an economical perspective to optimize production.

Most industrial *Aspergillus oryzae* strains are from safe strain lineages that have been repeatedly tested according to the criteria laid out in the Pariza & Johnson publication (Pariza and Johnson 2001).

Already since decades, *Aspergillus oryzae* strains are being safely used to produce a wide variety of food enzymes.

Furthermore, it should be noted that the toxicological tests (see **Section #6.2.1**) performed on the pectin esterase produced by *Aspergillus oryzae* AR-962 confirm the absence of toxic secondary metabolites.

*Aspergillus oryzae* is principally found in some locations in China and Japan, where it is used for the fermentation of certain foods. Outside this area the fungus may be sporadically found in soil or on decaying plant material.

Invasive growth or systemic infections by *A. oryzae* in healthy humans have never been reported. In a few cases, however, isolates identified as *A. oryzae* have been recovered from debilitated patients which are immunocompromised. Factors that may lead to immunosuppression include an underlying debilitating disease (e.g., chronic granulomatous diseases of childhood), chemotherapy, and the use of supraphysiological doses of adrenal corticosteroids (Bennett 1980; EPA 1997). *A. oryzae* has therefore low pathogenic potential but may, like many other harmless microorganisms, grow in human tissue under exceptional circumstances (Barbesgaard et al. 1992).

**Conclusion**:

*Aspergillus oryzae* has a long history of safe use in industrial-scale and can be considered as a safe production organism for enzymes for food as well as feed processing and numerous other industrial applications. As genetic engineering technology has improved over the last decades, updates to the industrial production strains of *Aspergillus oryzae* and considerable experience on

the safe use of recombinant *Aspergillus oryzae* strains at industrial scale has accumulated. With the proper monitoring in place, as described above, secondary metabolites are of no safety concern in fermentation products derived from *Aspergillus oryzae*.

### 6.1.2 Safety of the genetic modification

The insertion of the expression and selection cassettes into the genome of the recipient strain *Aspergillus oryzae* results in the recombinant strain *Aspergillus oryzae* AR-962 after completion of genetic modification. The production strain only differs from the recipient strain by the production of pectin esterase from the donor pectin esterase gene from *Aspergillus tubingensis*. 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 AR-962 found in this application.

**Pectin methylesterase gene**

Enzymes including pectin esterase have a long history of use in food (Pariza M.W. and Foster E.M. 1983; Pariza and Johnson 2001; Sharma et al. 2013) and have been evaluated worldwide, with multiple national (US GRAS, DK, France…) and international (JECFA) bodies concluding that these food enzymes do not constitute a toxicological hazard.

As the pectin esterase is not toxic our evaluation of the genetically modified *A. oryzae* strain is comparable to that of the recipient strain and the produced food enzyme is non-pathogenic for healthy humans and animals.

***AmdS***

The origin of the *amd*S marker gene is *Aspergillus nidulans*. The *amd*S gene in the expression cassettes is identical to the original *A. nidulans amd*S gene. *A. nidulans* is closely related to *Aspergillus niger*, which is used in industrial production of food enzymes. The product of the *amd*S gene, acetamidase (AmdS) can degrade acetamide which enables the strain to grow on media

without any other nitrogen sources. The AmdS is not harmful or dangerous; the *amd*S marker gene has been widely used as a selection marker in fungal transformations without any disadvantage for more than 30 years.

**Nitrate reductase gene**

The origin of the nitrate reductase gene marker gene is *Aspergillus tubingensis*. The nitrate reductase marker gene is identical to the original *A. tubingensis* nitrate reductase gene. *Aspergillus tubingensis* is closely related to *Aspergillus niger* as mentioned in section 2.2.2. The nitrate reductase produced can degrade chlorate and is not harmful or dangerous. The *niaD* marker gene has been widely used as a selection marker in fungal transformations without any disadvantage for more than 30 years.

AB Enzymes limits the possibilities of mutations through the inoculation of the seed culture for the fermentation with controlled spore stocks that have been stored at -80 ° C. Internal risk assessments have been done to rectify that the gene protein is not turning toxic because of any potential genetic modification of *Aspergillus oryzae*. There have not been any additional growth/mutagenesis cycles performed since the production strain has been constructed and thereafter deposited in CBS. The production strain differs from the original recipient strain in the high production of pectin esterase due to an overexpression of the pectin esterase.

