

GRAS Notice (GRN) No. 558

<http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/default.htm>

ORIGINAL SUBMISSION

558

AB Enzymes GmbH – Feldbergstrasse 78 , D-6412 Darmstadt



November 17, 2014

Office of Food Additive Safety (HFS-255),
Center for Food Safety and Applied Nutrition,
Food and Drug Administration,
5100 Paint Branch Parkway, College Park, MD 20740.

**RE: GRAS NOTICE FOR PECTIN ESTERASE ENZYME PREPARATION FROM
*TRICHODERMA REESEI***

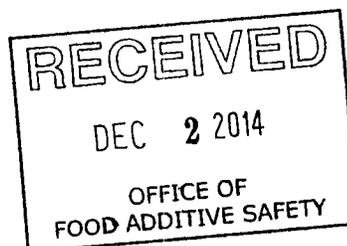
Pursuant to proposed 21 C.F.R § 170.36, AB Enzymes GmbH is providing in electronic media format (determined to be free of computer viruses), based on scientific procedures – a generally recognized as safe (GRAS) notification for pectin esterase enzyme from *Aspergillus tubingensis* expressed in *Trichoderma reesei* for use in fruit, vegetable and coffee processing, wine and flavor production, and grain treatment at a level not higher than the necessary dosage to achieve the desired enzymatic reaction – according to Good Manufacturing Practices.

The pectin esterase enzyme preparation described herein when used as described above and in the attached GRAS notice is exempt from the premarket approval requirements applicable to food additives set forth in Section 409 of the Food, Drug, and Cosmetic Act and corresponding regulations.

Please contact the undersigned by telephone or email if you have any questions or additional information is required.

(b) (6)

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Toronto, Ontario
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November 17, 2014

RE: GRAS Notification – Exemption Claim

Dear Sir or Madam:

Pursuant to the proposed 21C.F.R. § 170.36 (c)(1) AB Enzymes GmbH hereby claims that pectin esterase enzyme from *Aspergillus tubingensis* expressed in *Trichoderma reesei* produced by submerged fermentation is Generally Recognized as Safe; therefore, they are exempt from statutory premarket approval requirements.

The following information is provided in accordance with the proposed regulation:

Proposed 21C.F.R. § 170.36 (c)(i) *The name and address of notifier.*

AB Enzymes GmbH
Feldbergstr. 78
D-64293 Darmstadt, Germany

Proposed 21C.F.R. § 170.36 (c)(ii) *The common or usual name of notified substance.*

Pectin esterase enzyme from *Aspergillus tubingensis* expressed in *T. reesei*.

Proposed 21C.F.R. § 170.36 (c)(iii) *Applicable conditions of use.*

The pectin esterase enzyme preparation is used in fruit and vegetable processing, wine, coffee and flavouring production, and grain treatment. The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements under current Good Manufacturing Practices.

Proposed 21C.F.R. § 170.36 (c)(iv) *Basis for GRAS determination.*

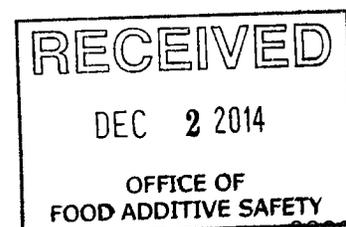
This GRAS determination is based upon scientific procedures.

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

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

Candice Cryne ✓
Regulatory Affairs Specialist

NOV 17 / 2014
Date



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Candice Cryne
Regulatory Affairs Specialist

Date

**GRAS NOTICE FOR PECTIN ESTERASE
FROM A GENETICALLY MODIFIED
STRAIN OF *TRICHODERMA REESEI***

AB ENZYMES GmbH

November 17, 2014

TABLE OF CONTENTS

1. GENERAL INTRODUCTION AND CLAIM OF EXEMPTION FROM PREMARKET APPROVAL REQUIREMENTS.....	3
1.1. NAME AND ADDRESS OF NOTIFIER.....	4
1.2. COMMON OR USUAL NAME OF SUBSTANCE	5
1.3. APPLICABLE CONDITIONS OF USE.....	5
1.3.1. <i>Food Products Used in</i>	5
1.3.2. <i>Levels of Use</i>	5
1.3.3. <i>Purposes</i>	6
1.3.4. <i>Consumer Population</i>	6
1.4. BASIS FOR GRAS DETERMINATION	7
1.5. AVAILABILITY OF INFORMATION FOR FDA REVIEW	7
2. PRODUCTION MICROORGANISM	9
2.1. DONOR, RECIPIENT ORGANISM AND PRODUCTION STRAIN.....	9
2.2. GENETIC MODIFICATION	12
2.3. STABILITY OF THE TRANSFORMED GENETIC SEQUENCE	12
2.4. GOOD INDUSTRIAL LARGE SCALE PRACTICE (GILSP).....	14
2.5. ABSENCE OF THE PRODUCTION ORGANISM IN THE PRODUCT	16
2.6. ABSENCE OF TRANSFERABLE RDNA SEQUENCES IN THE ENZYME PREPARATION	16
2.7. ABSENCE OF ANTIBIOTIC GENES AND TOXIC COMPOUNDS.....	17
3. ENZYME IDENTITY AND SUBSTANTIAL EQUIVALENCE.....	18
3.1. ENZYME IDENTITY.....	18
3.2. AMINO ACID SEQUENCE	18
3.3. ENZYMATIC ACTIVITY	18
4. MANUFACTURING PROCESS	21
4.1. OVERVIEW	21
4.2. FERMENTATION	21
4.2.1. <i>Raw materials</i>	21
4.2.2. <i>Materials used in the fermentation process (inoculum, seed and main fermentation)</i>	22
4.2.3. <i>Inoculum</i>	22
4.2.4. <i>Seed fermentation</i>	22
4.2.5. <i>Main fermentation</i>	23
4.3. RECOVERY.....	23
4.3.1. <i>Materials</i>	24
4.3.2. <i>Pre-Treatment</i>	24
4.3.3. <i>Primary solid/liquid separation</i>	24
4.3.4. <i>Concentration</i>	24
4.3.5. <i>Polish and germ filtration</i>	25

4.4. FORMULATION AND PACKAGING	25
4.5. QUALITY CONTROL OF FINISHED PRODUCT	25
5. COMPOSITION AND SPECIFICATIONS.....	26
5.1. FORMULATION	26
5.2. TYPICAL FINAL ENZYME PREPARATION SPECIFICATIONS	27
5.3. GENERAL PRODUCTION CONTROLS AND SPECIFICATIONS.....	27
6. APPLICATION	30
6.1. MODE OF ACTION.....	30
FIGURE #2 PECTIN ESTERASE SCHEMATIC	32
6.2. APPLICATION.....	33
6.3. USE LEVELS.....	43
6.4. ACTIVE AND INACTIVE ENZYME RESIDUES IN THE FINAL FOOD	45
6.4.1. <i>Possible Effects on Nutrients</i>	47
7. SAFETY EVALUATION	47
7.1. SAFETY OF THE PRODUCTION STRAIN	47
7.2. SAFETY OF THE PECTIN ESTERASE GENE.....	48
7.2.1. <i>Allergenicity</i>	49
7.2.2. <i>Leading Publications on the Safety of Pectin Esterase Enzyme or Enzymes that are Closely Related</i>	53
7.3. SAFETY OF THE MANUFACTURING PROCESS.....	54
7.4. SAFETY STUDIES.....	55
7.4.1. <i>Summary of Safety Studies</i>	55
7.4.2. <i>Results of the Safety Studies</i>	55
7.5. ESTIMATES OF HUMAN CONSUMPTION AND SAFETY MARGIN	60
7.5.1. <i>Estimate of Dietary Exposure</i>	60
8. CONCLUSION.....	64
9. LIST OF APPENDICES	66
10. LIST OF REFERENCES.....	67

1. GENERAL INTRODUCTION AND CLAIM OF EXEMPTION FROM PREMARKET APPROVAL REQUIREMENTS

Pursuant to the regulatory and scientific procedures established by proposed regulation 21 C.F.R. § 170.36 (see 62 Fed. Reg. 18,938 (April 17, 1997)), AB Enzymes GmbH ("AB Enzymes") has determined that the Pectin esterase enzyme preparation from *Trichoderma reesei* (*T.reesei*) strain expressing the gene pectin esterase from *Aspergillus tubigiensis* is a GRAS substance for the intended applications based on scientific procedures and is therefore exempt from the requirement for premarket approval. Information on the enzyme and the production organism providing the basis for this GRAS determination is described in the following sections. General and specific information identifying and characterizing the enzyme, its applicable conditions for use, AB Enzymes' basis for its GRAS determination and the availability of supporting information and reference materials for FDA's review can be found here in Section 1.

Section 2 also describes the genetic modifications implemented in the development of the production microorganism to create a safe standard host strain resulting in a genetically well-characterized production strain, free from known harmful sequences.

Section 3 shows the enzymatic activity of the enzyme, along with a comparison to other similar enzymes. The safety of the materials used in manufacturing, and the manufacturing process itself is described in Section 4. Section 5 reviews the hygienic measurements, composition and specifications as well as the self-limiting levels of use for pectin esterase. Section **Error! Reference source not found.** provides information on the mode of action, applications, and use levels of pectin esterase and enzyme residues in final food products. The safety studies outlined in Section 7 indicate that the pectin esterase enzyme preparation from *T. reesei* RF6201 show no evidence of pathogenic or toxic effects. Estimates of human consumption and an evaluation of dietary exposure are also included in Section 7.

1.1. Name and Address of Notifier

Notifier:

AB Enzymes GmbH
Feldbergstr. 78
D-64293 Darmstadt
Germany

Manufacturer:

Roal Oy¹
Tykkimäentie 15
FIN-05200 Rajamäki
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Person(s) Responsible for the Dossier:

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Dr. Hans-Juergen Schepers
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¹ ROAL is a Joint Venture between Associated British Foods (UK) and Altia OY (Finland). Manufacturing and research and development activities are performed for AB Enzymes by ROAL OY in Finland. ROAL coordinates its R&D activities independently while taking into account the market requirements reported by their sole distributor AB Enzymes GmbH.

1.2. Common or Usual Name of Substance

The food enzyme is a biological isolate of variable composition, containing the enzyme protein, as well as organic and inorganic material derived from the microorganism and fermentation process. The enzyme preparation is known as pectin esterase or pectin methylesterase (PME).

1.3. Applicable Conditions of Use

For an enzyme to perform a technological function in the final food, certain conditions have to be met, such as the enzyme must be in its native, non-denatured form, and must be free to move, a substrate must be present and conditions such as pH, temperature and water content must be favourable for the particular enzyme.

Temperature: Range: 20°C to 50°C

pH value: Range: 2.5-6.0

1.3.1. Food Products Used in

This dossier is specifically submitted for the use of pectin esterase in fruit, vegetable and coffee processing, wine and flavor production, and grain treatment.

1.3.2. Levels of Use

Maximum use levels of pectin esterase enzyme preparation from *T.reesei* RF6201/kg raw material, this will depend on the type of product and application utilized, as detailed in this dossier.

Application		Raw material (RM)	Maximal recommended use levels (mg TOS/kg RM)
Coffee processing		Coffee cherries	0.5
Flavouring production		Fruits/Vegetables	265
Fruit and vegetable processing	Fruit juices	Fruit/Vegetable	3
	Fruit purees	Fruit/Vegetable	24
	Fruit firming	Fruit/Vegetable	12
Grain treatment		Cereals	5
Wine production		Grapes	1

1.3.3. Purposes

Like most of the food enzymes, pectin esterase performs its technological function during food processing. Pectin esterase from *Aspergillus tubingensis* expressed in *T.reesei* RF6201 is mainly intended to be used in fruit and vegetable processing, wine production, flavouring production, coffee processing and grain treatment.

Pectin esterase is used in fruit and vegetable processing to improve extraction yield of nutrients, clarification of juices, and increased consistency of purees; in wine production to lower viscosity, improve filterability, wine clarification, and improve extraction rates; in grain processing for better separation of the bran from the endosperm, better extraction rates, and shorter processing times; in coffee processing to improve environmental impact and process improvement.

1.3.4. Consumer Population

Pectin esterase catalyzes the de-esterification of pectin into pectate and methanol and plays an important role in pectin disassembly in ripening fruit and by some bacteria (*Erwinia carotovora*) and fungi (*Aspergillus niger*) involved in maceration and soft-rotting of plant tissue. Since the pectin esterase enzyme is naturally present in nature, most notably in fruits, which are consumed by humans, it is expected that the enzyme from *T. Reesei* RF6201 will be digested as any other protein in the human.

Furthermore, pectin esterase and other pectin-degrading enzymes such as pectin lyase and pectate lyase are important enzymes and have been used in the food industry for many years (Sharma *et al.*, 2013). In the Australian New Zealand Food Standards Code – Standard 1.3.3 – processing aids, polygalacturonase or pectinase (multicomponent enzyme) is listed as safe for use in food from *Aspergillus niger*, *Aspergillus oryzae*, and *T.reesei*².

It is AB Enzyme's conclusion since the pectin esterase enzyme is removed or denatured during food processing and as such the denatured protein may be present in processed final foods at a very low concentration, and the fact that it naturally occurs in commonly consumed fruits and vegetables, that the consumer population will be unaffected by the potential negligible presence of the denatured enzyme preparation in food.

1.4. Basis for GRAS Determination

Pursuant to 21 C.F.R. § 170.30, AB Enzymes GmbH has determined, through scientific procedures, that its pectin esterase enzyme from *Aspergillus tubingensis* expressed in *T.reesei* is GRAS for use as an enzyme for fruit and vegetable processing, wine, coffee and flavouring production, and grain treatment.

