ORIGINAL SUBMISSION

AB Enzymes GmbH – Feldbergstrasse 78 , D-6412 Darmstadt

AB Enzymes
an ABF Ingredients company

GRN 000557

November 17, 2014

RECEIVED
DEC 1 2014
OFFICE OF
FOOD ADDITIVE SAFETY

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 POLYGALACTURONASE 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 polygalacturonase enzyme from *Aspergillus tubingensis* expressed in *Trichoderma reesei* for use in fruit and vegetable processing, wine, coffee and flavouring 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 polygalacturonase 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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AB Enzymes GmbH – Feldbergstrasse 78 , D-6412 Darmstadt

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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 polygalacturonase 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.*
Polygalacturonase enzyme from *Aspergillus tubingensis* expressed in *T. reesei.*

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

(b) (6)

Nov 17/14

Candice Cryne Date
Regulatory Affairs Specialist

POLYGALACTURONASE ENZYME PREPARATION FROM *TRICHODERMA REESEI*

AB ENZYMES GmbH

November 17, 2014

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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 polygalacturonase enzyme from *Aspergillus tubingensis* expressed in *Trichoderma reesei* 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 polygalacturonase. Section 6 provides information on the mode of action, applications, and use levels of polygalacturonase and enzyme residues in final food products. The safety studies outlined in Section 7 indicate that the polygalacturonase enzyme preparation shows no evidence of pathogenic or toxicoge 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 Oy[1]
Tykkimäentie 15
FIN-05200 Rajamäki
Finland

Person(s) Responsible for the Dossier:

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And

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 is known as polygalacturonase or pectin polygalacturonase.

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.

Some of the conditions for polygalacturonase are:

pH value: 4.5

Temperature: 50°C

1.4. Food Products Used in

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

1.5. Levels of Use

Maximum use levels of polygalacturonase enzyme preparation (expressed in mg TOS/kg raw material (RM)) 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 production		Coffee cherries	0.5
Flavouring production		Fruits/Vegetables	300
Fruit and vegetable processing	Fruit juices	Fruit/Vegetable	3
	Fruit purees	Fruit/Vegetable	6
Grain treatment		Cereals	5
Wine production		Grapes	2

1.6. Purposes

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

Polygalacturonase 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.

1.7. Consumer Population

Polygalacturonase is a major enzyme responsible for pectin disassembly in ripening fruit, and by some bacteria and fungi involved in the rotting process. Since the polygalacturonase enzyme is naturally present in nature, most notably in fruits, which are consumed by humans, it is expected that the enzyme from *T. reesei* will be digested as any other protein in the human body.

Furthermore, polygalacturonase and other pectin-degrading enzymes such as pectinesterase, 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 *Trichoderma reesei*[2].

It is AB Enzyme's conclusion that since the polygalacturonase enzyme from *A. tubingensis* expressed in *T. reesei* 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.

1.8. Basis for GRAS Determination

Pursuant to 21 C.F.R. § 170.30, AB Enzymes GmbH has determined, through scientific procedures, that its polygalacturonase enzyme preparation from *A. 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.

1.9. 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:

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2. PRODUCTION MICROORGANISM

2.1. Donor, Recipient Organism and Production Strain

Donor:

Polygalacturonase 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:	*Aspergillus*
Species:	*Aspergillus tubingensis*
Subspecies (if appropriate):	not applicable
Generic name of the strain:	RH3544

Recipient Organism:

The recipient (host) strain used for the genetic modification is *Trichoderma reesei* strain RF5455. *T. reesei* RF6197 strain was developed for polygalacturonase 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.

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 (Parzia and Johnson, 2001).

Based on the available data, it is concluded that the organism *T. reesei* is non-pathogenic and non-toxigenic and is safe to use as the production organism for polygalacturonase enzyme preparation from *A. tubingensis*.

Production Strain:

The transformed production strain containing the polygalacturonase gene from *A. tubingensis Mosseray* RH3544 is *T. reesei* strain RF6197 which is deposited in the "Centraalbureau voor Schimmelcultures" (CBS) in the Netherlands with the deposit number CBS 123986.

The production strain (RF6197) differs from its recipient strain (RF5455) in its high polygalacturonase production capacity due to expression of the *A. tubingensis* polygalacturonase gene from the expression cassettes integrated into the RF6197 genome (see Section 2.2). RF6197 secretes high amounts of polygalacturonase into its culture supernatant, resulting in high polygalacturonase activity in the cultivation broth. The heterologous polygalacturonase is the main component of the enzyme mix produced by RF6197. In addition to the heterologous polygalacturonase, the RF6197 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 (2001) (Decision Tree Analysis - *Appendix #4*). *T.reesei* strains are non-pathogenic and non-toxigenic 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 ^{0}C. No additional growth cycles have been performed after the *T. reesei* RF6197 strain deposition to the culture collection.

2.2. Genetic Modification

Trichoderma reesei RF6197 for *Aspergillus tubingensis* polygalacturonase production was constructed by transforming the *A. tubingensis* polygalacturonase gene expression cassettes to *T. reesei* RF5455, as several copies. The polygalacutronase gene and its native terminator were ligated to pUC19 plasmid. The transformation of RF5455 strain with the polygalacturonase expression cassettes was performed as described by Penttilä *et al*. (1987) with the modifications described in Karhunen *et al*. (1993).

Polygalacturonase Expression Cassette

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

- endogenous *T. reesei* promoter
- *A. tubingensis* polygalacturonase gene
- *A. tubingensis* polygalacturonase gene terminator
- *amdS* promoter and terminator *Aspergillus nidulans amdS* gene. The gene has been isolated from *Aspergilus 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 *amd*S gene, acetamidase, can degrade acetamide and is neither harmful or nor dangerous. The *amd*S marker gene has been widely used as selection marker in fungal transformation.
- (SpeI)-StuI linker

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 RF6197 stay genetically stable over the time necessary for the industrial fermentation process of the RF6197 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 RF6197 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 expression cassettes integrated into the genome of the *T. reesei* strain RF6197 was confirmed by Southern blot hybridization. The result obtained shows that the genome of RF6197 at the end of industrial fermentations corresponds to that

of the original strain. Thus, it can be concluded that the expression cassettes (recombinant polygalacturonase) are stably integrated into the genome of the production strain.

Furthermore, the transferred DNA contains no mobilizable elements, is stably integrated into the *T. reesei* chromosome and is mitotically stable.