The transformed expression and selection cassettes are fully characterized and free from potential hazards. There is no reason in which the genetic modification done to *Aspergillus oryzae* to construct the production strain have a negative effect on the safety properties of the strain. Based on the reasoning above, production strain from *Aspergillus oryzae* can be regarded as safe to be used to produce food enzymes.

## 6.2 Data for Risk Assessment

### 6.2.1 Toxicological testing

All the studies conducted on the pectin esterase from AR-962 were performed with the basic principles of Good Laboratory Practices (GLP) and under current guidelines of OECD and the European Union.

The following studies were performed for strain AR-962:

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

Pectin esterase sample that has been tested is a liquid ultra-filtrated concentrate before its formulation into a food enzyme preparation. The test sample is comparable to the commercial batch, the test sample is collected after concentration and filtration but not containing any diluent or other formulation ingredient.

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

**Reverse Mutation Assay using Bacteria (*Salmonella typhimurium*) with Pectin Esterase produced with *Aspergillus oryzae***

The assay, based on OECD Guidelines No. 471, was run at Eurofins BioPharma Product Testing Munich GmbH Behringstraße 6/8 82152 Planegg Germany during April 2019 – July 2020.

In order to investigate the potential of Pectin esterase produced with *Aspergillus oryzae* 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, with one exception: In experiment II toxic effects of the test item were observed in tester strain TA98 at concentrations of 2500 µg/plate and higher (**without** metabolic activation).

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

Conclusion:

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

Therefore, Pectin esterase produced with *Aspergillus oryzae* is considered to be non-mutagenic in this bacterial reverse mutation assay.

***In vitro* Mammalian Micronucleus Assay in Human Lymphocytes with Pectin esterase produced with *Aspergillus oryzae***

The assay, based on OECD Guidelines No. 473, was run at Eurofins BioPharma Product Testing Munich GmbH Behringstraße 6/8 82152 Planegg Germany during April 2019 - November 2019.

In order to investigate a possible potential of Pectin esterase produced with *Aspergillus oryzae* to induce micronuclei in human lymphocytes an *in vitro* micronucleus assay was carried out. The following study design was performed:

| | **Without S9 mix** | | **With S9 mix** |
|---|---|---|---|
| | **Experiment I** | **Experiment II** | **Experiment I** |
| **Exposure period** | 4 h | 44 h | 4 h |
| **Cytochalasin B exposure** | 40 h | 43 h | 40 h |
| **Preparation interval** | 44 h | 44 h | 44 h |
| **Total culture period*** | 92 h | 92 h | 92 hrs |

*Exposure started 48 h after culture initiation

The selection of the concentrations was based on data from the pre-experiment. In the first main experiment **without** and **with** metabolic activation 700 µg/mL and 900 µg/mL test item, respectively, and in experiment II 150 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: 500, 600 and 700 µg/mL

**with** metabolic activation: 500, 700 and 900 µg/mL

**Experiment II** with long-term exposure (44 h):

**without** metabolic activation: 50, 100 and 150 µg/mL

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

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** and **with** metabolic activation no increase of the cytostasis above 30% was noted up to 500 µg/mL. **Without** metabolic activation cytostasis was noted at 600 µg/mL (48%) and 700 µg/mL (53%). **With** metabolic activation cytostasis was noted at 700 µg/mL (39%) and 900 µg/mL (58%).

In experiment II no increase of the cytostasis above 30% was observed up to 100 µg/mL. At 150 µg/mL a cytostasis of 58% was noted.

In experiment I **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 concentration 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 (Table 14).

Methylmethanesulfonate (MMS, 50 and 65 µg/mL) and cyclophosphamide (CPA, 12.5 µg/mL) were used as clastogenic controls. Colchicine (Colc, 0.02 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.

Conclusion:

In conclusion, it can be stated that during the study described and under the experimental conditions reported, the test item Pectin esterase produced with *Aspergillus oryzae* did not induce structural and/or numerical chromosomal damage in human lymphocytes.

Therefore, Pectin esterase produced with *Aspergillus oryzae* 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 Wister Rats with Pectin Esterase produced with *Aspergillus oryzae***

The assay, based on OECD Guidelines No. 408, was run at BSL BioPharma BIOSERVICE Scientific Laboratories Munich GmbH Behringstraße 6/8 82152 Planegg Germany during September 2019 – June 2020.