1.5. Availability of Information for FDA Review

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

² Australia New Zealand Food Standards Code - Standard 1.3.3 - Processing Aids - F2012C00064, accessed June 3, 30214:
<http://www.comlaw.gov.au/Details/F2012C00064>

Please direct all inquiries regarding this GRAS determination to:

Candice Cryne
155 Claremont St
Toronto, Canada
M6J2M7
647-919-3964
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2. PRODUCTION MICROORGANISM

2.1. Donor, Recipient Organism and Production Strain

Donor:

Pectin esterase gene described in this application derives from *Aspergillus tubingensis* Mosseray RH3544 which is a filamentous fungus belonging to *Aspergillus* section *Nigri* (the black *aspergilli*; Samson et al., 2006), as are the already sequenced *A. niger*, *A. carbonarius*, and *A. aculeatus*. These filamentous fungi are common in causing food spoilage and biodeterioration of other materials. All black *Aspergilli* grow well on wheat bran, a crude plant biomass, but their protein (SDS-PAGE) and enzyme activity profiles are significantly different between these species. *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.

Aspergillus RH3544 was originally identified at DSMZ as *Aspergillus niger* van Tieghem 1867 (1982, Appendix #1) but was recently (2012) identified at CBS as *Aspergillus tubingensis* Mosseray (Appendix #2).

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

Genus:	<i>Aspergillus</i>
Species:	<i>Aspergillus tubingensis</i>
Subspecies (if appropriate):	not applicable
Generic name of the strain:	RH3544

Recipient Organism:

The recipient (host) strain used for the genetic modification is *T.reesei* strain RF5455. *T. reesei* RF6201 strain was developed for pectin esterase production. The strain RF5455 originally derives from a host strain that derives from QM6a with classical mutagenesis steps and has a higher capacity for protein production than the original *T. reesei* isolate QM6a. This host strain was further modified to derive strain RF5455 by deleting two endogenous *T. reesei* genes encoding cellulases.

Therefore the recipient can be described as followed:

- Genus: *Trichoderma*
- Species: *T.reesei*
- Subspecies (if appropriate): not applicable
- Generic name of the strain: RF5455

T. reesei is an aerobic filamentous fungus (an ascomycete). It grows in mycelium form but starts to sporulate when cultivation conditions do not favor growth (e.g. due to lack of nutrients). *T. reesei* is a mesophilic organism which means that it prefers to grow at moderate temperatures. The cultures are typically fast growing at about 30° C (above 20°C and below 37°C). *T. reesei* prefers acidic to neutral pH (about 3.5 to 6) for growth. The colonies are at first transparent or white on agar media such as potato dextrose agar (PDA). The conidia are typically forming within one week of growth on agar in compact or loose tufts in shades of green. Sporulation is induced by daylight. Yellow pigment may be secreted into the agar by the growing fungal colonies, especially on PDA.

The taxonomic classification of the *T. reesei* is: *Hypocreaceae, Hypocreales, Hypocreomycetidae, Sordariomycetes, Pezizomycotina, Ascomycota, Fungi*, according to Index *Fungorum* database

AB Enzymes GmbH has been using *T. reesei* as an enzyme producer for many years without any safety problems. A GRAS notice was filed for pectin lyase enzyme preparation produced with *T. reesei* containing a gene from *Aspergillus niger* and FDA had no question and designated it as GRAS Notice No. GRN 000032 (*Appendix #3*). *T. reesei* has a long history (more than 30 years) of safe use in industrial-scale enzyme production (e.g. cellulases and xylanases produced by this fungus are used in food, animal feed, pharmaceutical, textile, detergent, bioethanol and pulp and paper industries). Currently, various *Trichoderma* enzymes are also used in the brewing process (β -glucanases), as macerating enzymes in fruit juice production (pectinases, cellulases, hemicellulases), as a feed additive to livestock (xylanases, endoglucanases) and for pet food processing. *T. reesei* - wild type or genetically modified - is widely accepted as safe production organism for a broad range of food enzymes.

Based on the available data, it is concluded that the organism *T. reesei* is non-pathogenic and non-toxicogenic and is safe to use as the production organism for pectin esterase from *Aspergillus tubingensis*.

Production Strain:

The transformed production strain containing the pectin esterase *pmeA* gene is *T. reesei* strain RF6201 which is deposited in the "Centraalbureau voor Schimmelcultures" (CBS) in the Netherlands with the deposit number CBS 131879.

The production strain (RF6201) differs from its recipient strain (RF5455) in its high pectin esterase production capacity due to expression of the *pmeA* gene from *A. tubingensis* integrated into the RF6201 genome. RF6201 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 RF6201. In addition to the heterologous pectin esterase, the RF6201 strain produces some endogenous *Trichoderma* enzymes, e.g. cellulase and xylanase as side activities.

The techniques used in transforming and handling *T. reesei* were as described in Penttilä *et al.* (1987) and Karhunen *et al.* (1993). The production organism also meets the criteria for safe production microorganism as described by Pariza and Johnson (2001) (Decision Tree Analysis - Appendix #4). *T. reesei* strains are non-pathogenic and non-toxicogenic and have been shown not to produce fungal toxins or antibiotics under conditions used for industrial enzyme production. Further they are considered a safe host for other harmless gene products (Nevalainen *et al.*, 1994).

The seed culture for the fermentation is inoculated with spores that have been stored at -80 °C. No additional growth cycles have been performed after the *T. Reesei* RF6201 strain deposition to the culture collection.

2.2. Genetic Modification

The construction of the pectin esterase production strain was achieved by the use of an expression cassette.

Expression Cassette(s)

The *pmeA* gene was isolated from the *Aspergillus tubingensis* Mosseray RH3544. *T.reesei* strain RF6201 was constructed for production of *Aspergillus tubingensis* pectin esterase by introducing the *pmeA* gene copies into the genome of the *T.reesei* strain RF5455 using an expression cassette and the transcription is terminated using the *pmeA* gene's native terminator. The transformation of RF5455 strain with the expression cassettes was performed as described by Penttilä *et al.* (1987) with the modifications described in Karhunen *et al.* (1993). The expression cassettes are integrated into the RF5455 genome as several copies.

The purified expression cassette fragment is free from any harmful sequences and contains the following genetic materials:

- endogenous *T. reesei* promoter
- *A tubingensis pmeA* gene
- *A tubingensis pmeA* gene terminator
- *amdS* promoter and terminator *Aspergillus nidulans amdS* gene. The gene has been isolated from *Aspergillus nidulans* VH1-TRSX6. The gene codes for the enzyme acetamidase that enables the strain to grow on acetamide as a sole nitrogen source. This characteristic has been used for selecting the transformants (Hynes *et al.* (1983); Kelly and Hynes (1985)). The product of the *amdS* gene, acetamidase, can degrade acetamide and is neither harmful or nor dangerous. The *amdS* marker gene has been widely used as selection marker in fungal transformation.

2.3. Stability of the Transformed Genetic Sequence

T. reesei strains are widely used in biotechnological processes because of their known stability. The transformed DNA does not contain any antibiotic resistance genes. Southern blot analysis performed

revealed that the *T. reesei* host strain RH5455 and production strain RF6201 stay genetically stable over the time necessary for the industrial fermentation process of the RF6201 production strain.

The inserted DNA does not include any mobile genetic elements. Additionally, it should be highlighted that *T. reesei* genome lacks a significant repetitive DNA component and no extant functional transposable elements have been found in the genome (Kubicek *et al.*, 2011; Martinez *et al.*, 2008). This results to low risk of transfer of genetic material.

The fermentation process starts always from the identical replica of the RF6201 seed ampoule. Production preserves at -80°C (“Working Cell Bank”) are prepared from the “Master Cell Bank” (culture collection maintained at -150°C) in the following manner: A Petri dish is inoculated from the culture collection preserve in such a way that single colonies can be selected upon germination. Altogether 30 individual colonies are inoculated into shake flasks. The shake flasks constitute the culture stage.

From each shake flask, one oblique tube and another shake flask are inoculated. The latter is subjected to a so-called productivity test, i.e. shake flask cultivation being completed; the enzymatic activity is measured, which must correspond to a given value. If this value is not reached, the culture is discarded. This test serves to determine the characteristic metabolic efficiency of each strain, i.e. to establish its identity. The tubes whose parallel shake flasks show the highest results are then flushed with glycerol solution. The suspensions thus obtained are frozen and stored divided into 0.5 ml aliquots at -80°C (20 productivity preserves each).

The annual production starts from these production preserves. Six of them are thawed for inoculation of six shake flasks and subsequent inoculation of the first process bioreactor is from these flasks. Mutation frequencies are low and they only occur in the vegetative state during cell division. Owing to the above-described procedure, this vegetative state of the cultures is reduced to an inevitable minimum during production.

Potential changes in the genome of the production strain could theoretically occur during the propagation in the fermentation process. The presence of the expression cassettes integrated into the genome of the *T.reesei* strain RF6201 was confirmed by southern blot hybridization. The results obtained shows that the genome of RF6201 at the end of industrial fermentations corresponds to that of the original strain. Thus, it can be concluded that the expression cassettes (recombinant pectin esterae) are stably integrated into the genome of the production strain.

The DNA fragments that have been transformed to *T. reesei* recipient strain RF5455 are well characterized, the sequences of the genes are known, and the fragments are free of any harmful sequences. The production strain RF6201 is stable in terms of genetic traits. The genetic materials in the expression cassettes have been integrated as part of the genome and are as stable as any natural gene. The integrated genetic materials are not acting as mobilisable elements and they do not contain mobilisable elements.

2.4. Good Industrial Large Scale Practice (GILSP)

In the USA, *Trichoderma reesei* is not listed as a Class 2 or higher Containment Agent under the National Institute of Health (NIH, 1998) Guidelines for Recombinant DNA Molecules. Data submitted in Generally Recognized as Safe (GRAS) petitions to the Food and Drug Administration (FDA) for numerous enzyme preparations from *T. reesei* for human and animal consumption demonstrate that the enzymes are nontoxic. The Environmental Protection Institute (EPA) completed a risk assessment on *T. reesei* in 2011 resulting in a Proposed Rule in 2012, concluding that it is appropriate to consider *T. reesei* as a recipient microorganism eligible for exemptions from full reporting requirements³, if this fungus was to be used in submerged standard industrial fermentation for enzyme production.

The *T. reesei* RF6197 pectin esterase enzyme production strain complies with all criteria for a genetically modified GILSP organism.

³ reporting procedures in place under the Toxic Substances Control Act (TSCA) for new micro-organisms that are being manufactured for introduction into the commerce.

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

The host organism is non-pathogenic, does not produce adventitious agents under the fermentation conditions employed and has an extended history of safe industrial use (see Section 7.1). Indeed, the strain *T. reesei* RF5455 derives from a host strain that derives from QM6a (from classical mutagenesis and targeted gene deletion steps). The QM6a strain has been isolated from soil only at low altitudes and within a narrow belt around the equator (Kubicek *et al.* (2008)). The mycoparasitism-specific genes have been shown to be lost in *T. reesei* (Kubicek *et al.* (2011)).

Overall, industrial microorganisms modified to produce high levels of enzymes, in fermentation conditions (e.g. no competitive microorganisms, optimal nutrients and aeration that are not present in the natural environment) are not expected to have any competitive advantage against other microorganisms in nature, which themselves are well-adapted in their natural environment. The fitness of the industrial strains to survive is very likely reduced by their high performance characteristic: most of the energy is needed for the production of proteins in high amounts.

From the genetic modification performed, there is no reason to believe that the survival of the genetically modified production organism would be different when compared to its ancestor. The DNA insert is fully characterized and is free from known harmful sequences. No antibiotic resistance markers or other heterologous markers are present in the strain. We consider thus that the colonization capacity of *T. reesei* RF6201 in the environment is rather low because of its adaptation to fermentation conditions.

Therefore, the *T. reesei* host strain RF5455 and production organism RF6201 are considered to be of low risk and the production organism can be produced with minimal controls and containment procedures in large-scale production. This is the concept of Good Industrial Large Scale Practice (GILSP), as endorsed by the Organization of Economic Cooperation and Development (OECD). The production

organism has been approved by the Finnish competent authorities for large-scale productions, under containment conditions not exceeding the GILSP level of physical containment.

2.5. Absence of the Production Organism 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 RF6201 pectin esterase enzyme production strain is recovered from the fermentation broth by a widely used process that results in a cell-free enzyme concentrate. The absence of the production strain is confirmed for every production batch, using an internal Roal method. This method has been recently validated in-house. The sensitivity of the method is 1 cfu/20 ml in liquid and 1 cfu/0,2 gram in dried semifinals.

2.6. Absence of Transferable rDNA Sequences in the Enzyme Preparation

As described above the expression cassette is well characterized and does not contain any undefined or harmful sequences (see section 2.2). A Southern blot was performed to further confirm that there was no pUC19 derived vector DNA integrated into the genome of RF6201. The integration of pUC19 could have happened in case there had been a contaminating pUC19 DNA in the DNA preparation used for the transformation. It produced negative results (no hybridization), demonstrating that no part of the plasmid vector removed to generate the linear transforming NotI DNA fragment was introduced into the *T.reesei* production host RF6201.

The pectin esterase is produced by an aerobic submerged microbial fermentation using a genetically modified *T. reesei* RF6201 strain. All viable cells of the production strain are removed during the down-stream processing; further the fermentation broth is filtered concentrated, resulting in a concentrated enzyme solution free of the production strain and insoluble substances.

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 semi-final concentrates) were tested for the presence of recombinant DNA using highly sensitive and specific PCR techniques. No recombinant DNA (rDNA) of the production strain was shown to be present above the detection limits.

The inserted DNA does not include any mobile genetic elements. Additionally, it should be highlighted that *T. reesei* genome lacks a significant repetitive DNA component and no extant functional transposable elements have been found in the genome (Kubicek *et al.*, 2011; Martinez *et al.*, 2008). This results to low risk of transfer of genetic material.

2.7. 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 Paper⁴ (Rome, 2006) has also been tested from the fermentation product of the *T. reesei* strain RF6197. The Food Chemicals Codex ("FCC", 9th edition), 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 (JECFA, 2006), was also confirmed from two RF6197 enzyme production batches.

⁴ In the General Specifications for enzyme preparations laid down by JECFA in 2006, the following is said: "Although nonpathogenic and nontoxicogenic 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.