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 requirements[3], if this fungus was to be used in submerged standard industrial fermentation for enzyme production.

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

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

[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.

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* RF6197 in the environment is rather low because of its adaptation to fermentation conditions.

Therefore, the *T. reesei* host strain RF5455 and production organism RF6197 is considered to be of low risk and can be produced with specific 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 RF6197 polygalacturonase 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 RF6197. 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 RF6197.

The polygalacturonase is produced by an aerobic submerged microbial fermentation using a genetically modified *T. reesei* RF6197 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 Paper[4] (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.

3. ENZYME IDENTITY AND SUBSTANTIAL EQUIVALENCE

3.1. Enzyme Identity

Systematic name	**(1->4)-alpha-D-galacturonan glycanohydrolase**
Common names	**pectin depolymerase; pectinase; endopolygalacturonase; pectolase; pectin hydrolase; pectin polygalacturonase; endo-polygalacturonase; poly-α-1,4-galacturonide glycanohydrolase; endogalacturonase; endo-D-galacturonase; poly(1,4-α-D-galacturonide) glycanohydrolase; (1→4)-α-D-galacturonan glycanohydrolase**
Enzyme Commission No.	**3.2.1.15**
CAS number	**9032-75-1**

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

3.2. Amino Acid Sequence

The *A. tubingensis* polygalacturonase mature protein is approximately 45 kDa as confirmed by SDS-PAGE.

3.3. Enzymatic Activity

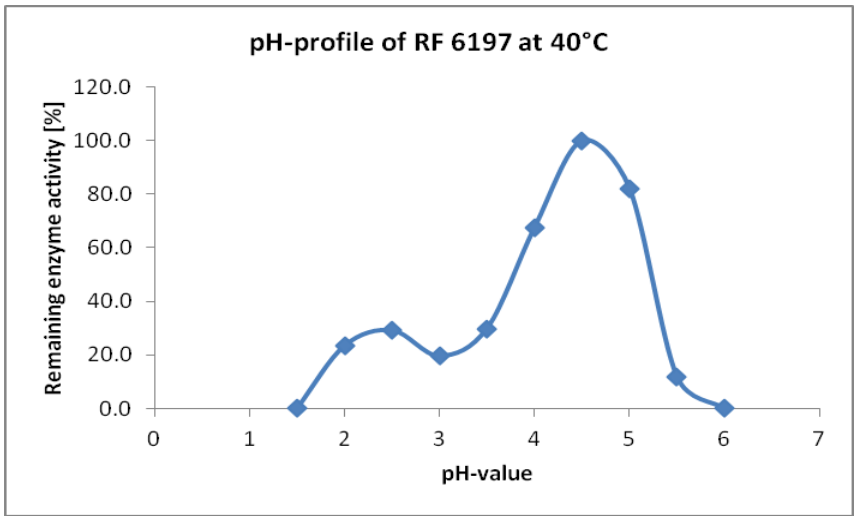
The main activity of the *T.reesei* RF6197 enzyme preparation is polygalacturonase (IUB 3.2.1.15). Polygalacturonase is a pectinolytic enzyme that breaks down pectin, and is found abundantly in plants, microorganisms, and animals. Pectin is a structural polysaccharide found in primary cell wall and middle 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 pathogensis, 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 polygalacturonase activity as defined by its IUBMB classification. The enzyme activity is usually reported in PGU/mg. Polygalacturonase activity is determined using in-house validated methods. Polygalacturonase causes a reduction of viscosity of a pectin substrate. The activity is calculated based on an enzymatic activity value of a known standard sample.

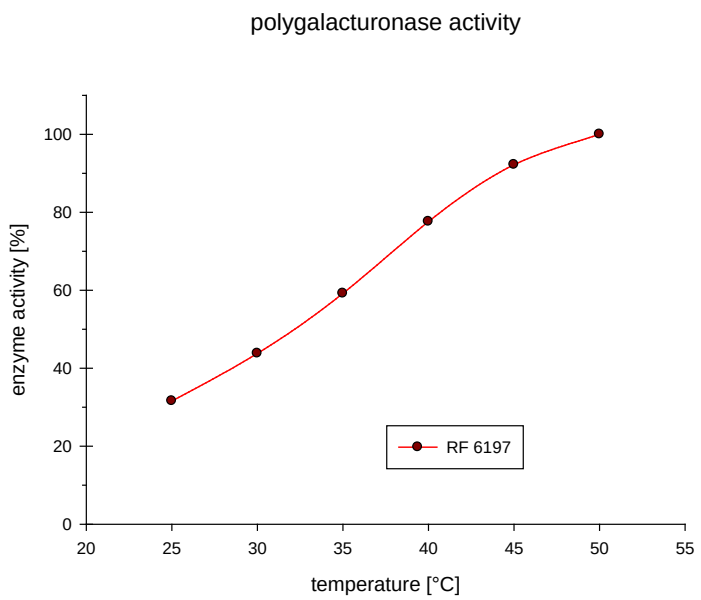
Apart from polygalacturonase, the *T.reesei* RF6197 enzyme preparation also contains other enzymatic side activities in small amounts, which are naturally and typically produced by the production organism *T. reesei*, including glucanase, cellulase and xylanase activities. However, these activities are not relevant from an application and/or safety point of view, due to the small amounts produced and the fact that such enzyme activities have been used and approved for decades in food processing.

The activity of the food enzyme polygalacturonase expressed in *T. reesei* was measured under various pH and temperature conditions. The effect of pH on enzyme activity was evaluated by measuring

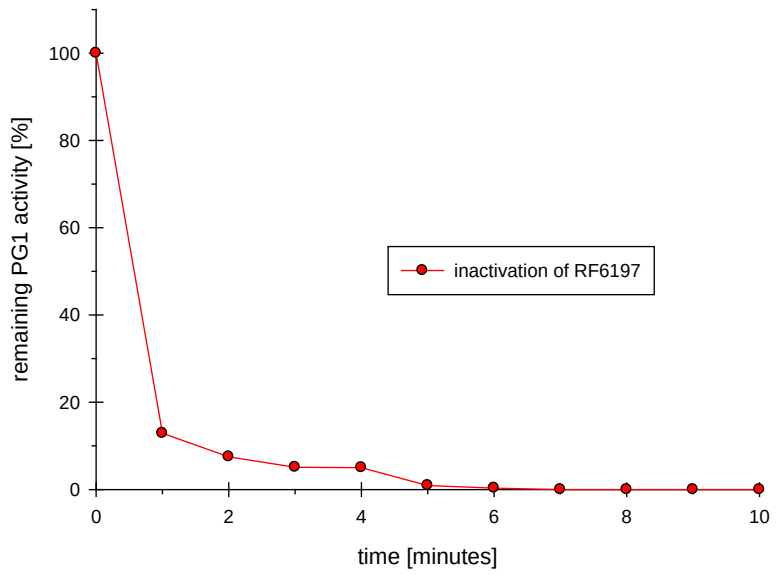
polygalacturonase activity at 40°C in different buffer solutions. The pH-optimum was measured to be 4.5. See curve below.