The aim of this study was to assess the possible health hazards which could arise from repeated exposure of Pectin esterase produced with *Aspergillus oryzae* via oral administration to rats over a period of 90 days.

The test item was administered daily in graduated doses to 3 groups of test animals, one dose level per group for a treatment period of 90 days. Animals of an additional control group were

handled identically as the dose groups but received *aqua ad injectionem* (sterile water), the vehicle used in this study. The 4 groups comprised of 10 male and 10 female Wistar rats. The control group C was shared with Eurofins Munich / BSL Munich Study No. 190108.
The following doses were evaluated:
Control: 0 mg/kg body weight
Low Dose: 100 mg/kg body weight
Medium Dose: 300 mg/kg body weight
High Dose: 1000 mg/kg body weight

The test item formulation was prepared at least every 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.

No mortality occurred in the controls or any of the dose groups during the treatment period of this study.

Slight or moderate salivation was observed immediately after the dose administration on few or single days in 2/10 male and 1/10 female HD animals and therefore considered to be a sign of discomfort after oral administration without toxicological relevance. Moving the bedding was observed on few observation days for 1/10 male and female MD animals and 3/10 male HD animals immediately after the dose administration and therefore considered to be a sign of discomfort after oral administration without toxicological relevance. Other findings such as lacrimation (1/10 male control, 1/10 male LD animal) and hairless area (1/10 female control animal) were observed in control groups or for one single animal in the LD group and considered to be not related to the treatment with test item.

No test item-related abnormalities occurred during weekly detailed clinical observation for all parameters in males and females. The statistical significances in single weeks (week 1, 2, 5 and 11)

for decreased or increased mean values of animal is sleeping/moving in the cage and increased response to handling observed in male or female LD and female MD or HD groups were considered to be of no toxicological relevance as no test item-related clinical findings were seen during the daily observation and no consistency was seen within the dose groups.

No test item-related findings were found in the functional observation battery for all parameters in the male and female dose groups. The statistical significances in males in the last week of the treatment period (decrease of animal is sleeping in the HD group, increase of response to handling in the LD group, increased rearing supported in the MD/HD group, increase of defecation/grooming in the HD group) and for females before start of the first treatment (decreased score of head touch in the LD/MD group, increase of equilibrium/positional passivity in the MD group, increase body temperature in MD/HD group) were considered to be not related to the treatment with test item as no test item related findings were noted during daily clinical observation or they were seen before start of the treatment.

The test item had no effect on body weight development in this study. Overall, the mean body weight increased during the observation period in the control and in all male and female dose groups. The statistically significantly increased body weight change in week 10 (male LD and HD group) and slightly and statistically significantly decrease mean body weight on days 22, 29, 36 and 50 (female LD group) were considered to be of no toxicological effect of the test item.

The test item had no effect on food consumption in this study.

No test item-related effects were found for all haematological and coagulation parameters for all male and female dose groups at the end of the treatment period. The statistically significantly changes from the control group in the male LD group (decrease of reticulocytes) and in the female LD (increase of RBC, HGB and HCT, decrease of MCV), MD (decrease of MCV, increase of MCHC) and HD groups (increase of MCHC) were seen without dose dependency within the male or female

dose groups or were seen only in one gender with a slight increase between the dose group (MCHC). Additionally, there were no considerable changes from the historical control data. No statistical significance was noted for coagulation parameters in any male and female dose group with exception of a statistical significant increase for aPTT in the female HD group. In the absence of statistical significance in males for this parameter and no test item-related findings at histopathological evaluation, no effect of the test item is considered.

There was no test item related effect on clinical biochemical parameters measured at the end of the treatment period. Statistical significant changes from the control group were observed for ALAT (decrease in LD/MD/HD males and HD females), Na (decrease in LD/MD/HD males), TP (decrease in MD males), ASAT (decrease in HD females), TBIL (increase in LD/MD/HD females), Crea (decrease in MD/HD females), Urea (decrease in LD/MD/HD females), K (increase in LD/MD/HD females) and LDL (increase in MD females). The histopathological evaluation of all organs in the male and female HD groups was found without test item-related changes and no findings in organ weights were seen. Therefore, no test item-related effects for the statistical significant differences in both genders were considered. No statistically significant changes were found for hormone analysis (T3, T4 and TSH) and no toxicological relevant differences to the control group were found.

There was no test item-related effect on urinary parameters measured at the end of the treatment period.