3. ENZYME IDENTITY AND SUBSTANTIAL EQUIVALENCE

3.1. Enzyme Identity

Systematic name	Pectin esterase
Common name	Pectin demethoxylase Pectin methoxylase Pectin methylesterase
Enzyme Commission No.	3.1.1.11
CAS number	9025-98-3

3.2. Amino Acid Sequence

Pectin esterase is a mature protein of 331 amino acids. The amino acid sequence was confirmed by SDS- page, amino-terminal protein sequencing, and amino acid composition analysis.

3.3. Enzymatic Activity

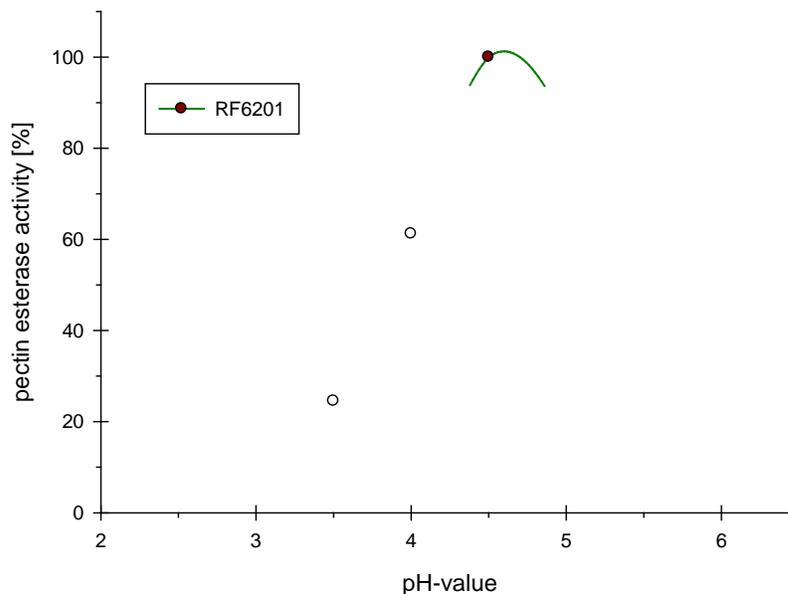
The main activity of RF6201 enzyme preparation is pectin esterase (IUB 3.1.1.11). Pectin esterase is a hydrolyase enzyme that catalyzes the deesterification of methyl ester linkages of galacturonan backbone of pectic substances to release acidic pectins and methanol. Pectin esterase is naturally present in plants (tomato, papaya, and grapes), plant pathogenic bacteria and fungi (Jayani, 2004). 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 esterase has 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 pectinlyases (PL).

The method to analyse 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/mg. 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.

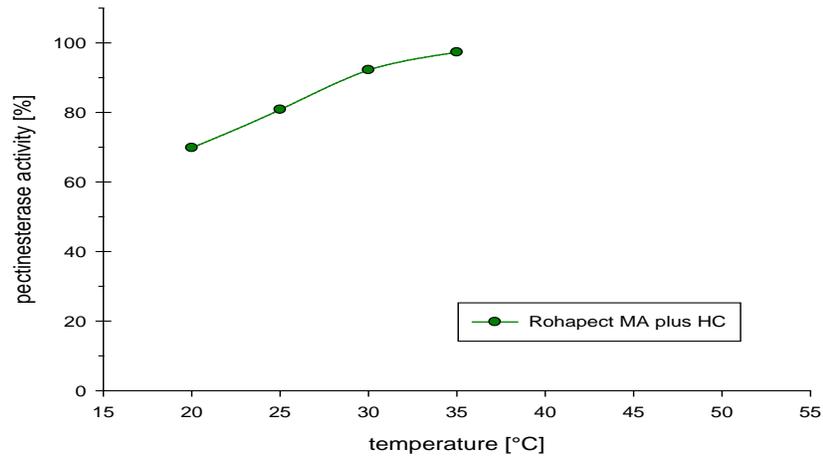
Apart from pectin esterase, the *T.reesei* RF6201 enzyme preparation also contains other enzymatic side activities in small amounts, which are naturally and typically produced by the production organism *T. reesei*, including glucanase, cellulase and xylanase activities. However, these activities are not relevant from an application and/or safety point of view, due to the small amounts and the fact that such enzyme activities have been used and approved for decades in food processing.

The activity of the food enzyme pectin esterase expressed in *T. reesei* was measured under various pH and temperature conditions.

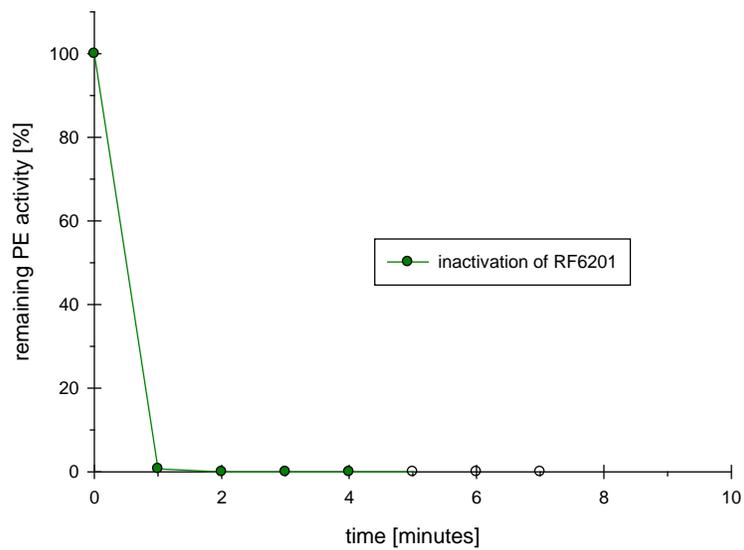
The effect of pH on enzyme activity was evaluated by measuring pectin esterase activity at 30°C in different buffer solutions (10 min incubation time). The pH-optimum was measured to be 4.5. See the curve below.



The effect of temperature on enzyme activity was evaluated by measuring pectin esterase activity at pH 4.5 (10 minutes incubation time). The temperature optimum of the pectin esterase is 40°C, but is applicable in a wide range of temperature:



Additionally, it can be concluded from the following curve that no enzyme activity is left after 1-2 minutes at 85°C.



4. MANUFACTURING PROCESS

4.1. Overview

Like all food enzymes, the pectin esterase enzyme preparation described in this dossier is manufactured in accordance with current Good Manufacturing Practices for Food (cGMPs) and the principals of Hazard Analysis of Critical Control Points (HACCP). Compliance to Food Hygiene Regulation is regularly controlled by relevant food inspection services in Finland.

The *T. reesei* RF6201 production strain described herein is produced by controlled submerged fermentation. The production process involves the fermentation process, recovery (downstream processing) and formulation and packaging. Finally, the measures taken to comply with cGMPs and HACCP are provided. A manufacturing flow-chart is given in Appendix #5.

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 vast majority of cases, the enzyme protein in question is only partially separated from the other organic material present in the food enzyme.

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

4.2.1. 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 ([Section 7.4](#)). The raw materials conform to either specifications set out

in the Food Chemical Codex, 6th edition, 2008 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 antifoam agents and flocculants used in the fermentation and recovery processes are used as described in the Enzyme Technical Association submission to FDA on antifoam and flocculants (April 24, 1998, Appendix #6). The maximum use levels of antifoam and flocculants are $\leq 0.15\%$ and $\leq 1.5\%$ respectively.

4.2.2. 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

4.2.3. Inoculum

A suspension of a pure culture of *T. reesei* RF6201 is aseptically transferred to a shake flask (1 liter) containing fermentation medium.

In order to have sufficient amount of biomass, the process is repeated several times. When a sufficient amount of biomass is obtained the shake flasks are combined to be used to inoculate the seed fermentor.

4.2.4. Seed fermentation

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

4.2.5. Main fermentation

Biosynthesis of the pectin esterase by the production strain occurs during the main fermentation. The content of the seed fermentor is aseptically transferred to the main fermentor containing fermentation medium. The fermentation in the main fermentor is run as normal submerged fermentation under well-defined process conditions (pH, temperature, mixing, etc.).

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.

4.3. 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.

4.3.1. 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.

4.3.2. 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 %.

4.3.3. 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 defined pH and temperature ranges in order 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.

4.3.4. Concentration

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

4.3.5. 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.

4.4. Formulation and Packaging

Following formulation, the final product is defined as a 'food enzyme preparation.' Food enzymes can be sold as dry or liquid preparations, depending on the final application where the enzyme is intended to be used. For all kinds of food enzyme preparations, the food enzyme is standardized and preserved with food ingredients or food additives which are approved in the USA according to ruling legal provisions.

T.reesei RF6201 pectin esterase enzyme preparation is tested by Quality Control for all quality related aspects, like expected enzyme activity and the general testing requirements for Food Enzyme Preparations, and released by Quality Assurance ([see section 4.5](#)). 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. Labels conform to relevant legislation.

4.5. Quality Control of Finished Product

The final enzyme product complies with the recommended 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 (Rome, 2006) and the Monograph "Enzyme Preparations" Food Chemicals Codex (FCC) 9th edition (2014) for food-grade enzymes. Specifications for the food enzyme preparation have been defined as follows:

Property	Requirement
Total viable counts	< 50000 g ⁻¹
Yeasts and fungi	each < 1000 g ⁻¹
<i>E. coli</i>	not present in 25 g
<i>Salmonella</i>	not present in 25 g
Coliform counts	< 30 g ⁻¹
Arsenic	< 3 ppm
Lead	< 2 ppm
Heavy metals	< 30 ppm
Antibacterial Activity	not detectable
Mycotoxins ⁵	No significant levels

5. COMPOSITION AND SPECIFICATIONS

5.1. Formulation

The composition of the final enzyme product is as follows:

Composition	
Constituent ⁶	Average %
PE activity (PE/g)	40,800
Water (%)	37.95
Ash (%)	16.65
Protein (%)	8.85
TOS (%)	19.4
PE activity/mg TOS	208

⁵ See JECFA specifications, <ftp://ftp.fao.org/docrep/fao/009/a0675e/a0675e00.pdf>, page 64: Although nonpathogenic and nontoxic 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.

⁶ The methods by which ash and dry matter content (to calculate the TOS) and protein values are measured are standardized and/or validated methods

5.2. Typical Final Enzyme Preparation Specifications

Property	Requirement	
Pectin esterase activity (PE mg ⁻¹)	Min	6000
pH	4.5	
Appearance	Light brown liquid	
Specific weight	~ 1.17 g ml ⁻¹	

5.3. General Production Controls and Specifications

In order 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). A Cell Bank is a collection of ampoules containing a pure culture. The cell line history and the production of a Cell Bank, propagation, preservation and storage is monitored and controlled. The MCB is prepared from a selected strain. The WCB is derived by sub-culturing of one or more ampoules of the MCB. 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 would immediately result in 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 fermentors, vessels and pipelines are washed after use with a CIP-system (Cleaning in Place), where hot caustic soda and nitric acid 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 downstream 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 (at 121°C for at least 20 min at pH 4.3 – 4.8.).
- 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 suitable 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 (Metso DNA) 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 both the seed and main fermentation and at regular intervals and at critical process steps during fermentation and recovery.

Monitoring of fermentation parameters may include:

- pH
- Temperature
- Dissolved oxygen content
- CO₂

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.

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.

Deviations from the pre-defined values at any of the preceding steps will lead to adjustment or actions ensuring an optimal enzyme product are achieved.

6. APPLICATION

6.1. Mode of Action

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, 2004). 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 (Liu, 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:

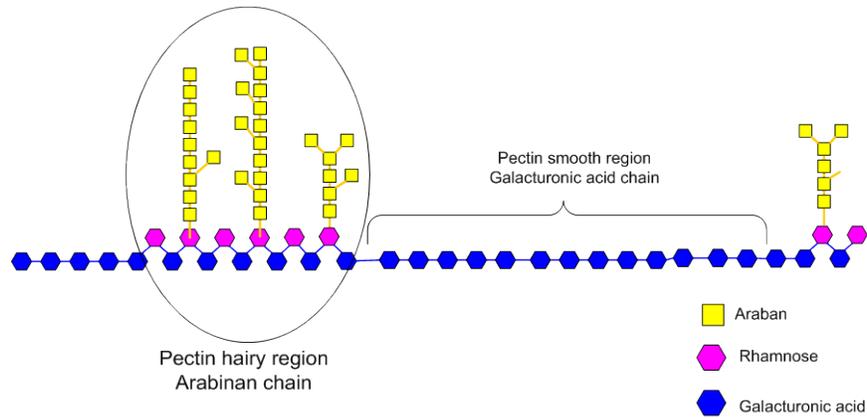


Figure #1: Pectin schematic

Pectin 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 catalyses 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:

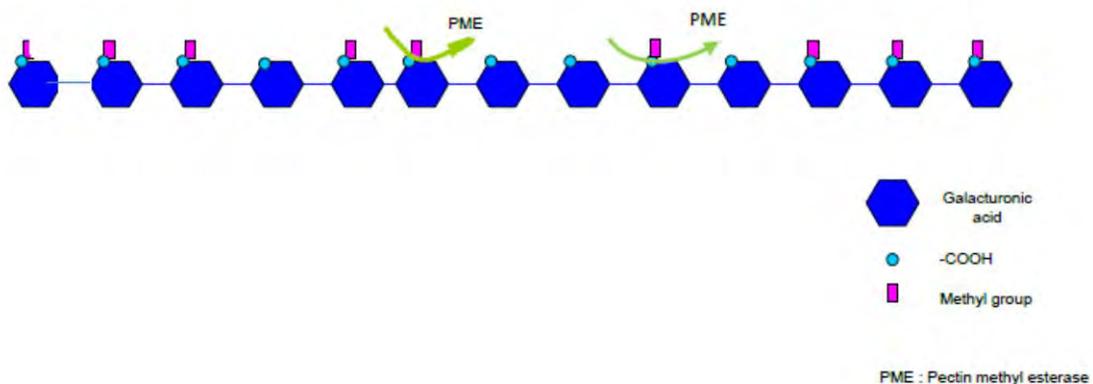


Figure #2 Pectin esterase schematic

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 to provide complete pectin breakdown.