The effect of temperature on enzyme activity was evaluated by measuring polygalacturonase activity at pH 3.9. The highest activty of polygalacturonase is 50°C. See curve below.



Additionally, it can be concluded from the following curve that no enzyme activity is left after several minutes at 85°C and pH of 4.45.



4. MANUFACTURING PROCESS

4.1. Overview

Like all food enzymes, polygalacturonase 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* RF6197 production strain described herein is produced by controlled submerged fermentation. The production process involves the fermentation process, recovery (downstream processing), 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 ≤0.15% and ≤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 (Ammonium sulphate, Monopotassium phosphate)
- pH adjustment agents
- Foam control agents

4.2.3. Inoculum

A suspension of a pure culture of *T. reesei* RF6197 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 polygalacturonase 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 below 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* RF6197 polygalacturonase 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}
E. coli	not present in 25 g
Salmonella	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] See JECFA specifications, ftp://ftp.fao.org/docrep/fao/009/a0675e/a0675e00.pdf, page 64: Although nonpathogenic and nontoxigenic microorganisms are normally used in the production of enzymes used in food processing, several fungal species traditionally used as sources of enzymes are known to include

5. COMPOSITION AND SPECIFICATIONS

Commercial enzymes, whether used in the production of food, feed or for technological purposes, are biological isolates of variable composition. Apart from the enzyme protein in question, microbial food enzymes also contain some substances derived from the producing micro-organism and the fermentation medium. These constituents consist of organic material (proteins, peptides, amino acids, carbohydrates, lipids) and inorganic salts. As has been established by JECFA (FAO/WHO, 2006), the percentages of these organic materials are summarized and expressed as Total Organic Solids (TOS). The TOS value is an internationally accepted method to describe the chemical composition of commercial food enzymes. The ratio between the enzyme activity and TOS is an indication of the relative purity of the enzyme.

5.1. Formulation

The TOS values, protein content and relative purity of the polygalacturonase expressed in *T. reesei* were measured in 2 stabilised liquid concentrates.

Composition	
Constituent[6]	**Average %**
PG activity (PGU/mg)	1,422,000
Water (%)	31.25
Ash (%)	6.9
Protein (%)	13.4
TOS (%)	26.5
PG activity/mg TOS	5,338,858

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	
Polygalacturonase activity (PGU mg^{-1})	Min	150.000
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 down-stream equipment are sanitized with disinfectants approved for food industry
- Sterilization of all fermentation media:
    - all the media are sterilized with steam injection in fermentors or media tanks (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

Pectinases are a complex heterogeneous group of different enzymes that act specifically on pectic substances. Pectinases act on and decrease the intracellular adhesivity and tissue rigidity. Pectinases are the acidic polysaccharides consisting of 3 main classes. They include polymethylesterase's (PME),

polygalacturonase's (PG), and pectate lyase's (PAL). Polygalacturonases causes the breakdown of α (1-4) glycosidic linkage between the galacturonic acid residues, pectate lyase acts on pectin eliminating oligosaccharides of α (1-4) linked galacturonic acid residues and poly methyl esterases act on pectin methyl esters releasing methanol (Kashyap, 2001).

Like any other enzyme, polygalacturonase 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.

Polygalacturonase is an enzyme widespread in nature and is involved in pectin degradation. It is found naturally in plants for cell elongation, growth and ripening, and is found in bacteria, yeast, insects, nematodes and protozoa for plant pathogenesis, symbiosis and decomposition of plant deposits (Lang and Dörnenburg, 2000). In unripe fruit, pectin is bound to cellulose microfibrils in the cell wall and is insoluble and confers rigidity on cell walls. During ripening process the pectin structure is altered by naturally occurring enzymes in the plant. These alternations involve the breakdown of the pectin, and the pectin becomes more soluble and loses its grip on the surrounding cell walls and the plant tissue begins to soften (Kashyap, 2001).

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::

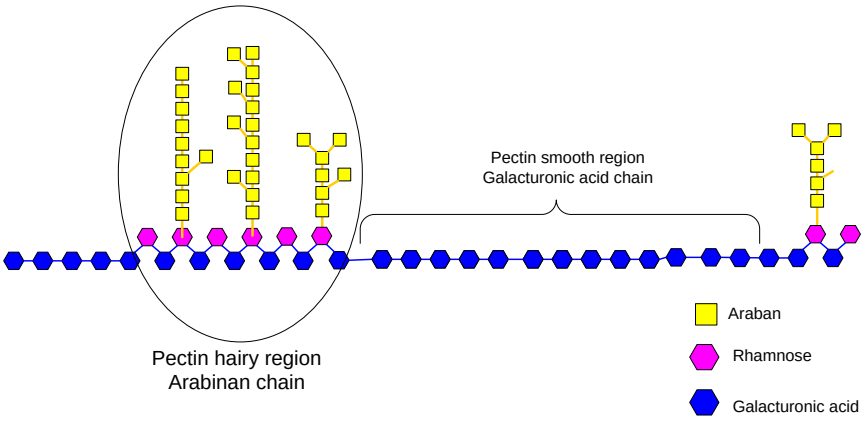


Figure #1: Pectin schematic

Because of the contribution of both ionic and covalent linkages, the structure of pectin may be modified by the ionic strength of the apoplast, by enzymes that modify the charge of the GalUA residues, or by enzymes that cleave either the α-(1–4)-linked GalUA backbone or side chains of the hairy pectin regions (Hadfield, 1998). Polygalacturonase catalyses the hydrolysis of "smooth" region pectin within the polygalacturonic acid chain (depolymerisation) to give oligosaccharides (mainly mono-galacturonic acid), which are already part of the human diet. See figure #2 below:

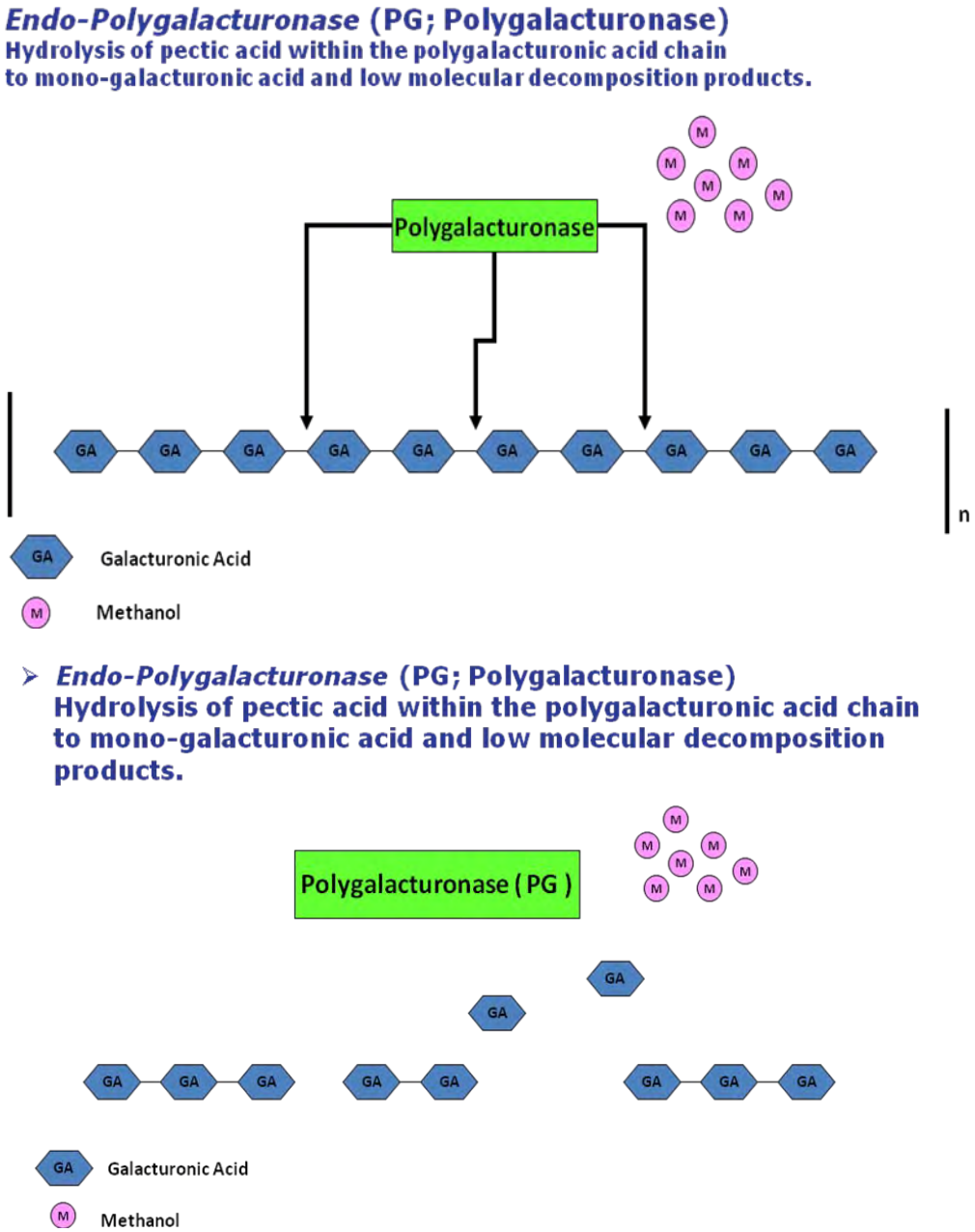


Figure #2: Reaction catalyzed by polygalacturonase

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 polygalacturonase is specific for the "smooth region" of the pectin molecule, it does not provide complete pectin enzymatic hydrolysis and is most often used with other enzymes. Thus, to achieve

complete pectin degradation, pectin (methyl) esterase is commonly combined in an enzyme preparation with polygalacturonase, as it removes the methyl-group from the pectin backbone, converting the pectin to a partially demethylated version (pectinic acid) or pectic acid.

6.2. Application

Polygalacturonase expressed in *T. reesei* RF6197, subject of this dossier, is intended to be used in fruit and vegetable processing, wine, coffee and flavouring production, and grain treatment.

Fruit and Vegetable Juice Extraction

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. Polygalacturonase is a pectinase and will assist in degradation of pectin in the processing of juice. Raw fruit and vegetables contain a naturally varied concentration of polygalacturonase, which has been shown to be involved in the disassembly of pectin that accompanies many stages of plant development, and particularly tissue deterioration in the late stages of fruit ripening (Hadfield, 1998). In industrial processing of fruit and vegetables, it is technological advantageous to employ the use of exogenous polygalacturonase 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 polygalacturonase, the viscosity of the juice drops, pressability improves, the jelly structure disintegrates and the fruit juice can be easily obtained with higher yields. See figure #3 and 4 below for description of juice and puree processing.

Furthermore, although raw fruits and vegetables contain endogenous polygalacturonase it is too variable in concentration and the specificity of the enzyme may not be optimal for the desired process.