Macroscopic examination of organs at necropsy showed a mass at the thymus (hard pale, size 0.2 cm) of one male HD animal and the lung with white numerous foci (size 1mm) for one male HD animal and with spots for one male control animal. The uterus of one female HD animal and one LD animal was completely dilated and for one control animal filled with gas. There were no pathologic findings observed at histopathological evaluation for thymus, lung and uterus. Therefore, no correlation to the treatment with the test item was considered.

There were no test item-related findings in organ weights for all male and female dose groups. No statistical significances were found for all male and female organ weights absolute or relative to brain/body weight when compared to control.

No test item related changes were observed during the histopathological evaluation. All recorded findings were deemed to be incidental or were within the range of background alterations that may be recorded in Wistar rats.

Therefore, the histopathological NOEL (no observed effect level) could be established at 1000 mg/kg bw/day.

Conclusion:

On the basis of the present study, the 90-Day Repeated Dose Oral Toxicity study with Pectin esterase produced with *Aspergillus oryzae* 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:

No mortality was observed, and no effects of the test item were found for male and female clinical observations, functional observations, body weight development, food consumption, hormone analysis, hematology and coagulation, clinical biochemistry, urinalysis, gross macroscopic findings at necropsy, organ weights and histopathology in all treated dose groups.

The no observed adverse effect level (NOAEL) of Pectin esterase produced with *Aspergillus oryzae* in this study is considered to be 1000 mg/kg body weight/day.

# 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-962 Composition Report
2. Flow Chart of the manufacturing process with control steps
3. Pariza and Johnson Decision Tree AR-962

## Publication bibliography


Aalberse, R. C. (2000): Structural biology of allergens. In *The Journal of allergy and clinical immunology* 106 (2), pp. 228–238. DOI: 10.1067/mai.2000.108434.

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

Barbesgaard, P.; Heldt-Hansen, H. P.; Diderichsen, B. (1992): On the safety of Aspergillus oryzae. a review. In *Applied microbiology and biotechnology* 36 (5), pp. 569–572.

Bennett, J. E. (1980): Aspergillosis. In Kurt J. Isselbacher (Ed.): Harrison's principles of internal medicine. 9. ed. New York: McGraw-Hill, pp. 742–743.

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

Campbell, E. I.; Unkles, S. E.; Macro, J. A.; van den Hondel, C.; Contreras, R.; Kinghorn, J. R. (1989): Improved transformation efficiency of Aspergillus niger using the homologous niaD gene for nitrate reductase. In *Current genetics* 16 (1), pp. 53–56.

Chen, Jun; Liu, Wei; Liu, Cheng-Mei; Li, Ti; Liang, Rui-Hong; Luo, Shun-Jing (2014): Pectin modifications. A review. In *Critical reviews in food science and nutrition* 55 (12), pp. 1684–1698. DOI: 10.1080/10408398.2012.718722.

Codex Alimentarius Commission (2003): Alinorm 04/34:Appendix III. Guideline for the conduct of food safety assessment of foods derived from recombinant DNA plants. Annex IV. Annex on the assessment of possible allergenicity.

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

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

Earl, Ashlee M.; Losick, Richard; Kolter, Roberto (2008): Ecology and genomics of Bacillus subtilis. In *Trends in microbiology* 16 (6), pp. 269–275. DOI: 10.1016/j.tim.2008.03.004.

EFSA (2010): Scientific Opinion on the assessment of allergenicity of GM plants and microorganisms and derived food and feed. In *EFSA Journal* 8 (7). DOI: 10.2903/j.efsa.2010.1700.

EPA (1997): Final risk assessment of Aspergillus oryzae.

Food and Agriculture Organization of the United Nations (January/2001): Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology,. Rome, Italy.

Food and Agriculture Organization of the United Nations (2006): Compendium of food additive specifications. Joint FAO/WHO Expert Committee on Food Additives : 67th Meeting 2006. Rome: FAO (FAO JECFA monographs, 1817-7077, 3).

Godfrey, T.; West, S. (1992): Industrial Enzymology Second edition: Chapter 2.13 Fruit juices pgs. 227-260

Goodman, Richard E.; Tetteh, Afua O. (2011): Suggested improvements for the allergenicity assessment of genetically modified plants used in foods. In *Current allergy and asthma reports* 11 (4), pp. 317–324. DOI: 10.1007/s11882-011-0195-6.