6.2. Application

The pectin esterase from *T. reesei* RF6201 object of this dossier is specifically intended to be used in fruit and vegetable processing (including apple and pear mash treatment, citrus juices, citrus pulp wash, carrot juices, etc, as well as fruit firming and production of fruit purees), wine production, grain treatment (before the grain is milled into flour), coffee processing and flavouring production.

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 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, 2004). 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, 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 behaviour 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 depolymerisation of pectin with the help of pectin esterase⁷ 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 solubilisation of pectin
- Increased cloud stability (reduced turbidity) of the clear concentrate

⁷ In most industrial processing of fruit and vegetable juice, pectin esterase is combined with other enzymes in order to complete the full pectin degradation.

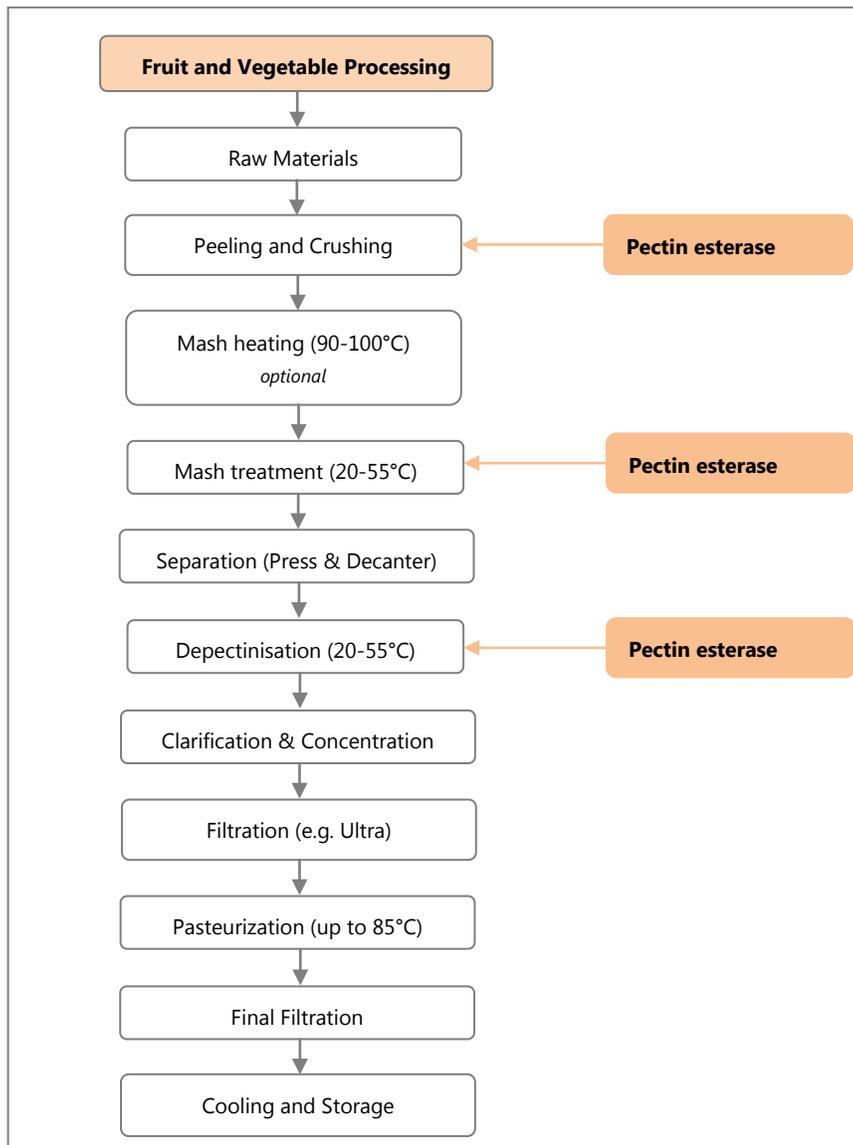


Figure #3 Enzymatic Fruit and Vegetable Processing

The benefits of the pectin backbone demethylation using pectin esterase (in presence of calcium⁸) to produce fruit pieces products and purees are:

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

⁸ 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.

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.

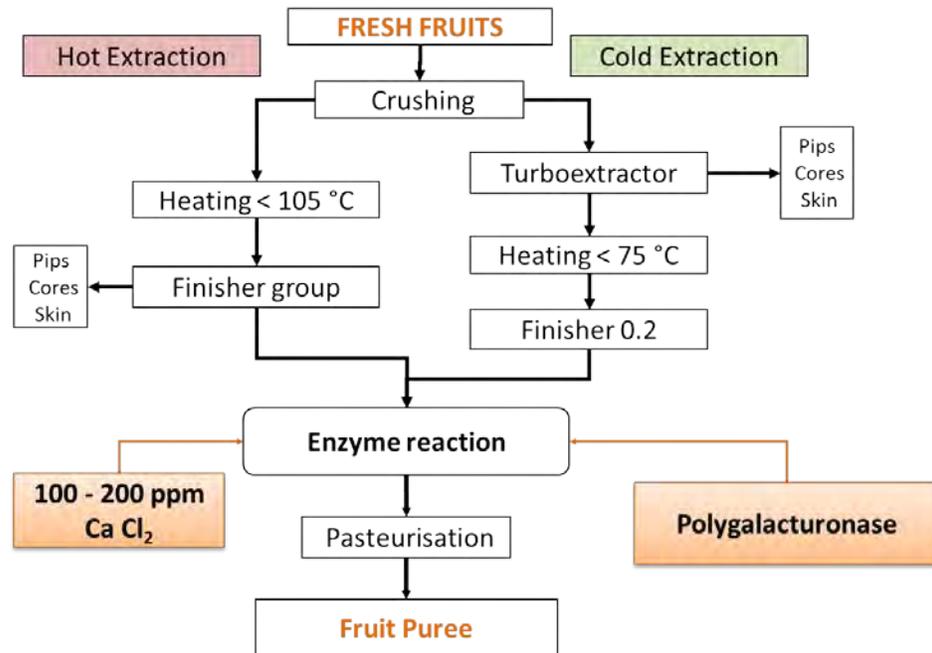


Figure #4 Enzymatic Fruit Puree Processing

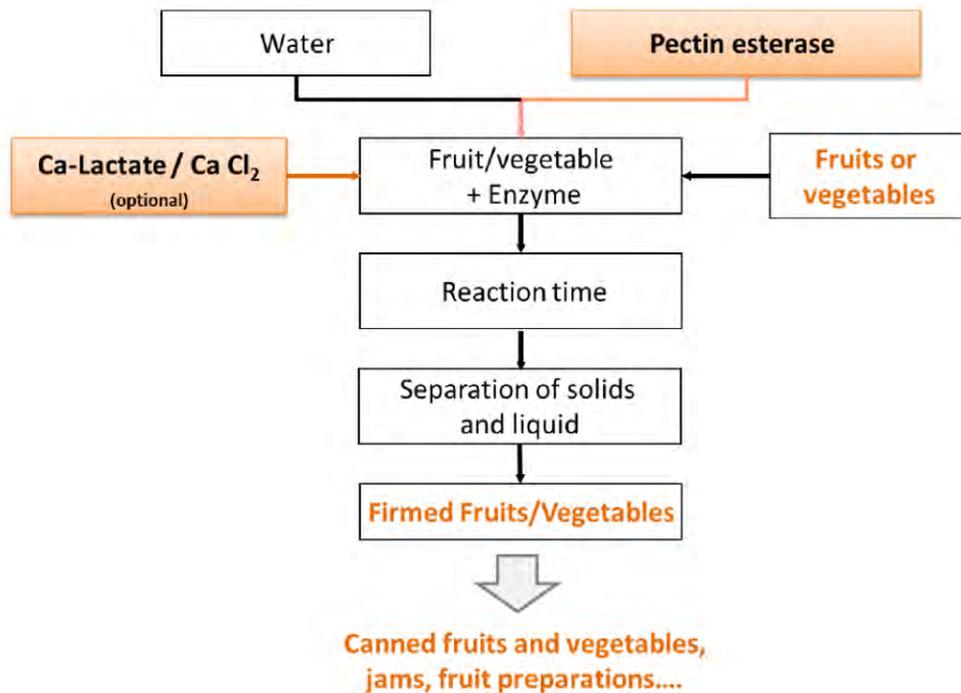


Figure #5 Enzymatic Fruit Firming

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, decolouration, dealcoholisation, enhance flavour development, or augment anthocyanin liberation. Pectinases have been used since the 1960's in wine production (Kashyap, 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 l⁻¹) and are difficult to crush and press. They are de-stemmed, crushed, and heated to 60°C or 80°C to release colour (red grapes) from the skins and to destroy endogenous polyphenoloxidase (Kashyap, 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, 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

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.

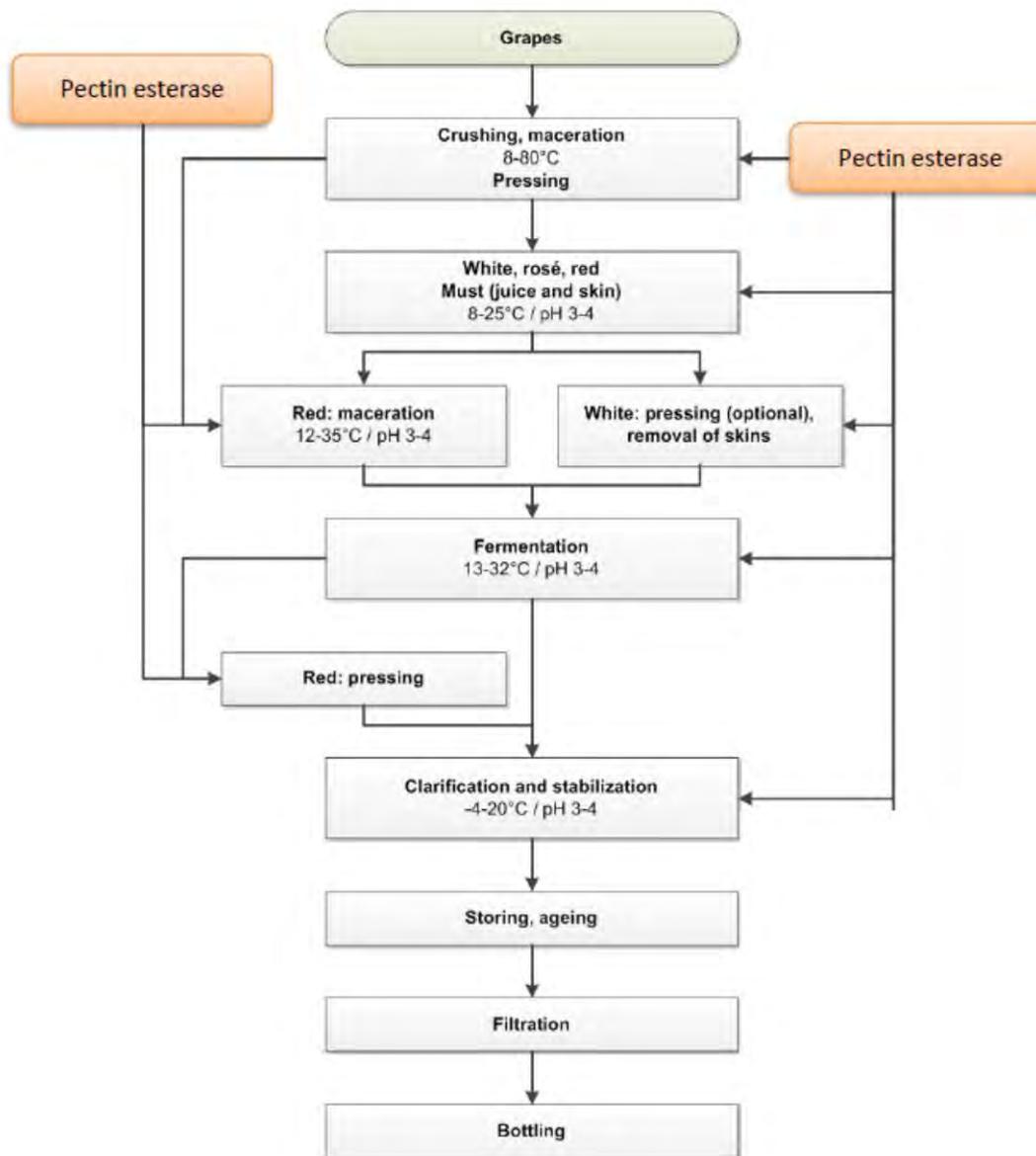


Figure #6: Enzymatic Wine Processing

Grain Treatment

Insufficiently hydrolysed grain (e.g. malt, barley, wheat but as well rye, oat, maize, rice) cell wall components reduce the effectiveness of the mechanical treatments such as milling and peeling to which these grains are further subjected. Degradation of cellulosic material with the help of cellulases into smaller molecules helps to eliminate this problem. As the cellulosic outer layers of grains are a

cross linked matrix of cellulose with embedded pectins, the use of pectin esterase (often together with other pectinases, like polygalacturonase) may be used in synergy with cellulases to help such enzyme to reach their substrate, and thus improve the cellulosic structure degradation.

Beneficial effects of the use of pectinases in grain treatment are:

- Improved processing, e.g. better separation of bran from endosperm, better extraction rates, shorter process times (during water soaking state the water may penetrate faster);
- This will lead to better production economy and environmental benefits such as the use of less raw materials, energy savings, and production of less waste.