The benefits of the depolymerisation of pectin with the help of polygalacturonase[7] in fruits and vegetable processing/purees are:

- Efficient peel removal
- Faster viscosity reduction leading to increased press/centrifugation capacity and filtration efficiency
- Increased concentrate of juice
- Higher juice extract yield, due to efficient solubilisation of pectin
- Increased cloud stability (reduced turbidity) of the clear concentrate
- Less use of raw materials
- Energy savings and production of less waste products

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

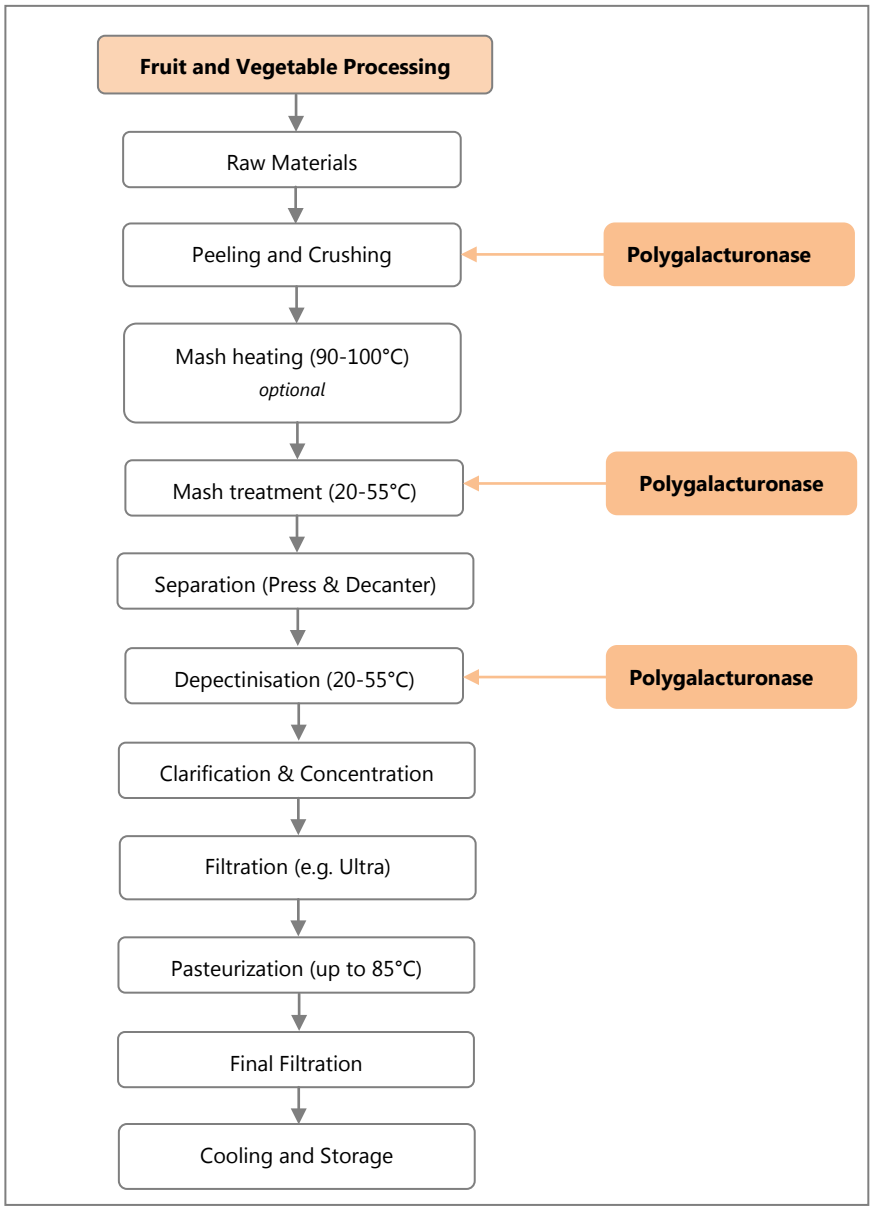


Figure #3 Enzymatic Fruit and Vegetable Processing

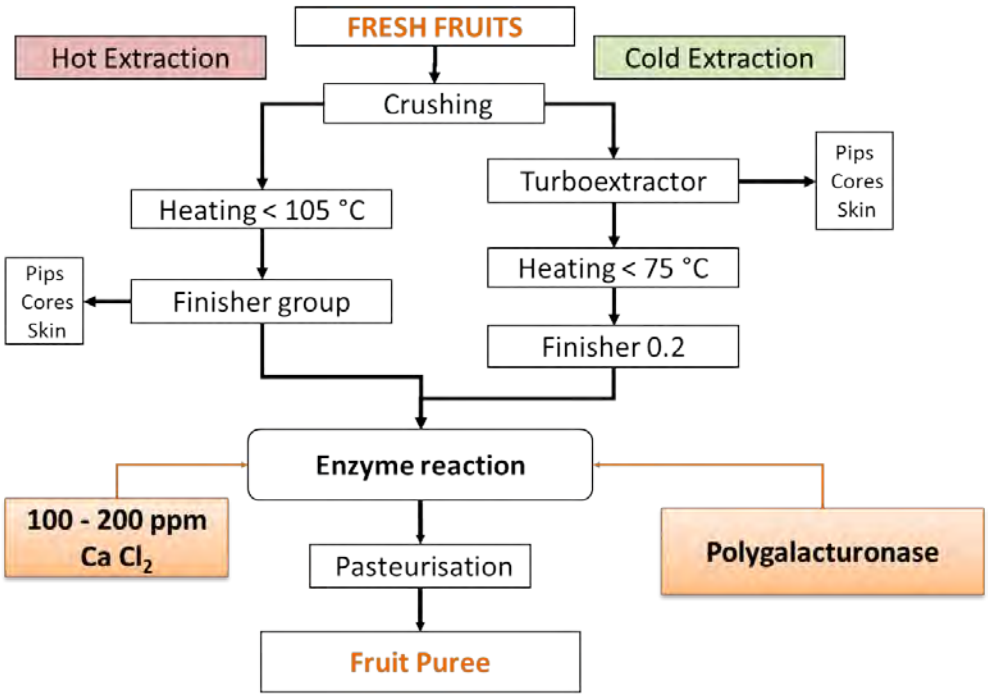


Figure #4 Enzymatic Fruit Puree Processing

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 agument 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 #5 below.

Grapes have high pectin content (5-10 g 1^{-1}) and are difficult to crush and press. They are de-stemmed, crushed, and heated to 60°C or 80°C to release colour (red grapes) from the skins and to destroy endogenous polyphenoloxidase (Kashyap, 2001). Polygalacturonase together with other pectinases,

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, polygalacturonase (in combination with pectin esterase) improves visual characteristics (color stability and turbidity) as compared to untreated wines.