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

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

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

Jayani, Ranveer Singh; Saxena, Shivalika; Gupta, Reena (2005): Microbial pectinolytic enzymes. A review. In *Process Biochemistry* 40 (9), pp. 2931–2944. DOI: 10.1016/j.procbio.2005.03.026.

Kashyap, D. R.; Vohra, P. K.; Chopra, S.; Tewari, R. (2001): Applications of pectinases in the commercial sector: a review. In *Bioresource technology* 77 (3), pp. 215–227.

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

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

Klein, D. A.; Paschke, M. W. (2004): Filamentous fungi: the indeterminate lifestyle and microbial ecology. In *Microbial ecology* 47 (3), pp. 224–235. DOI: 10.1007/s00248-003-1037-4.

Ladics, Gregory S.; Bannon, Gary A.; Silvanovich, Andre; Cressman, Robert F. (2007): Comparison of conventional FASTA identity searches with the 80 amino acid sliding window FASTA search for the elucidation of potential identities to known allergens. In *Mol. Nutr. Food Res.* 51 (8), pp. 985–998. DOI: 10.1002/mnfr.200600231.

Löffler, F.; Nguyen, K. Q.; Schuster, E.; Sprössler, B.; Thomas, L.; Wolf, S. (1999): Lysophospholipase produced from Aspergillus by recombinant methods. Applied for by Röhm GmbH, Darmstadt. Patent no. US5965422.

Nakazawa, Takuya; Takai, Toshiro; Hatanaka, Hideki; Mizuuchi, Eri; Nagamune, Teruyuki; Okumura, Ko; Ogawa, Hideoki (2005): Multiple-mutation at a potential ligand-binding region decreased allergenicity of

a mite allergen Der f 2 without disrupting global structure. In *FEBS letters* 579 (9), pp. 1988–1994. DOI: 10.1016/j.febslet.2005.01.088.

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

Parekh, S.; Vinci, V. A.; Strobel, R. J. (2000): Improvement of microbial strains and fermentation processes. In *Applied Microbiology and Biotechnology* 54 (3), pp. 287–301. DOI: 10.1007/s002530000403.

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

Pariza M.W.; Foster E.M. (1983): Determining the safety of enzymes used in food processing. In *Journal of Food Protection* 46 (5), pp. 453–468. Available online at http://www.nal.usda.gov/.

Pooja Kohli, Manmohit Kalia and Reeba Gupta (2015): Pectin Methylesterases. A Review. In *J Bioprocess Biotech* 05 (05). DOI: 10.4172/2155-9821.1000227.

Samson, Robert A.; Hong, Seung-Beom; Frisvad, Jens C. (2006): Old and new concepts of species differentiation in Aspergillus. In *Med Mycol* 44 (s1), pp. 133–148. DOI: 10.1080/13693780600913224.

Sanchez, Sergio; Demain, Arnold L. (2002): Metabolic regulation of fermentation processes. In *Enzyme and Microbial Technology* 31 (7), pp. 895–906. DOI: 10.1016/S0141-0229(02)00172-2.

Sharma, Nevadita; Rathore, Madhu; Sharma, Mukesh (2013): Microbial pectinase. Sources, characterization and applications. In *Rev Environ Sci Biotechnol* 12 (1), pp. 45–60. DOI: 10.1007/s11157-012-9276-9.

Sowbhagya, H. B.; Chitra, V. N. (2010): Enzyme-assisted extraction of flavorings and colorants from plant materials. In *Critical reviews in food science and nutrition* 50 (2), pp. 146–161. DOI: 10.1080/10408390802248775.

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

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

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

**To: Jannavi R. Srinivasan**

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Regulatory Affairs
E-Mail info@abenzymes.com
Date: 2021-08-26

# RE: Questions for Notifier of GRN 979

1. Please provide a statement and supporting narrative to demonstrate the non-toxigenicity of the production strain.

## AB Enzymes' Response:

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

- Results of the toxicological studies presented in section 6.2.1 of the dossier;
- Safety and history of use of the production organism *Aspergillus oryzae;*
- Mycotoxin testing results presented in the composition report (Appendix #1 of this notification).

One of the primary methods to demonstrate non-toxigenicity is to conduct toxicological studies on the notified substance. AB Enzymes has conducted three toxicological studies on the *Aspergillus oryzae* production strain to demonstrate non-toxigenicity. The toxicological studies conducted include, a reverse mutation assay using bacteria, an *in vitro* micronucleus assay in human lymphocytes and a 90-day repeated dose oral toxicity study in Wister rats. All three toxicological studies showed negative findings demonstrating the production strain to be non-mutagenic, to not induce structural and/or numerical chromosomal damage in human lymphocytes, 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 page 63-71 of the GRAS dossier.