The process flow is given in the figure #7 below:

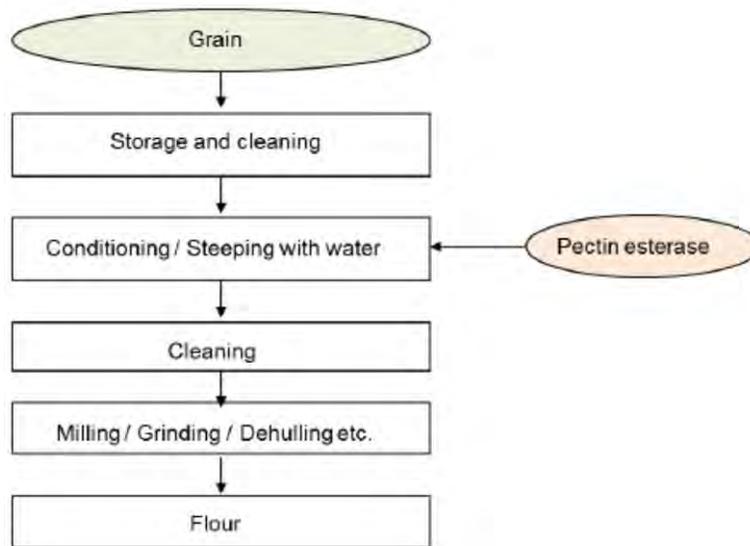
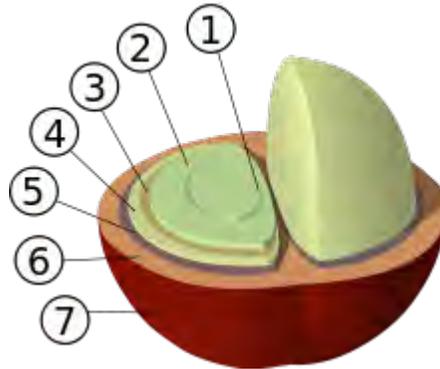


Figure #7: Enzymatic Grain Processing

Coffee Production

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" flavour.

- 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 waste water is achieved.
- As the complete removal of the mucilage layer reduces the drying time, a significant saving of energy is achieved.

The process flow is presented below in diagram #8:

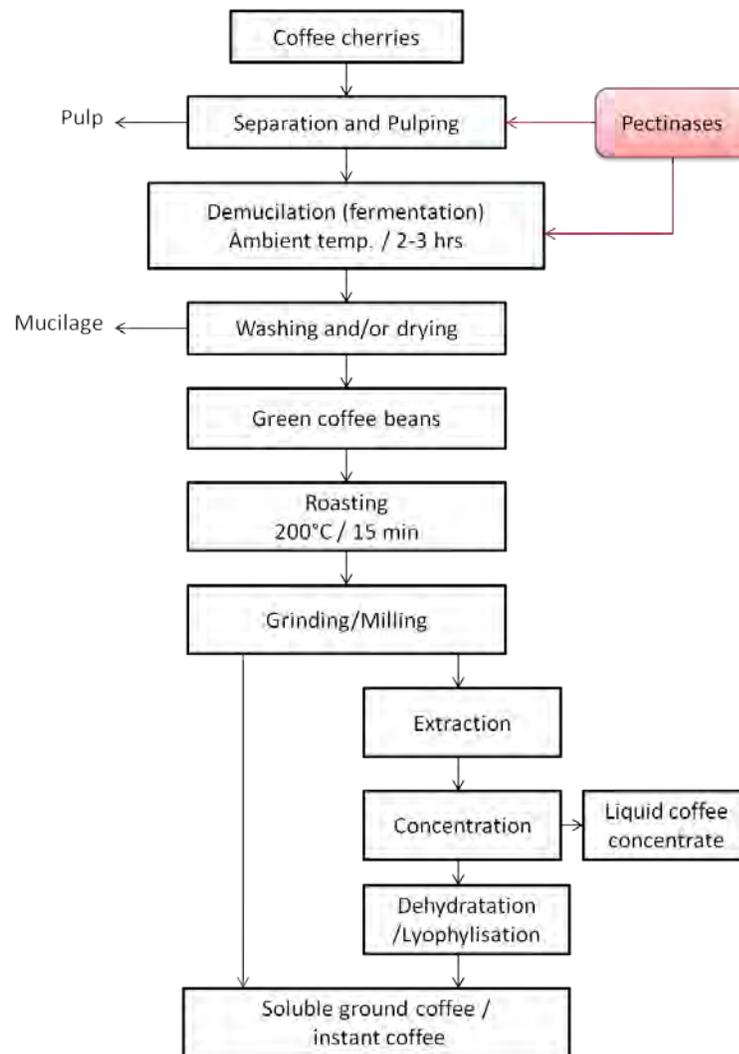


Figure #8 Coffee Production Flow

Flavouring Production

Pectin esterase may be used in the production of flavouring substances and/or preparations. Flavouring 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 flavour 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 (as cited in Sowbhagya, 2010).

6.3. 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 Practices. The amount of enzyme activity added to the raw material by the individual food manufacturer has to be determined case by case, based on the desired effect and process conditions.

Therefore, the enzyme manufacturer can only issue a recommended enzyme dosage range. Such a dosage range is the starting point for the individual food producer to fine-tune his process and determine the amount of enzyme that will provide the desired effect and nothing more.

Consequently, from a technological point of view, there are no 'normal or maximal use levels' and pectin esterase is used according to the QS principle. A food producer who would add much higher doses than the needed ones would experience untenable costs as well as negative technological consequences.

Food enzymes also contain substances derived from the producing microorganism and the fermentation medium, and the presence of all organic material is expressed as Total Organic Solids⁹ (TOS, FAO/WHO, 2006). This distinguishes the proportion of the enzyme preparation derived from the source material from that contributed by diluents, and other additives and ingredients.

Whereas the dosage of a food enzyme depends on the enzyme activity present in the final food enzyme preparation, the dosage on basis of TOS is more relevant from a safety point of view. Therefore, the use levels are expressed in TOS.

The Table below shows the range of recommended use levels for each application where the pectin esterase from *T. reesei* RF6201 may be used:

Application		Raw material (RM)	Maximal recommended use levels (mg TOS/kg RM)
Coffee production		Coffee cherries	0.5
Flavouring production		Fruits/Vegetables	265
Fruit and vegetable processing	Fruit juices	Fruit/Vegetable	3
	Fruit purees	Fruit/Vegetable	24
	Fruit firming	Fruit/Vegetable	12
Grain treatment		Cereals	5
Wine production		Grapes	1

⁹ In the case of food enzymes, which are – per legal definition – not formulated, TOS is the same as Dry Matter minus ash. The amount of ash (e.g. mineral salts used in the fermentation) does generally not exceed a few percent.

6.4. Active and Inactive Enzyme Residues in the Final Food

In principle, the hydrolysis of pectin with the help of pectin esterase can be used in the processing of all fruits and vegetables based foods and food ingredients which naturally contain pectin. In these processes, the pectin esterase is used as a processing aid in food manufacturing and is not added directly to final foodstuffs.

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 favourable

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

Inactivation conditions in pasteurized products:

- Fruit-own pectin esterase: $>80^{\circ}\text{C}$ / $>2\text{min}$
- Pectin esterase: $>75^{\circ}\text{C}$ / $>2\text{min}$

Removal of pectin esterase in non-pasteurized products:

- Precipitation by bentonite
- Removal by filtration processes

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 stabilised 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, sulphur 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.

After grain treatment, the enzyme is mainly removed with the separated bran. In cases where some inactivated enzyme remains, the treated grain is milled into flour prior to the baking process, where the baking (where temperatures inside the dough reach between 95°C and 100°C) will denature the enzyme.

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.

Due to the above mentioned reasons, it can be concluded that pectin esterase enzyme and it's residues from *T.reesei* RF6201 has no technological function in the final food products.

6.4.1. Possible Effects on Nutrients

As the catalytic activity of the enzyme preparation is very specific, i.e. hydrolysis of the polygalacturonic acid chain in the pectin molecule, it is not to be expected that the enzyme preparation will have any significant effect on other constituents or nutrients in food.

Such enzyme activity is widely present in nature and in particular in food ingredients such as fruits and vegetables (broccoli, tomato, papaya, strawberry, etc.) along with bacteria (bacillus) and fungi (*Aspergillus sp. and Saccharomyces*)¹⁰. The substrates and the reaction products are themselves present in food ingredients. No reaction products which could not be considered normal constituents of the diet are formed during the production or storage of the enzyme treated food. Consequently, no adverse effect on nutrients is expected.

7. SAFETY EVALUATION

7.1. Safety of the Production Strain

The insertion of the expression cassette into the genome of the recipient strain *T. reesei* RF5455, results in the recombinant *T. reesei* strain RF6201. The production strain only differs from its recipient strain by its production of pectin esterase gene from *A. tubingensis*.

T. reesei is an industrially important filamentous fungus and has been used as producer of different hydrolases such as xylanase and cellulase for food, animal feed, and pulp and paper industries. It is also used as host for production of heterologous proteins in the same areas. Like many other organisms with a long safe history of industrial use, *T. reesei* strains have been and are being used by many commercial companies in the construction of production strains by genetic engineering.

Trichoderma are metabolically versatile aerobic mesophilic imperfect fungi and are common in soil in all climate zones (Nevalainen *et al.* (1994)). According to Kuhls *et al.* (1996), *T. reesei* is a clonal, asexual derivative of the ascomycete *Hypocrea jecorina* and can be identified by PCR-fingerprinting assay and

¹⁰ <http://www.brenda-enzymes.org/enzyme.php?ecno=3.1.1.11>

sequence analyses of the nuclear ribosomal DNA region containing the internal transcribed spacers (ITS-1 and ITS-2) and the 5.8S rRNA gene (Kuhls et al. (1996)).

T. reesei is regarded as non-pathogenic and non-toxicogenic. The safety of this organism as an enzyme producer has been reviewed by Nevalainen *et al.* (1994), Blumenthal (2004), and Olempska-Beer (2006).

The transformed expression cassettes, fully characterized and free from potential hazards, are stably integrated into the fungal genome (see section 2.3) and are no more susceptible to any further natural mutations than any other genes in the fungal genome. Also, the transformation does not increase the natural mutation frequency. If there were any mutations in the genes affecting the relevant characteristics of the fungus, this would likely be noticed in the growth characteristics in the fermentation and/or in the product obtained, and no such changes have been observed. The possibility of mutations is further decreased by inoculating the seed culture for the fermentation with controlled spore stocks that have been stored at -80°C. There is no indication that this genetic modification will have a negative effect on the safety properties. Therefore, it can be concluded that the *T. reesei* strain RF6201 can be regarded as safe as the recipient or the parental organism to be used for production of enzymes for food processing.

7.2. Safety of the Pectin Esterase Gene

Pectin esterase is a hydrolyase enzyme that catalyzes the deesterification of methyl ester linkages of galacturonan backbone of pectic substances to release acidic pectins and methanol. Pectin esterases are naturally present in plants (tomato, papaya, and grapes), plant pathogenic bacteria and fungi (Jayani, 2004).

Pectin esterase and other pectin-degrading enzymes such as polygalacturonase, pectin lyase and pectate lyase are important enzymes and have been used in the food industry for many years (Sharma *et al.*, 2013). In the Australian New Zealand Food Standards Code – Standard 1.3.3 – processing aids, pectin esterase or pectinase (multicomponent enzyme) is listed as safe for use in food from *Aspergillus niger*, *Aspergillus oryzae*, and *T.reesei* .

Commercial pectin enzyme preparations from various micro-organisms (including genetically modified ones) are widely accepted and *T.reesei* – whether or not genetically modified is widely accepted as a safe production organism for a broad range of enzymes, that have been used as processing aids in the beverage industry for several decades. Pectin esterase from *T. reesei* RF6201 is a commercial enzyme preparation from AB Enzymes, formerly Röhm Enzymes, which has been widely used in food processing for many years and has not been reported to produce any negative impact in either workers or consumers.

To further confirm that the pectin esterase enzyme preparation does not have any toxic properties and to ensure the toxicological safety of the use of the enzyme preparation from *A. tubingensis*, the following studies were conducted:

- Sub-chronic (90 day) oral toxicity study
- Ames test
- Chromosomal aberration test, in vitro

Based upon the results of these studies, it can be concluded that the pectin esterase enzyme preparation does not product adverse effects in rodents, nor was there any mutagenic or clastogenic activity detected, details are provided in section 7.4

7.2.1. Allergenicity

Virtually all food allergens are proteins, although only a small percentage of proteins are allergens. Any food containing protein has the potential to cause allergic reactions, however a few food groups are known to cause allergies more frequently than others. These major allergenic food groups are: milk, eggs, fish, crustacea (shrimp, lobster, and crab), soybeans, peanuts, tree nuts and wheat. Allergens from these food groups account for more than 90% of food allergic reactions. The prevalence of allergic sensitivities to specific foods varies between countries, depending on the frequency with which the foods are consumed and the age at which it is introduction into the diet. Although no general characteristics can be defined that make a protein an allergen, size and structure, glycosylation,

solubility, resistance to heat and sensitivity to enzymatic and acidic degradation are believed to play a role. Most food allergens, perhaps especially those that do cause systemic effects, are resistant to digestion, proteolysis, and other forms of hydrolysis.

Previously, the AMFEP Working Group on Consumer Allergy Risk from Enzyme Residues in Food performed an in-depth analysis of the allergenicity of enzyme products (Dauvrin *et al.*, 1998). The overall conclusion was that – as opposed to exposure by inhalation – there are no scientific indications that the small amounts of enzymes in food can sensitize or induce allergy reactions in consumers.

To evaluate the potential allergenicity of *T.reesei* RF6201 expressed in *Aspergillus tubingensis* pectin esterase, a sequence comparison with known allergenic proteins was done using a database of allergens from the Food Allergy Research and Resource Program (FARRP), University of Nebraska, Allergen Database (Version 14, January 20, 2014), which contains the amino acid sequences of known and putative allergenic proteins.

The analyses were performed in the form of 1) a FASTA generated full-length alignment of the query sequence (the mature 314 amino acid molecule) with the proteins in the database, 2) by searching for identities in all possible 80 amino acid sequence “windows” covering the entire query protein (“Sliding Window”) and 3) by searching for a perfect match of a stretch of eight amino acids anywhere in the query protein to proteins in the database, as recommended in the most recent literature (FAO/WHO, 2001; Ladics *et al.*, 2007; Goodman *et al.*, 2008).

- 1) The alignments resulting from a FASTA search for sequence identities of the full-length pectin esterase protein to any allergenic protein in the Allergen Database (V14) showed no matches greater than 31.5% identity. 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.