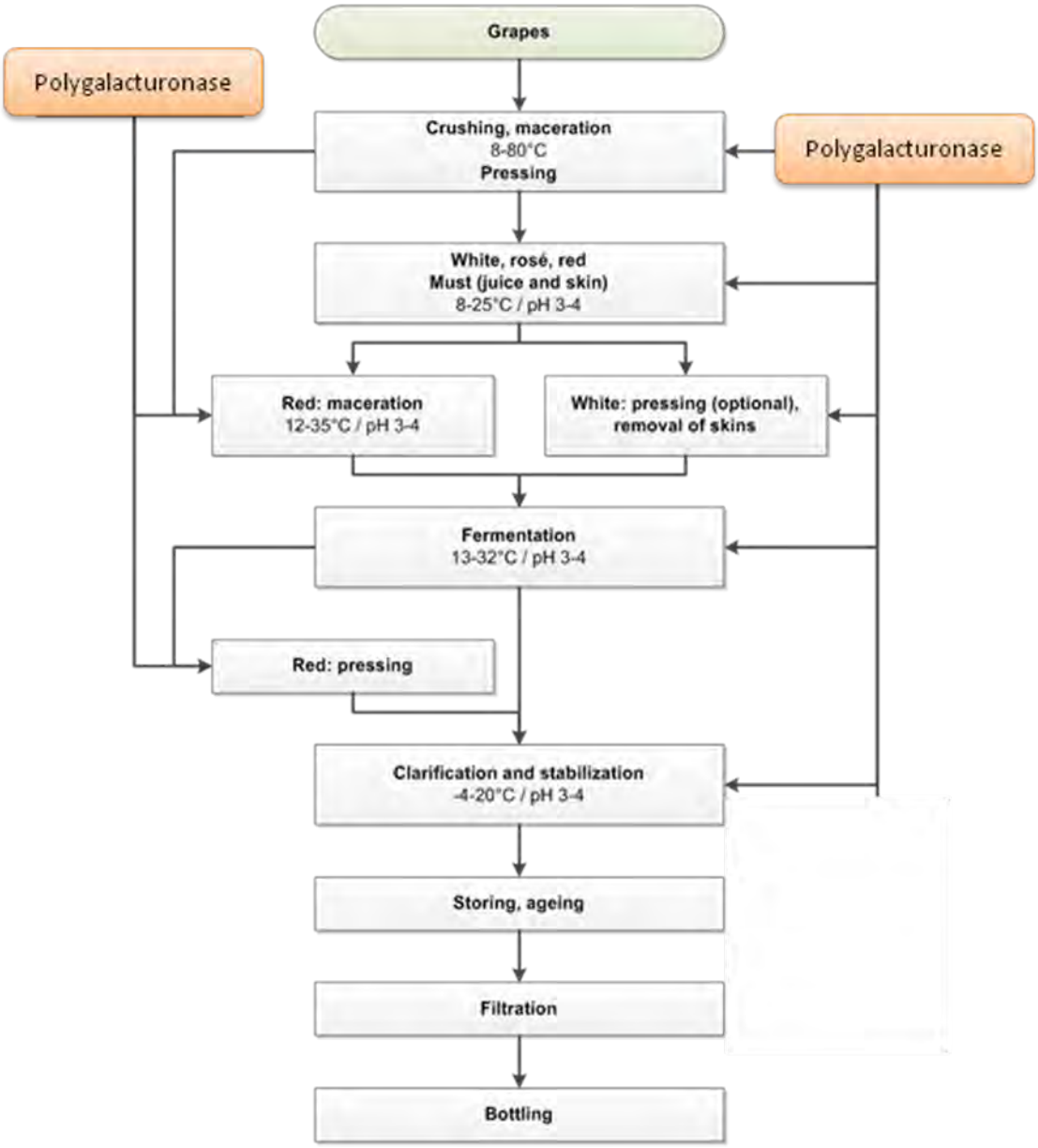


Figure #5 Enzymatic wine processing

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 polygalacturonase (often together with other pectinases, like pectin esterase) 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 below:

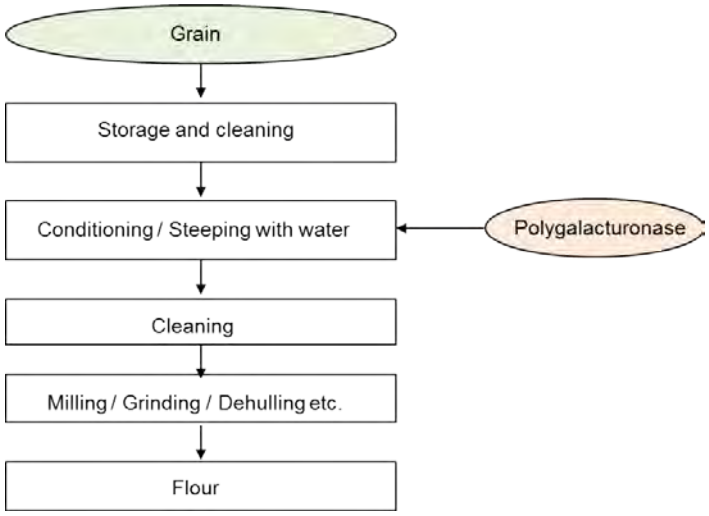
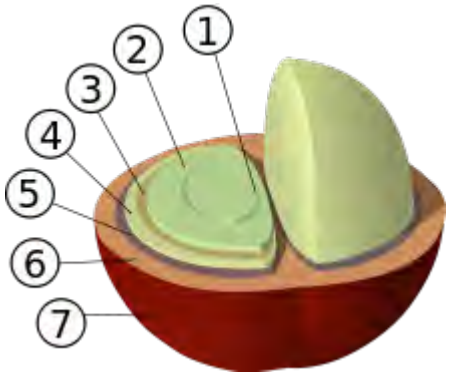


Figure #6 Enzymatic Grain Treatment

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.