To add on, as mentioned in section 6.1 of the dossier (begins on page 54), the *Aspergillus oryzae* as a production organism has a long history of use for the production of industrial food enzymes. Food enzymes, including those derived from

recombinant *Aspergillus oryzae* 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 *Aspergillus* 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 *Aspergillus oryzae* 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.

2. From your narrative, it appears that the pectin esterase gene and the nitrate reductase marker gene are on the same plasmid for expression and selection, respectively. It also appears that a separate plasmid was used with the A. nidulans amdS marker gene. Please clarify the intended integration in the final production strain, and if you are using two selection markers.

**AB Enzymes' Response:**

To clarify on the explanation provided on page 8 of the dossier for the steps used in the construction of the production strain, there seems to be some confusion. There are two different cassettes integrated into the final production strain. The contents of each cassette are listed on pages 8 - 9 of the dossier. The pectin esterase gene is present in the same cassette as the *A. nidulans amdS* marker gene – the expression cassette, while the nitrate reductase gene is present in a separate cassette – selection cassette. We confirm that two selection markers are used. The final production strain contains the components of the cassettes listed on pages 8 - 9 of the dossier.

To recap, the first plasmid including the pectin methylesterase expression cassette, contains the pectin methylesterase gene from *Aspergillus tubingensis* under the control of an *Aspergillus oryzae* derived promoter and the *amdS* selection marker gene from *Aspergillus nidulans*. The second plasmid contains a selection cassette with the nitrate reductase selection marker gene (*niaD*). The selection cassette including *niaD* gene was co-transformed to the recipient together with the pectin methyl esterase expression cassette as the recipient strain had an inactive *niaD* gene in its genome.

3. Please clarify how the insertion of the expression cassettes in the production strain was confirmed.

**AB Enzymes' Response:**

The insertion of the expression cassettes in the production strain was confirmed using various methods. The methods used are listed below:

- Southern blot and bioinformatics methods: The presence of the expression cassettes in the genome was confirmed by using Southern blot method and by analyzing the genome sequence of the production strain. To perform the Southern blot, the genomic DNAs from the recipient strain and the production strain were digested with three different restriction enzymes and the labelled pectin methyl esterase gene was used as a probe in hybridization. The DNA fragments of expected lengths, deriving from the expression cassette were hybridizing in the genomic DNA digestions from the production strain. No hybridizing bands were detected from the genomic digestions of the recipient strain. This result confirms the integration of the expression cassette into the fungal genome. The integration of the sequences of the expression and selection cassettes was also confirmed from the sequence of the genomic DNA of the production strain.
- SDS Page and Western blot: Presence of protein bands having expected molecular mass of pectin methyl esterase enzyme protein were detected as the main proteins from the culture supernatants of the production strain. The corresponding bands were not included in the culture supernatants of the recipient strain. The identity of the protein bands as pectin methyl esterase was confirmed using a Western blot assay. In the assay, a peptide antibody was used for detection. This peptide antibody was prepared basing on the amino acid sequence encoded by the gene in the expression cassette.
- Enzyme activity method: As mentioned in section 2.6 of the dossier (page 23), by using an in-house validated method for pectin esterase activity, we were able to confirm high pectin methyl esterase activity from the culture supernatants of the production strain thereby confirming the insertion of the expression cassettes.

4. Please confirm that the production strain does not contain any functional or transferable antibiotic resistance genes.

**AB Enzymes' Response:**

We confirm that the production strain does not contain any functional or transferable antibiotic resistance genes. As demonstrated in the composition report provided as appendix #1 for this notification, for the three liquid enzyme concentrate batches presented no antibiotic activity was detected. The DNA preparations transformed to the recipient strain did not contain any antibiotic marker genes but only contained isolated expression cassettes (no vector DNA with the ampicillin resistance gene was transformed). The lack of vector derived DNA (including the ampicillin resistance gene) has been confirmed from the production strain using Southern blot analysis.

5. You state that the genetic stability was demonstrated by monitoring the ability of the production strain to generate the enzyme. Please provide a narrative on additional confirmational methods, if any, were employed to monitor the integration and stability of the introduced gene.