- 2) 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 recommended 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 (FAO/WHO 2001). This recommendation was challenged however recently. 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 judgment 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 approach.
- 3) In addition, the pectin esterase protein sequence showed no perfect match to any known allergen when searching for a straight stretch of eight amino acids that could serve as potential IgE binding sites.

As it was reported that the allergenicity prediction based on similarities of protein motifs was superior to that based on amino acid sequence comparison (Stadler *et al.*, 2003) we also performed a motif-based sequence analysis of the mature 314 amino acid molecule using the tools and data provided by ADFS (Allergen Database for Food Safety; <http://allergen.nihs.go.jp/ADFS/index.jsp?pagen=motif>). Again no hits were found.

Furthermore, besides egg lysozyme, the enzyme industry is not aware of enzyme proteins used in food that are homologous to known food allergens. Pectin esterase is used in very small amounts during food processing and as such negligible amounts of the enzyme protein may be found in final food products. A high concentration generally equals a higher risk of sensitization, whereas a low level in the final food equals a lower risk (Goodman et al., 2008). When the proteins are denatured, due to food processing conditions, the tertiary conformation of the enzyme molecule is destroyed. In general, these alterations in conformation are associated with decrease in the antigenic reactivity in humans: in the vast majority of investigated cases, denatured proteins are much less immunogenic than the corresponding native proteins (Valenta and Kraft, 2002; Valenta, 2002; Takai et al., 1997; Takai et al., 2000). In addition, residual enzyme proteins still present in the final food will be subjected to digestion in the gastro-intestinal system, which reduces further the risk of enzyme allergenicity. While stability to digestion is considered as a potential risk factor of allergenicity, it is believed that small protein fragments resulting from digestion are less likely to be allergenic (FAO/WHO, 2001; Goodman et al., 2008). Lastly, food enzymes have a long history of safe use, with no indication of serious adverse effects or reactions. Moreover, a wide variety of enzyme classes (and structures) are naturally present in food. This is in contrast with most known food allergens, which are naturally present in a narrow range of foods.

With respect to the fermentation media used to produce the enzyme preparation it is AB Enzyme's conclusion based on available scientific evidence and the employed manufacturing process that there will not be any allergy causing protein from the fermentation media found in the enzyme preparation. Firstly, the Enzyme Technical Association (ETA) conducted a survey of its members in 2004 to obtain information on the potential presence of protein from the fermentation media in the final enzyme preparations. This information is made public on ETA's website and was provided to FDA in the form of a letter in 2005. The statement concludes that no allergens protein from the fermentation medium has been found in the finished enzyme, and states that regulatory bodies in both the EU and Japan have concluded that enzyme preparations do not pose an allergen risk that would require allergen labeling on the final product (appendix #7). Secondly, the manufacturing process of enzymes ensures that the biomass and fermentation media is separated from the enzyme during filtration - see [section 4](#). Lastly,

the Food Allergy Research and Resource Program (FARRP) issued a report in 2013 which concluded due to the fermentation media being consumed during the enzymatic process and the fact that *de minimis* amount of fermentation media protein survives; there is no significant public health risk to consumers. FARRP also concludes that any protein allergen present in the final enzyme product would not be present at a level that requires allergen labelling.

In summary, the bioinformatics approach to estimate potential allergenicity 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 *T.reesei* RF6201 is of no concern.

Pectinases of microbial origin have been used in food for decades (Grassin, C. and Fauquembergue, P., 1996; Sharma *et al.*, 2013). We have no knowledge of any reports of allergic reactions to the residues of pectin esterase in food as well as to the residues of other enzymes (except for egg lysozyme) used in food processing.

By analogy, and on the basis of the results obtained from a sequence homology comparison test, and on the fact that the enzyme is typically denatured during the food manufacturing process and that any residual enzyme still present in the final food will be subject to digestion in the gastro-intestinal system, it is not likely that the pectin esterase produced by *T.reesei* RF6201 under evaluation will cause allergic reactions after ingestion of food containing the residues of these enzymes.

7.2.2. Leading Publications on the Safety of Pectin Esterase Enzyme or Enzymes that are Closely Related

The production organism *T.reesei* RF6201 has been demonstrated to be non-toxicogenic and non-pathogenic and any food ingredient (enzyme) from that organism will exhibit the same safety properties if manufactured under current Good Manufacturing Practices ("cGMPs"). Pariza and Foster (1983) noted that a non-pathogenic organism was very unlikely to produce a disease under ordinary circumstances. In their publication, *T. reesei* is included in the authors' listing of the organisms being

used in the industry. The evaluation of the safety of the genetic modification should be examined based on the concepts outlined in the Pariza and Foster (1983) paper. Their basic concepts were further developed by the JFBC in 1990, the EU Scientific Committee for Food in 1991, the OECD in 1993, ILSI Europe Novel Food Task Force in 1996 and FAO/WHO in 1996. Basically, the components of these evaluations start with an identified host strain, descriptions of the plasmid used and the source and fraction of the material introduced, and an outline of the genetic construction of the production strain. This information is found in Section 2.

The FDA has also accepted the GRAS Notifications stating that pectinlyase (GRN 32), chymosine (GRN 230), transglucosidase (GRN 315), protease (GRN 333), glucoamylase (GRN 372) enzyme preparations from *T. reesei* are generally recognized as safe. *T. reesei* is listed as a production organism for enzymes (Pariza and Johnson (2001)) and has a long history of safe use (also see Section 7.1).

As is clear from the information provided in this notification, there have been genetic modifications to *T. reesei* used by AB enzymes, but these genetic modifications are thoroughly well characterized and specific in that the DNA encoded does not express any harmful or toxic substance. The safety studies described in Section 7.4 of this dossier support the fact that the genetic modification did not result in any toxic effects.

7.3. Safety of the Manufacturing Process

T. reesei RF6201 meets the general and additional requirements for enzyme preparations as outlined in the monograph on Enzyme Preparations in the Food Chemicals Codex.

As described in Section 4, the *T. reesei* RF6201 pectin esterase production strain is produced in accordance with cGMPs using ingredients that are acceptable for general use in foods, under conditions that ensure a controlled fermentation. These methods are based on generally available and accepted methods used for the production of microbial enzymes.

7.4. Safety Studies

This section describes the studies performed to evaluate the safety of the *T.reesei* RF6201 enzyme preparation. All safety studies were performed according to internationally accepted guidelines (OECD or FDA) and are in compliance with the principles of Good Laboratory Practice (GLP) according to the FDA/OECD.

7.4.1. Summary of Safety Studies

The following studies were performed with *T.reesei* RF6201 pectin esterase production strain:

- Ames Test
- Chromosome aberration test
- 90-day oral toxicity study in rats

These safety studies were conducted using the enzyme preparation concentrate, not with the diluted final product. All studies were conducted using the same production batch, Batch No. PE 10025 A3, with 94.2% TOS. Dose calculations for the experiments were adjusted to account for TOS.

The composition of the test material is as follows:

Batch No	PE 10025 A3
Ash (%)	0.81
Water (%)	5.0
TOS (%)	94.2
Activity (PE/g)	382,000
Activity /mg TOS	405
Protein (%)	65.3

7.4.2. Results of the Safety Studies

7.4.2.1. Ames Test

The test, based on OECD Guidelines No. 471 (OECD, 2000a), was run at Harlan, Cytotest Cell Research GmbH (Harlan CCR) Rossdorf – Germany, during June 28th, 2010 – July 15th, 2010.

This study was performed to investigate the potential of pectin esterase from *T.reesei* RF6201 to induce gene mutations according to the plate incorporation test (experiment I) and the pre-incubation test (experiment II) using the *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, TA 100, and TA 102.

The assay was performed in two independent experiments both with and without liver microsomal activation. Each concentration, including the controls, was tested in triplicate.

The test item was tested at the following concentrations (dose calculation was adjusted to TOS):

— Pre-Experiment/Experiment I: 3; 10; 33; 100; 333; 1,000; 2,500; and 5,000 µg/plate

— Experiment II: 33; 100; 333; 1,000; 2,500; and 5,000 µg/plate

The plates incubated with the test item showed normal background growth up to 5,000 µg/plate with and without S9 mix in all strains used.

No toxic effects, evident as a reduction in the number of revertants (below the indication factor of 0.5), occurred in the test groups with and without metabolic activation.

No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment with pectin esterase produced with *T.reesei* at any dose level, neither in the presence nor absence of metabolic activation (S9 mix). There was also no tendency of higher mutation rates with increasing concentrations in the range below the generally acknowledged border of biological relevance.

Appropriate reference mutagens were used as positive controls and showed a distinct increase of induced revertant colonies.

In conclusion, it can be stated that during the described mutagenicity test and under the experimental conditions reported, the test item did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used.

Therefore, the pectin esterase from *T.reesei* RF6201 was considered to be non-mutagenic in this *Salmonella typhimurium* reverse mutation assay.

7.4.2.2. Chromosomal Aberration Test

The test, based on OECD Guidelines No. 473 (OECD, 2000b), was run at Harlan, Cytotest Cell Research GmbH (Harlan CCR) Rossdorf – Germany, during 27 May 2010 –30 June 2010.

The pectin esterase from *T.reesei* RF6201 was assessed for its potential to induce structural and numerical chromosome aberrations in V79 cells of the Chinese hamster in vitro in two independent experiments.

In each experimental group two parallel cultures were set up. At least 100 metaphases per culture were evaluated for structural chromosome aberrations, except for the positive control in Experiment II without metabolic activation, where only 50 metaphases were evaluated. The highest applied concentration (5,310 µg/mL adjusted to TOS) was chosen with respect to the current OECD Guideline 473. Dose selection for the cytogenetic experiments was performed considering the toxicity data.

In the absence and presence of S9 mix no cytotoxicity was observed up to the highest applied concentration.

No clastogenicity was observed at the concentrations evaluated either with or without metabolic activation.

No relevant evidence of an increase in polyploid metaphases was noticed after treatment with the test item as compared to the control cultures.

Appropriate mutagens were used as positive controls. They induced statistically significant increases ($p < 0.05$) in cells with structural chromosome aberrations.

In conclusion, it can be stated that under the experimental conditions reported, no biologically relevant increases of chromosomal aberrations were observed.

Therefore the pectin esterase from *T.reesei* RF6201 is classified as non-clastogenic, when tested up to the highest concentration required by the guideline and adjusted to TOS.

In vivo tests were not performed, as there was no in vitro mutagenicity detected.

7.4.2.3. 90-Day Sub-Chronic Toxicity Study

The test was performed according to the following guidelines: OECD No. 408 (OECD, 2000c) at Harlan Laboratories Ltd (Itingen, Switzerland) in February-April 2013.

In this subacute toxicity study, pectin esterase from *T.reesei* RF6201 was administered daily by oral gavage to SPF-bred Wistar rats of both sexes at dose levels of 100, 300 and 1,000 mg TOS/kg body weight/day for a period of 91 days. A control group was treated similarly with the vehicle, bi-distilled water, only.

The groups comprised 10 animals per sex which were sacrificed after 91 days of treatment. Clinical signs, outside cage observation, food consumption and body weights were recorded periodically during the acclimatization and treatment periods. Ophthalmoscopy, clinical laboratory diagnostics, functional observational battery, locomotor and grip strength were performed during week 13.

At the end of the dosing, blood samples were withdrawn for hematology and plasma chemistry analyses. Urine samples were collected for urinalyses. All animals were sacrificed, necropsied and examined post mortem. Histological examinations were performed on organs and tissues from all control and high dose animals, and all gross lesions from all animals.

Mortality / Viability: There were no test item-related deaths.

Clinical Signs (Daily and Weekly): There were no test item-related findings of toxicological relevance during the daily observations or during the weekly behavioral observations (weeks 1 to 12) at any dose.

Functional Observational Battery: There were no clinical observations evident during the functional observational battery (week 13) at any dose level.

Grip Strength: The mean fore- and hind limb grip strength values of the test item-treated rats compared favorably with those of the respective control rats.

Locomotor Activity: Minor differences in the locomotor activity of some males were not seen in the female rats of the same groups and therefore were not considered to be findings of toxicological relevance.

Food Consumption: There were no test item-related differences in the mean daily food consumption of the males and females at any dose level.

Body Weights: There were no test item-related differences in the mean body weights of the males and females at any dose level. Males treated with 1000 mg/kg/day had reduced mean body weight gain from day 8 onwards that was considered to be related to the treatment with the test item.

Ophthalmoscopic Examinations: There were no test item-related ophthalmoscopic changes at any dose level.

Clinical Laboratory Investigations:

Hematology: There were no test item-related differences in the mean hematology parameters at any dose level.

Clinical Biochemistry: There were no test item-related differences in the mean clinical biochemistry parameters at any dose level.

Urinalysis: There were no test item-related differences in the mean urinalysis parameters at any dose level.

Organ Weights: There were no test item-related changes in the mean absolute or relative organ weights at any dose level.

Macroscopic / Microscopic Findings: There were no test item-related macroscopical changes. At the end of the treatment period, microscopic changes related to the treatment with the test item were observed in the adrenal cortex of males treated with 1000 mg/kg/day, and consisted of an increased incidence and severity of diffuse fatty changes in the adrenal cortices. The higher incidence of diffuse fatty change in the adrenal cortex of males treated with 300 mg/kg/day when compared to control was still within the historical data of control male rats of this strain and age in this laboratory and, therefore, not regarded to be test item related.

The remainder of findings recorded was within the range of normal background lesions which may be recorded in rats of this strain and age.

Conclusion:

Based on the results of this study, 300 mg TOS /kg body weight/day of pectin esterase from *T.reesei* RF6201 was established as the no-observed-effect-level (NOEL) and 1,000 mg/kg body weight/day as the no-observed-adverse-effect-level (NOAEL).

7.5. Estimates of Human Consumption and Safety Margin

7.5.1. Estimate of Dietary Exposure

As described herein the *T.reesei* RF6201 production strain is used in the manufacturing of pectin esterase. The most appropriate way to estimate the human consumption in the case of food enzymes is using the so-called Budget Method, originally known as the Danish Budget Method (Hansen (1966); Douglass *et al.* (1997)). This method enables one to calculate a Theoretical Maximum Daily Intake

(TMDI) based on conservative assumptions regarding physiological requirements for energy from food and the energy density of food rather than on food consumption survey data.