Polygalacturonase (often together with other pectinases, such as pectin lyase and/or pectin esterase) 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 #7:

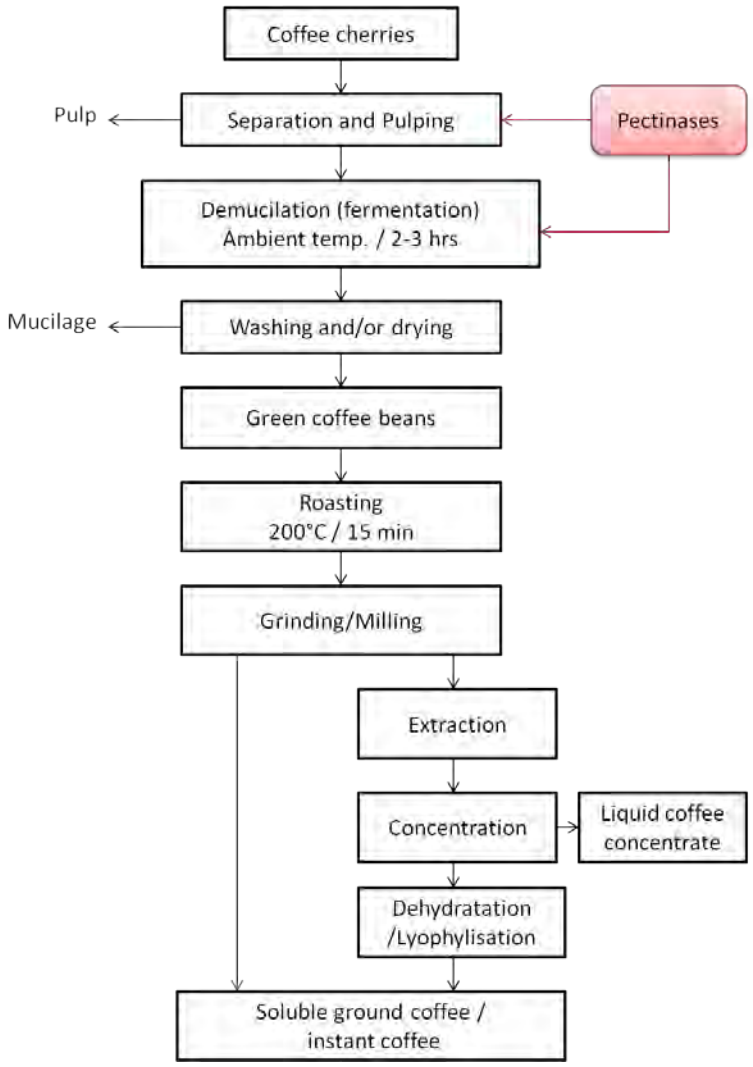


Figure #7 Coffee Production flow

Flavouring Production

Polygalacturonase 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 polygalacturonase 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 Solids[8]

[8] 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.

(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 polygalacturonase from *T. reesei* RF6197 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	300
Fruit and vegetable processing	Fruit juices	Fruit/Vegetable	3
	Fruit purees	Fruit/Vegetable	6
Grain treatment		Cereals	5
Wine production		Grapes	2

6.4. Active and Inactive Enzyme Residues in the Final Food

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

Polygalacturonase (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 polygalacturonase: >80°C / >2min
- Polygalacturonase: >75°C / >2min

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).

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 polygalacturonase enzyme and it's residues from *T.reesei* RF6197 have 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)*[9]. 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.

[9] http://www.brenda-enzymes.org/enzyme.php?ecno=3.2.1.15

7. SAFETY EVALUATION

7.1. Safety of the Donor and 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 RF6197. The production strain only differs from its recipient strain by its production of polygalacturonase 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 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-toxigenic. 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 RF6197 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 Polygalacturonase Enzyme

Polygalacturonase are hydrolytic depolymerases with endo and exo activities. Endo-PGases (E.C. 3.2.1.15) are important enzymes involved in fruit ripening and in fungal/bacterial attack on plants and are naturally present in plants.

Furthermore, polygalacturonase and other pectin-degrading enzymes such as pectinesterase, 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 *Trichoderma reesei*[10].

Commercial pectin enzyme preparations from various micro-organisms (including genetically modified ones) are widely accepted and *Trichoderma 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. Polygalacturonase from *T. reesei* RF6197 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 polygalacturonase 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

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

- Ames test
- Chromosomal aberration test, in vitro

Based upon the results of these studies, it can be concluded that the polygalacturonase 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 further evaluate the potential allergenicity of *T.reesei* RF6197, the sequence comparison with known allergenic proteins was conducted using a database of allergens from the Food Allergy Research and Resource Program (FARRP), University of Nebraska, Allergen Database (Version 12, February 7, 2012), 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 350 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).

The alignments search for sequence identities of the full-length polygalacturonase protein to any resulting from a FASTA allergenic protein in the Allergen Database (V14) showed no matches greater than 33.1% 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 polygalacturonase in question can be presumed to be allergenic based on full-length sequence relatedness to known allergens.

In the 80-mer sliding window analysis the polygalacturonase protein sequence did show degrees of identity from 35.8 % to 46.3% with pollen allergens of different species such as maize pollen allergen, pollen allergen of the subtropical Bahia grass, Japanese cedar pollen, pollen allergen of conifer Cryptomeria japonica. 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 judgement on the predictive inferiority of the 80-mer (35% threshold) approach was supported recently by Goodman and Tetteh (2011) who suggested: "*Because the purpose of the bioinformatics search is to identify matches that may require further evaluation by IgE binding, full-length sequence evaluation or an increase in the threshold from 35% identity toward 50% for the 80 amino acid alignment should be considered*" (Goodman and Tetteh, 2011). Using the latter

recommendation the polygalacturonase in question would be below threshold even using the 80-mer sliding window approach.

In addition, the polygalacturonase 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 350 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.

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 polygalacturonase produced by *T. reesei* RF6197 is of no concern.

Furthermore, besides egg lysozyme, the enzyme industry is not aware of enzyme proteins used in food that are homologous to known food allergens. Polygalacturonase 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.

Thus, due to the aforementioned criteria, and that no indication of an allergenic potential of the polygalacturonase was detected, it is concluded that the polygalacturonase enzyme preparation is not a potential allergen and no further allergenicity studies are necessary.

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

The production organism *T.reesei* RF6197 has been demonstrated to be non-toxigenic 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 the *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 RF6197 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* RF6197 polygalacturonase 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* RF6197 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* RF6197 polygalacturonase production strain:

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

These safety studies were conducted using the dry ultra-filtrated concentrate, not with the diluted final product. All studies were conducted using the same production batch, Batch No. PG 10089 B3 with 92.41% TOS. Dose calculations for the experiments were adjusted to account for TOS.

The composition of the test material is as follows:

Batch No	PG 10089 B3
Ash (%)	0.9
Water (%)	6.7
TOS (%)	92.4
Activity (PGU/mg)	6,680,000
Activity /mg TOS	7,229,437
Protein (%)	75.7

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 24 November 2010 – 10 December 2010.