**AB Enzymes' Response:**

Apart from the enzyme activity values presented in Appendix #1 of this GRAS notification, as mentioned in section 2.3.1. another conformational method was used to determine genetic stability of the production strain. The production strain was cultivated for five generations on solid agar by transfer of spores from one slant to the next one for five times, starting from the original strain deposit at -80°C ("generation 0"). After this, shake flask cultivations were inoculated from the slants representing each generation. After the cultivations the pectin methyl esterase activities were analyzed from the culture supernatants and samples were run on SDS-PAGE gels. The genomic DNAs were isolated from mycelia (representing each generation) and were analyzed by using Southern blot. The results from the analysis showed that the production strain remained stable through the generations: the enzyme activities and protein patterns remained similar in all culture supernatants and the hybridization patterns were identical from all mycelia. It is expected that in

case the strain is unstable, the enzyme activities would decrease from generation to the next and, also, differences in the banding patterns (in SDS-PAGE and Southern blot) would be detected.

6. Please provide a statement to demonstrate that the fermentation medium used in the manufacturing of pectin esterase enzyme preparation does not contain any major food allergens or no major allergens are expected to be in the final ingredient.

**AB Enzymes' Response:**

AB Enzymes expects no major food allergens to be in the final enzyme preparation. A rigorous allergen risk assessment is routinely conducted during the manufacturing of the final ingredient (i.e. enzyme preparation) for the purpose of determining and avoiding cross-contamination of food allergens into the final enzyme concentrate (before formulation). AB Enzymes uses a wheat-based fermentation ingredient for production of the pectin esterase enzyme preparation from AR-962 production strain. We routinely test our enzyme products for gluten traces at an external testing partner using an R5 antibody-based ELISA (Codex Alimentarius specifies in Codex Standard 118-1979 (2008)) and recent analysis has detected gluten traces in the pectin esterase enzyme preparation below the limit of quantification (LoQ) <5ppm.

7. It appears from the batch data that you provide, the values for lead are well below 5 mg/kg specification. Please consider reducing this specification to a level representing the manufacturing process.

**AB Enzymes' Response:**

We will consider reducing the lead specification to a level representing the manufacturing process for future GRAS notifications. The purity specifications provided in section 2.5.4. of the dossier are based on JECFA's General Specifications for Enzyme Preparations to demonstrate compliance with the international standard.

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8. Please provide the highest dose tested in the 90-d oral toxicity studies, in mg TOS/kg bw/d.

**AB Enzymes' Response:**

The highest dose tested in the 90-day oral toxicity studies was 1000 mg TOS/kg bw/day.

9. In discussing allergenicity, you state that the pectin esterase shares >35% sequence identity with pollen allergens of a common weed Salsola kali and olive tree Olea europaea across a window of 80 amino acids. You support your safety conclusion by citing opinions by Ladics et al. (2007) and Goodman and Tetteh (2011). Please provide a narrative including either 1) evidence to support general recognition of these cited viewpoints that proteins having homology greater than 35% but less than 50% identity in a 80-mer window analysis lack allergic potential, or 2) a rationale using other specific information to the pectin esterase notified in GRN 979 such as its low dietary exposure, digestibility through the gastro intestinal tract etc. to demonstrate the unlikelihood of any significant risk for allergic reaction from its intended use in spite of potential cross-reactivity to pollen allergens.

**AB Enzymes' Response:**

It is very unlikely that the pectin esterase notified in GRN 979 will cause any significant risk for allergenic reaction during oral consumption for the following reasons:

- Fate of the enzyme in final food
- pH conditions in the gastro-intestinal tract
- Occupational use
- Literature research results on pectin esterase allergenic potential

The pectin esterase serves as a processing aid in the manufacturing of the final food. As mentioned in section 2.10 of the dossier, the enzyme does not have a technical function in the final food itself. In the food applications for which this enzyme is to be used in, the removal conditions of pectin esterase are also mentioned in section 2.10. These removal conditions place the enzyme in an environment with unfavorable conditions such as high temperatures, and pH levels above or below the

pH optimum of the enzyme. Given the conditions in which the pectin esterase is exposed to in the manufacturer of finals foods, there is a low chance of active enzyme protein carryover into the final food. In the case where there is a carryover of pectin esterase in the final food, we expect the enzyme to be digested when reaching the gastro-intestinal track due to the harsh pH conditions. The pH level of gastric juice is known to be 2, the pectin esterase notified cannot survive at a pH level of 2.