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

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

Consumption of food patterns:

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

In Section 6.3, the recommended use levels of pectin esterase are given based on the raw materials used in the food processes. For the calculation of the TMDI, the maximum use levels are chosen. Furthermore, the calculation takes into account how much food (or beverage) is obtained per kg raw material and it is assumed that all the TOS will end up in the final product and the wide variety of food products based on edible oils that are available to consumers.

Applications		Raw material (RM)	Maximal recommended use level (mg TOS/kg RM)	Final food (FF)	Ratio RM/FF*	Maximal level in final food (mg TOS/kg food)
Liquid foods	Coffee production	Coffee cherries	0.5	Coffee	40	20
	Flavouring production	Fruit/vegetable	265	Various beverages	0.01	2.65
	Fruit and vegetable processing	Fruit/vegetable	3	Juices	1.3	3.9
	Wine production	Grape	1	Wine	1.6	1.6
Solid foods	Flavouring production	Fruit/vegetable	265	Various solid foods	0.01	3
	Fruit and vegetable processing	Fruit/vegetable	24	Purees	1	24
			12	Processed fruits (canned fruits, jams)	1	12
	Grain treatment	Cereals	5	Bread	0.56	2.8

* Assumptions behind Ratio of Raw Material / Final Food:

- *Flavourings are generally used in small amounts in final foods. Depending on the composition of the flavouring 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 flavouring per kg of final food.*
- *For fruit juices, we assume that a RM/FF ratio of 1.3 kg fruit per L of fruit juice will be used (typically 0.75-0.9 l juice is produced per kg of fruit thus the range for RM/FF will be 1.1-1.3 kg fruit per L of fruit juice).*

- For fruit purees and fruit pieces (fruit firming application), we assume a RR/FF of 1 (1 kg of fruits / kg of processed fruit, ie puree or fruit piece).
- For coffee processing, we assume that a RM/FF of 40 will be used (100 kg de-pulped coffee cherries lead to 330 g green coffee and 1kg green coffee leads to the production of 0.38 kg ground coffee, 0.38 kg ground coffee is 5% of the final food).
- For grain treatment, we assume that a RM/FF ratio of 0.56 kg bread per kg treated grain will be used (corresponding to a ratio of 0.8 kg flour per kg treated grain and a ratio of 0.7 kg bread per kg flour).
- 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 TMDI can be calculated on basis of the maximal values found in food and beverage (in this case, purees and juices respectively) multiplied by the average consumption of food and beverage/kg body weight/day.

The Total TMDI will consequently be calculated as follows:

TMDI in food (mg TOS/kg body weight/day)	TMDI in beverage (mg TOS/kg body weight/day)	Total TMDI (mg TOS/kg body weight/day)
24 x 0.0125 = 0.3	20 x 0.025=0.5	0.8

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

- It is assumed that ALL producers use pectin esterase from *T.reesei* RF6201 in all food stuff mentioned above;
- 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 as a result of the food production process;

- It is assumed that the final food and beverages 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)).

7.5.2 Safety Margin

Summarizing the results obtained from the several toxicity studies the following conclusions can be drawn:

- No mutagenic or clastogenic activity under the given test conditions were observed;
- The sub-chronic oral toxicity study showed a No Observed Adverse Effect Level (NOAEL) of at least 1000 mg TOS/kg body weight/day.

The Margin of Safety (MoS)¹¹ for human consumption can be calculated by dividing the NOAEL by the Total Theoretical Maximal Daily Intake (TMDI). The Total TMDI of the food enzyme is 0.8 mg TOS/kg body weight/day. Consequently, the MoS is:

$$\text{MoS} = 1,000 / 0.8 = 1250$$

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

8. Conclusion

Results of the toxicity and mutagenicity tests described in [Section 7.4](#) demonstrate the safety of pectin esterase enzyme preparation from *T. reesei* RF6201, which showed no toxicity or mutagenicity across a

¹¹ A "margin of safety" (MOS) is calculated as the ratio of the outcomes of the effect assessment and the exposure assessment, by dividing the N(L)OAE (usually given in mg/kg bodyweight/day) by the corresponding measured or predicted exposure (also in mg/kg bw/day).

variety of test conditions. The data resulting from these studies is consistent with the long history of safe use for *T. reesei* and pectin esterase in food processing, and in keeping with the conclusions found in a review of relevant literature. Based upon these factors, as well as upon the limited and well characterized genetic modifications allowing for safe production of the enzyme preparations, it is AB Enzymes' conclusion that pectin esterase enzyme preparation from *T. reesei* RF6201 expressing the gene encoding pectin esterase from *Aspergillus tubingensis* is GRAS for the intended conditions of use described herein.

9. List of Appendices

#1 *Aspergillus* RH3544 - DSMZ Certificate, 1982

#2 *Aspergillus tubingensis* Mosseray RH3544 – CBS Certificate, 2012

#3 FDA GRAS Notice #32, 2000

#4 Pariza Johnson decision tree

#5 Manufacturing flow chart

#6 FDA Response to ETA Letter – Defoaming agents and flocculants, 2003

#7 ETA Letter “ETA Position on Food Allergen Labelling of Microbially Derived Enzymes Under FALCPA as it applies to Fermentation Media Raw Materials, 2005

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Centraalbureau voor Schimmelcultures

Fungal Biodiversity Centre

Institute of the Royal Netherlands Academy of Arts and Sciences (KNAW)

AB Enzymes GmbH
i.V. Dr. Patrick Lorenz
Feldbergstraße 78
D-64293 Darmstadt
Germany

MGT	FC	SC	HR	GBO
R&D	AB Enzymes GmbH			KAM
QM	II			RM
IPM	29. AUG. 2012			BUFS
RA	MGT			BUTE
				BUFE

Utrecht, August 24, 2012

CBS IDENTIFICATION SERVICE

Your ref.: **RH3544**

Our ref.: **Det 12.060**

Please state always our reference number when you contact us.

Dear Dr. Patrick Lorenz,

Herewith we inform you about the results of our identification of your strain(s).

RH3544 = *Aspergillus tubingensis* Mosseray

The invoice for this identification will be sent separately.

Yours sincerely,


M. Meijer, Bsc.

DSM ■ DEUTSCHE SAMMLUNG VON MIKROORGANISMEN

German Collection of Microorganisms

GESELLSCHAFT FÜR BIOTECHNOLOGISCHE FORSCHUNG MBH

DSM · Grisebachstrasse 8 · D-3400 Göttingen, Germany

Tel. (05 51) 39 38 22 / 39 38 23

Datum/Date 08.03.19

Röhm GmbH
Chemische Fabrik
Kirschenallee

6100 Darmstadt

BESTÄTIGUNG

Der von Ihnen eingesandte Pilzstamm mit der Bezeichnung **RH 3544** ist von uns an Hand morphologischer Merkmale als

Aspergillus niger van Tieghem 1867

bestimmt worden.

Göttingen, den 08.03.1982

Stempel und Unterschrift

(b) (6)

DEUTSCHE SAMMLUNG VON MIKROORGANISMEN
der

Gesellschaft für Biotechnologische Forschung mbH
Grisebachstraße 8
D-3400 Göttingen

Gesellschaft für Biotechnologische Forschung mbH, Mascheroder Weg 1, 3300 Braunschweig, Tel.: (05 31) 70 08—1, Telex: 9—5 26 67

Vorsitzender des Aufsichtsrats:
Min. Dir. Dr. Friedrich Bischoff

Geschäftsführer:
Dr. Maria-Regina Kula
Dr. Helmut Zeitträger

Bankkonto:
Gebr. Löbbecke, Braunschweig
Konto 23 781 (BLZ 270 305 00)

Registergericht:
Amtsgericht Braunschweig
HRB 477
000077

Identifizierung eines Pilzstammes nach morphologischen Merkmalen

Einsender: Dr. Schuster, Röhm GmbH, Darmstadt

Stammbezeichnung: RH 3544

Anzucht: Czapek-Dox und Malzextrakt Agar, 26 C

Kolonie: 40-45 mm Ø in 14 d, 3-4 mm Randzone ohne Luftmyzel;
Luftmyzel reichlich, weiß, zentral leicht gelblich
Sporulation schwach, Köpfchen schwarz, radiär, später
in mehrere Säulen gespalten, vereinzelt über 1 mm
hoch; Rückseite gelblich.

Morphologie:

Konidiophoren: glattwandig, farblos-bräunlich, meist vom
Substrat ausgehend, ca. 20 µm Ø.

Vesikel: kugelig, farblos-schwach bräunlich, -40 µm Ø

Metulae: vorhanden, zylindrisch, 5 x 15 µm

Phialiden: flaschenförmig, farblos, 2-4 x 8 µm

Konidien: kugelig, fein stachelig, bräunlich, 4-5 µm Ø

Bestimmung: Nach Raper & Fennell The Genus Aspergillus 1965

Gruppe: Aspergillus niger

Art: Aspergillus niger van Tieghem 1967

Anmerkung: Die Sporulation dieses Stammes ist schwächer als bei
typischen Isolaten.



U.S. Food & Drug Administration



[Home](#) [Food](#) [Food Ingredients & Packaging](#) [Generally Recognized as Safe \(GRAS\)](#)

Agency Response Letter GRAS Notice No. GRN 000032

CFSAN/Office of Premarket Approval

April 20, 2000

Gary L. Yingling
McKenna and Cuneo, L.L.P.
1900 K Street, N. W.
Washington, D.C. 20006-1108

Re: GRAS Notice No. GRN 000032

Dear Mr. Yingling:

The Food and Drug Administration (FDA) is responding to the notice, dated October 13, 1999, that you submitted on behalf of Rohm Enzyme GmbH in accordance with the agency's proposed regulation, proposed 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS)). FDA received the notice on October 14, 1999 and designated it as GRAS Notice No. GRN 000032.

The subject of the notice is the pectin lyase enzyme preparation obtained from a *Trichoderma reesei* (formerly classified as *Trichoderma longibrachiatum*), which contains a recombinant gene encoding pectin lyase (also called pectin transeliminase) from *Aspergillus niger* var. *awamori*. The notice informs FDA of the view of Rohm Enzyme GmbH that the pectin lyase enzyme preparation is GRAS, through scientific procedures, for use as a processing aid for the preparation of fruit and vegetable juices, purees and concentrates by various production processes, including the pulp wash processes and in-line pulp wash processes in the processing of citrus fruits. According to Rohm Enzyme GmbH, the pectin lyase preparation will be used for the degradation of the pectin to lower the viscosity and to clarify juices at a dose of 5 to 100 parts per million (ppm) to accomplish the intended technical effect in accordance with current good manufacturing practices.

The notice describes (1) published information pertaining to the safety of the various components of the production organism, including the host (*T. reesei*) and the donor (*Aspergillus niger*) organisms; (2) published information about the safety evaluation of microbial-derived food-grade enzyme preparations, including commercial pectinase enzyme preparations; (3) published information about the technical effect of the enzyme preparation; (4) published information related to the production process of the pectin lyase enzyme preparation; (5) scientific publications and recommendations issued by international organizations on the safety of enzymes used in food processing including enzymes derived from genetically modified microorganisms; and (6) unpublished studies conducted with the production strain and the pectin lyase enzyme preparation from *A. niger*.

The notice states that the pectin lyase preparation meets the specifications for enzyme preparations provided in the Food Chemicals Codex (4th ed., 1996) and the specifications for enzyme preparations provided by the Joint Expert Committee on Food Additives (JECFA; a joint committee of the Food and Agriculture Organization/World Health Organization).

Based on the information provided by Rohm Enzyme GmbH, as well as other information available to FDA, the agency has no questions at this time regarding the conclusion of Rohm Enzyme GmbH that the pectin lyase enzyme preparation is GRAS under the intended conditions of use. The agency has not, however, made its own determination regarding the GRAS status of the subject use of the pectin lyase enzyme preparation. As always, it is the continuing responsibility of Rohm Enzyme GmbH to ensure that food ingredients that the firm markets are safe, and are otherwise in compliance with all applicable legal and regulatory requirements.

In accordance with proposed 21 CFR 170.36(f), a copy of the text of this letter, as well as a copy of the information in your notice that conforms to the information in proposed 21 CFR 170.36(c)(1), is available for public review and copying on the Office of Premarket Approval's homepage on the World Wide Web.

Sincerely,

Alan M. Rulis, Ph.D.
Director
Office of Premarket Approval
Center for Food Safety and Applied Nutrition

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U.S. Food and Drug Administration
10903 New Hampshire Avenue
Silver Spring, MD 20993
Ph. 1-888-INFO-FDA (1-888-463-6332)

ANALYSIS OF SAFETY BASED ON PARIZA/JOHNSON DECISION TREE

Pariza and Johnson have published updated guidelines for the safety assessment of microbial enzyme preparations (2001)¹ from the 1991 IFBC Decision Tree². The safety assessment of a given enzyme preparation is based upon an evaluation of the toxigenic potential of the production organism. The responses below follow the pathway indicated in the decision tree as outlined in Pariza and Johnson, 2001. The outcome of this inquiry is that pectin esterase enzyme preparation from *A. tubingensis* expressed in *T. reesei* RF6201 is "ACCEPTED" as safe for its intended use.