In more general terms it is remarkable that in 2006, a group of scientists in Denmark investigated the possible allergenicity of 19 different commercial enzymes used in the food industry such as a-amylase, maltogenic amylase, glucoamylase, pectin esterase, glucose oxidase, lipase, protease and xylanase. Four hundred adult patients with a diagnosed allergy to inhalation allergens, food allergens, bee or wasp venoms were included in the study. The Investigation demonstrated that "none of the patients was positive to any of the commercial enzymes in the subsequent oral challenge using exaggerated dosages of the enzymes compared to normal daily intake" (Bindslev-Jensen et al. 2006). And the authors conclude" that ingestion of food enzymes in general is not considered to be concern with regard to food allergy". However, it has been demonstrated that inhalation of enzyme aerosols and dust may cause allergic reactions especially for workers and anyone repeatedly inhaling high concentrations of enzyme containing aerosols. Therefore, the handling of large amounts of enzyme preparations must be done with care to avoid dust and aerosols and to reduce occupational allergic disease. These engineering controls have been effectively used so that pectin esterase which has been produced by *Aspergillus oryzae* for many years, there are no public reports of allergic reactions in workers caused by inhalation of enzyme dust.

It was concluded that the "respiratory enzyme allergy is an occupational risk only and effectively prevented by complying with product formulation, hazard labeling and handling standards. Many years of experience and numerous studies have

shown that enzymes present no risk of causing allergies to the consumer" (AMFEP 2009)[1].

Lastly, we conducted a comprehensive literature search for data on possible adverse reaction or allergenicity after oral exposure to pectin esterase, particularly with relation to cedar pollen. PCM database by the US National Library of Medicine and National Institutes of Health (Home - PMC - NCBI (nih.gov)), containing global scientific literature (more than 6 million records) as well as the Pubmed database from the National Library of Medicine, National Center for Biotechnology (PubMed (nih.gov); more than 32 million records) were searched with the following terms:
"pectin esterase*" AND "allergen*" AND "oral"
"pectin esterase*" AND "adverse" AND "oral"
"pectin esterase*" AND "exposure" AND "oral"
"pectin esterase*" AND "pollen" AND "oral"
"pectin methylesterase*" AND "allergen*" AND "oral"
"pectin methylesterase*" AND "adverse" AND "oral"
"pectin methylesterase*" AND "exposure" AND "oral"
"pectin methylesterase*" AND "pollen" AND "oral"
The searches were performed on June 30th, 2021 for references up to this date.
After removal of duplicates, 77 publications were retrieved. The publications were assessed for their relevance by stepwise means:

First, the title and abstract, and
Second, the whole text was assessed.

Papers were considered relevant if they included oral exposure related data on adverse reactions or allergenicity to pectin esterases.

After assessment of title, abstract, 1 publication remained possibly relevant and was fully assessed.

[1] Amfep 09 17 - Amfep Fact Sheet on Protein Engineered Enzymes

(Ghiani *et al.* 2016) describe two pectin esterases from tomato as being putative allergens present in tomato. The rest of the article mainly focusses on a possible difference in IgE recognition between peel and pulp and subsequently differences between tomato lines. According to the authors, 27 potential tomato allergens, including different isoforms, have been reported and only six tomato allergens were recognized by the International Union of Immunological Society, pectin esterases not being part of them. The article does not show clear evidence of pectin esterases actually being an allergen in humans.

The results from (Ghiani *et al.* 2016) do not challenge the initial risk assessment as presented in the dossier and after consideration of the full article is not considered relevant.

**In conclusion, as already concluded in the initial dossier, no indication of allergenic risk was identified.**

## Publication bibliography

AMFEP (2009): Amfep Fact Sheet on Protein Engineered Enzymes. Available online at https://amfep.org/_library/_files/Amfep-Fact-Sheet-on-Protein-Engineered-Enzymes.pdf, checked on 8/20/2021.

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

Ghiani, Alessandra; D'Agostino, Nunzio; Citterio, Sandra; Raiola, Assunta; Asero, Riccardo; Barone, Amalia; Rigano, Maria Manuela (2016): Impact of Wild Loci on the Allergenic Potential of Cultivated Tomato Fruits. In *PloS one* 11 (5), e0155803. DOI: 10.1371/journal.pone.0155803.

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