Decision Tree:

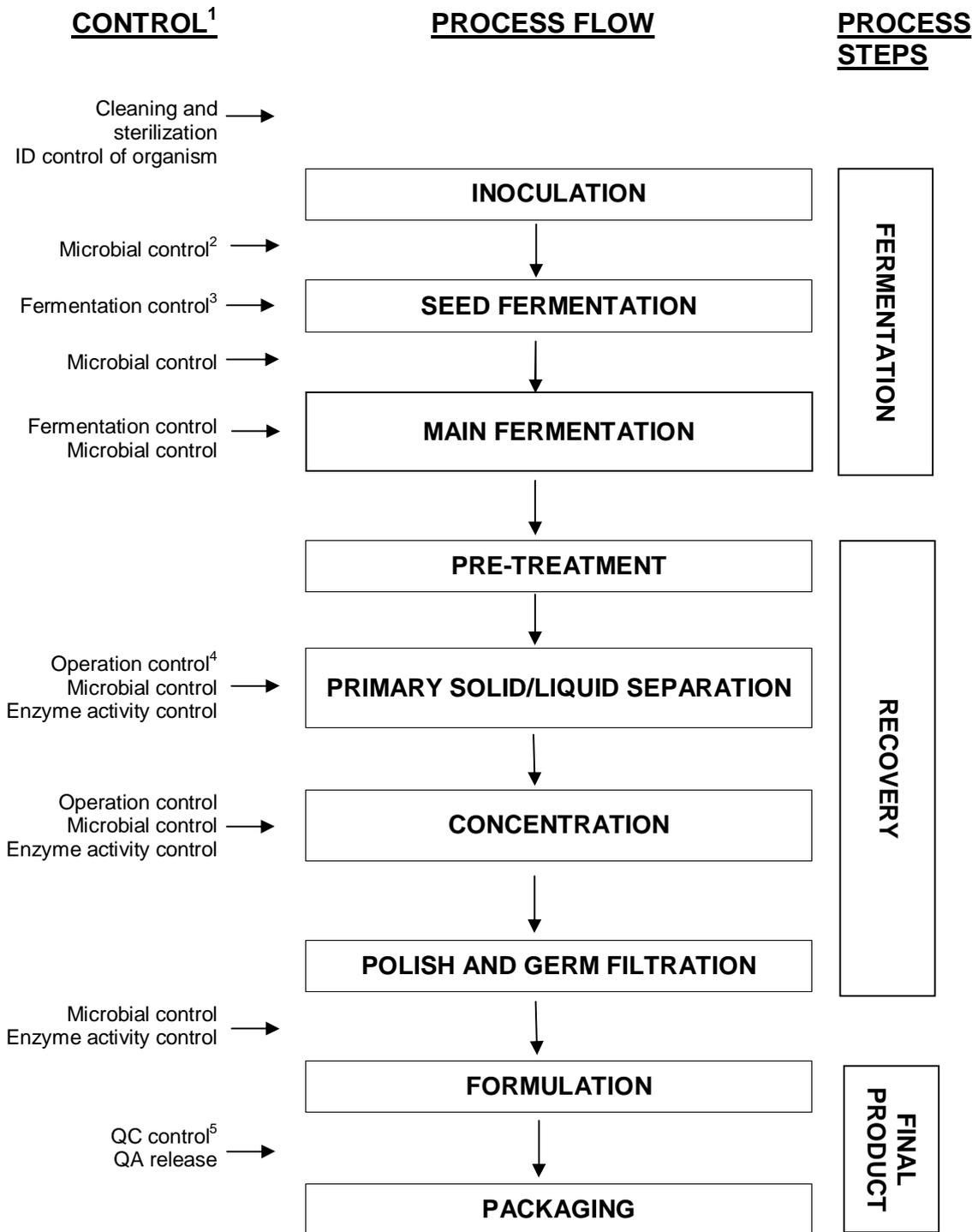
- 1. Is the production strain genetically modified?** *Trichoderma reesei* RF6201 for *Aspergillus tubingensis* pectin esterase production was constructed by transforming the *A. tubingensis* pectin esterase gene expression cassettes to *T. reesei* RF5455, as several copies.
Yes go to #2;
- 2. Is the production strain modified using rDNA techniques?** Yes go to #3a;
- 3.**
 - 3a. Does the expressed enzyme product which is encoded by the introduced DNA have a history of safe use in food?** Yes, Go to 3c;
 - 3c. Is the test article free of transferable antibiotic resistance gene DNA?** Yes, transferable DNA was not detected in the pectin esterase preparation manufactured using *T. reesei* and production process described herein. Additionally, no antibiotic resistance gene has been integrated. Go to 3e;
 - 3e. Is all other introduced DNA well characterized and free of attributes that would render it unsafe for constructing microorganisms to be used to produce food-grade products?** Yes, inserted DNA is well characterized. Go to 4;
- 4. Is the introduced DNA randomly integrated into the chromosome?** Yes, go to #5;

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

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

5. **Is the production strain sufficiently well characterized so that one may reasonably conclude that unintended pleiotropic effects which may result in the synthesis of toxins or other unsafe metabolites will not arise due to the genetic modification method that was employed?** Yes, there is no concern for pleiotropic effects. Go to #6;
6. **Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure?** Yes, *T. reesei* has been demonstrated as a safe production host and methods of modification have been well documented. Safety of this organism has been evaluated and confirmed through toxicological testing as described herein. **ACCEPTED**

Production Process of Food Enzymes from Fermentation



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

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

³ During fermentation parameters like e.g. pH, temperature, oxygen, CO₂, sterile air overflow are monitored / controlled.

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

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



September 11, 2003

Mr. Gary Yingling
Kirkpatrick & Lockhart LLP
1800 Massachusetts Avenue, NW
Second Floor
Washington, DC 20036-1221

Dear Mr. Yingling:

You requested, on behalf of the Enzyme Technical Association, that OFAS review the use of certain defoaming and flocculating agents in the manufacture of enzyme preparations used in food. You provided information related to these compounds in your letters of December 20, 1996 (to Dr. Alan Rulis), 4-24-1998 (to Dr. Zofia Olempska-Beer), and 11-30-99 (to Dr. Zofia Olempska-Beer). You also arranged for a teleconference between ETA members and OFAS representatives, facilitated telephone contacts with technical experts from ETA member companies, and responded to numerous requests for clarification. We appreciate your and ETA's cooperation.

We reviewed the information on defoaming and flocculating agents that you submitted as well as the information provided in GRAS affirmation petitions and GRAS notices for enzyme preparations. The enclosed attachment provides a brief overview of our evaluation and itemizes the evaluated defoamers (Table 1) and flocculants (Table 2). We conclude that these compounds are used by enzyme manufacturers in accordance with the principles of good manufacturing practice (GMP).

Sincerely yours,

(b) (6)

Laura M. Tarantino, Ph.D.
Acting Director
Office of Food Additive Safety, HFS-200
Center for Food Safety and Applied Nutrition

Defoaming and Flocculating Agents Used in the Manufacture of Enzyme Preparations Used in Food

Enzyme Preparations

Most enzymes currently used in food are derived from microorganisms. The manufacturing process of such enzymes includes three major steps: fermentation, enzyme recovery, and enzyme formulation. The formulated products are generally referred to as enzyme preparations. In addition to the enzymes of interest, enzyme preparations contain added substances such as diluents, preservatives, and stabilizers. They may also contain metabolites derived from the production microorganism and the residues of substances used in the manufacturing process, such as components of the fermentation medium or defoaming and flocculating agents used during fermentation and recovery. When FDA reviews safety data on enzyme preparations, it considers all components of the preparation.

Defoaming Agents

Defoaming agents (defoamers) are used by enzyme manufacturers to reduce or prevent foaming during fermentation and recovery. They are formulated with ancillary ingredients such as surface-active agents or carriers. Defoamers currently used in the manufacture of food enzymes are listed in Table 1. The Table includes five major defoamers that are identified by a double asterisk and several compounds that are used either as secondary defoamers or ancillary ingredients in defoamer formulations.

The major defoamers are added to the fermentation broth at levels within the range of 0.05-1% on a weight basis. Some of these defoamers, for example, polyoxyethylene-polyoxypropylene block copolymer, may contain trace levels of ethylene oxide, propylene oxide, and 1,4-dioxane which are known to cause cancer in laboratory animals. The Office of Food Additive Safety (OFAS) has evaluated the use of defoamers listed in Table 1 and determined that human exposure to the residues of these defoamers in enzyme preparations does not present human safety concern.

Flocculating Agents

Flocculating agents (flocculants) are used in the enzyme recovery step to separate microbial cells and cell debris from the fermentation broth containing the dissolved enzyme. The flocculation typically consists of two steps - primary flocculation and secondary flocculation. In the primary flocculation, inorganic salts (such as calcium chloride or aluminum sulfate) or "low molecular weight" polymers (such as polyamines) are used to agglomerate the cellular debris. The primary flocculation is usually followed by the secondary flocculation in which "high molecular weight" polymers are used to aid the formation of larger agglomerates that are subsequently removed by centrifugation or filtration. The polymers used as flocculants can be either cationic or anionic. The cationic polymers are added to the fermentation broth at levels not higher than 1% on a

weight basis. The anionic polymers are used at levels at or below 0.025%.

The flocculants used in the manufacture of food enzymes are listed in Table 2. They include inorganic salts, polyamines, and polyacrylamides. Several of these compounds are regulated in 21 CFR either as food additives or GRAS substances. Certain polyamines may contain traces of epichlorohydrin and 1,3-dichloro-2-propanol. Polyacrylamides usually contain very low levels of acrylamide. These contaminants of polyamines and polyacrylamides are known to cause cancer in laboratory animals. OFAS has evaluated all polymers included in Table 2 and determined that human exposure to the residues of these flocculants in enzyme preparations does not present human safety concern.

Sources of Information on Defoamers and Flocculants

OFAS compiled data on defoamers and flocculants listed in Tables 1 and 2 using information voluntarily submitted by the Enzyme Technical Association. OFAS also relied on the information provided in GRAS affirmation petitions and GRAS notices for enzyme preparations. Other sources of information included published articles, computer searches, and Material Safety Data Sheets issued by manufacturers of defoamers and flocculants.

Table 1. Defoamers Used in the Manufacture of Food Enzymes

Compound	CAS Reg. No.	Supplemental Information
Polypropylene glycol**	25322-69-4	Average MW: 2000
Polyglycerol polyethylene-polypropylene glycol ether oleate**	78041-14-2	
Polyoxyethylene-polyoxypropylene block copolymer**	9003-11-6	Average MW: 2000
Polypropylene glycol monobutyl ether**	9003-13-8	
Polydimethylsiloxane**	63148-62-9 68083-18-1	
Silica	7631-86-9 63231-67-4	
Stearic acid	57-11-4	
Sorbitan sesquioleate	8007-43-0	
Glycerol monostearate	123-94-4	
Polysorbates (polyoxyethylene sorbitan fatty acid esters)		Polysorbate 60 (CAS No. 9005-67-8), Polysorbate 65 (CAS No. 9005-71-4), and polysorbate 80 (CAS No. 9005-65-6) are regulated as food additives and components of defoamer formulations
Rape oil mono- and diglycerides	93763-31-6	
White mineral oil	64742-47-8	

Table 2. Flocculants Used in the Manufacture of Food Enzymes

Compound	CAS Reg. No.	Supplemental Information
Dimethylamine-epichlorohydrin copolymer	25988-97-0	Cationic polyamine
Methylamine-epichlorohydrin copolymer	31568-35-1	Cationic polyamine
Dimethylamine-epichlorohydrin-ethylenediamine terpolymer	42751-79-1	Cationic polyamine
Polyacrylamide modified by condensation with formaldehyde and dimethylamine	67953-80-4	Cationic polyacrylamide
Acrylamide-acryloxyethyl-trimethylammonium chloride copolymer	69418-26-4	Cationic polyacrylamide
Acrylamide-acrylic acid copolymer	25987-30-8 9003-06-9	Anionic polyacrylamide
Aluminum sulfate	10043-01-3	
Calcium chloride	10035-04-8 10043-52-4	



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POSITION PAPER

ETA Position On Food Allergen Labeling of Microbially Derived Enzymes Under FALCPA as it Applies to Fermentation Media Raw Materials

It is the position of the Enzyme Technical Association (ETA) that microbially derived enzymes do not fall within the scope of the Food Allergy Labeling and Consumer Protection Act (FALCPA) and that labeling for food allergens is not triggered by the use of a microbially derived enzyme preparation. There may be other reasons why a manufacturer labels a food product with regard to allergen content, but the use of a microbially derived enzyme preparation is not a reason for such labeling.

Enzymes are not one of the eight major allergenic foods, often referred to as the big 8, so they do not fit within the first requirement of FALCPA. In addition, microbial enzymes are not byproducts of nor are they derived from the major food allergens. Although enzymes are not major food allergens,¹ many enzymes are produced with microorganisms and the nutrient media used to feed these microorganisms may contain protein from one or more of the major food allergens. The enzymes are not derived from raw materials containing major food allergens, but rather are obtained from the microorganisms which are used to produce the enzyme proteins. In other words, enzymes obtained from fermentation are directly derived from microorganisms fed on media that may include protein obtained from one or more of the major food allergens. Proteins and other nitrogenous material are consumed by the microorganisms for cell growth, cell maintenance, and production of enzyme protein. It is the intent of the enzyme manufacturer to supply enzymes, therefore it is critical that the ratio of nutrient to enzyme yield is carefully controlled. It is also the intent of the manufacturer that these raw materials are added to the fermentation as food to be consumed by the microorganism and are not added as formulation ingredients.

In arriving at its position ETA also considered that:

- The regulatory agencies in the EU and Japan have determined that enzyme preparations are not required to have allergen labeling for the raw materials used in the fermentation process. Indeed, the European Commission's Health & Consumer Protection Directorate General has clearly stated that enzymes

¹ To the extent the enzyme producer uses an allergenic material, such as wheat flour diluent in the final product formulation, labeling may be required.

are outside the scope of the Directive 2003/89/EC which amended the EU Food Labelling Regulations.

- Enzyme broths are normally processed to separate biomass and fermentation materials from the enzyme, to concentrate the enzymatic activity, and formulated to achieve a uniform and stable enzyme product.
- The unique role of enzymes in food processing is as a catalyst. Due to the specific nature of enzymes, only small amounts are required to make desired modifications to the property of a food.
- Many enzymes do not become a component of the food ingredient or final food. Some enzymes are used in an immobilized form or are denatured during processing. Further, processing of the food ingredient after the enzyme catalyst has performed the expected function often reduces or eliminates the enzyme from the product.
- ETA has made an extensive review of the published scientific literature and has found no reports that even suggest there has been an allergenic reaction to a component of the fermentation media which was used to feed the microorganism that produced the enzyme.

The above position paper and accompanying report were provided to FDA on September 12, 2005 and to date ETA has received no comment.

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SUBMISSION END