This study was performed to investigate the potential of polygalacturonase enzyme preparation from *T. reesei* RF6197 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 S9 liver microsomal activation. Each concentration, including the controls, was tested in triplicate. The test item was tested at the following concentrations calculated to the total organic substance (TOS):

Pre-Experiment/Experiment I: 3; 10; 33; 100; 333; 1000; 2500; and 5000 µg/plate

Experiment II: 33; 100; 333; 1000; 2500; and 5000 µg/plate

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 Polygalacturonase from *Aspergillus niger* produced with *T. reesei* at any dose level, neither in the presence nor absence of metabolic activation (S9 mix). 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, p
olygalacturonase from *Aspergillus niger* produced with *Trichoderma reesei* is 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 3 November 2010 – 16 December 2010.

This in vitro test was performed to assess the potential of polygalacturonase from *Aspergillus niger* produced with *T. reesei* to induce structural chromosome aberrations. Evaluation of cytogenetic damage induced in V79 cells (cell line from the lung of the Chinese Hamster) in the absence and presence of metabolic activation was performed in two independent experiments at one preparation interval (18 hours).Phenobarbital/β-naphthoflavone induced rat liver S9 was used as the metabolic activation system. The S9 was prepared from 8 - 12 weeks old male Wistar rats.

	Without S9 mix		With S9 mix
	Exp. I	Exp. II	Exp. I
Exposure period	4 hrs	18 hrs	4 hrs
Recovery	14 hrs	-	14 hrs
Preparation interval	18 hrs	18 hrs	18 hrs

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 5000.0 µg/mL (5411.0 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 in Experiment I and II. In Experiment I no clastogenicity was observed at the concentrations evaluated either with or without metabolic activation. In Experiment II in the absence of S9 mix two statistically significant increases in the number of aberrant cells, excluding gaps (3.5 and 3.0

%, respectively) were observed at the test item concentrations 3607.3 and 4509.2 µg/mL. These values were within the range of the total laboratory´s historical solvent control and are therefore regarded as biologically not relevant.

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, the test item did not induce structural chromosome aberrations in V79 cells (Chinese hamster cell line) in vitro.

Therefore, polygalacturonase from *Aspergillus niger* produced with *T. reesei* is considered to be non-clastogenic in this chromosome aberration test in the absence and presence of metabolic activation, when tested up to the highest required concentration adjusted to TOS.

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-May 2012.

In this subchronic toxicity study, polygalacturonase from *Aspergillus niger* produced with *a T. reesei* was administered daily by oral gavage to SPF-bred Wistar rats of both sexes at dose levels of 100, 300 and 1000 mg/kg body weight/day for a period of 91/92 days. A control group was treated similarly with the vehicle, bidistilled water, only.

The groups comprised 10 animals per sex which were sacrificed after 91/92 days of treatment. Clinical signs, outside cage observation, food consumption and body weights were recorded periodically during the acclimatization and treatment periods. Functional observational battery, locomotor activity and grip strength were performed during week 13.

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 level.

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 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.

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

All macroscopic findings were considered to be within the range of normal background alterations and no microscopic findings could be attributed to treatment with the test item. All findings recorded were within the range of normal background lesions which may be recorded in animals of this strain and age.

Based on the results of this study, 1000 mg/kg body weight/day of Polygalacturonase from *Aspergillus niger* produced with *T. reesei* was established as the no-observed-effect level (NOEL) and 1000 mg/kg/day as the no-observed-adverse-effect-level (NOAEL).

7.5. Estimates of Human Consumption and Safety Margin

7.5.1. Estimate Dietary Exposure

The estimated daily intake of the polygalacturonase enzyme preparation was calculated based on the level of resultant TOS in food from the addition of the enzyme preparation and the intake of those foods based on US population. According to current FDA guidance, in circumstances where the actual TOS level in the final food is not available/measurable, the estimated daily intake should be calculated based on the use level of the enzyme expressed on a TOS basis. Therefore, a dietary exposure assessment was performed. 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:

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

In Section 6.3, the recommended use levels of polygalacturonase 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 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	300	Various beverages	0.01	3
	Fruit and vegetable processing	Fruit/vegetable	3	Juices	1.3	3.9
	Wine production	Grape	2	Wine	1.6	3.2
Solid foods	Flavouring production	Fruit/vegetable	300	Various solid foods	0.01	3
	Fruit and vegetable processing	Fruit/vegetable	6	Purees	1	**6**
	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, we assume a RM/FF of 1 (1 kg of fruits / kg of processed fruit).*
- *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 both food and beverages (in this case, coffee production and fruit juices respectively) multiplied by the average consumption of food /kg body weight/day. Consequently, the Total TMDI will be:

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)**
6 x 0.0125 = 0.075	3.9 x 0.025=0.5	**0.575**

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 polygalacturonase from *T.reesei RF6197* 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 0.575 mg TOS/kg body weight/day. Consequently, the MoS is:

MoS = 1,000 / 0.575 = 1739

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 polygalacturonase enzyme preparation from *T. reesei* RF6197, which showed no toxicity or mutagenicity across a variety of test conditions. The data resulting from these studies is consistent with the long history of safe use for *T. reesei* and polygalacturonase in food processing, and in keeping with the

[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)OAEL (usually given in mg/kg bodyweight/day) by the corresponding measured or predicted exposure (also in mg/kg bw/day).

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 polygalacturonase enzyme preparation from *T. reesei* RF6197 expressing the gene encoding polygalacturonase 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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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

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 mmm 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.

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 II			KAM
QM				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

(b) (6)

M. Meijer, Bsc.

U.S. Department of Health & Human Services

U.S. Food & Drug Administration

Food

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

Links on this page:

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- Site Map
- Transparency
- Website Policies

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 Tree[2]. The safety assessment of a given enzyme preparation is based upon an evaluation of the toxigenic potential of the production organism. The responses below follow the pathway indicated in the decision tree as outlined in Pariza and Johnson, 2001. The outcome of this inquiry is that polygalacturonase enzyme preparation from *A. tubingensis* expressed in *T. reesei* RF6197 is "ACCEPTED" as safe for its intended use.

Decision Tree:

1. **Is the production strain genetically modified?** *Trichoderma reesei* RF6197 for *Aspergillus tubingensis* polygalacturonase production was constructed by transforming the *A. tubingensis* polygalacturonase gene overexpression 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 polygalacturtonase 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;

---

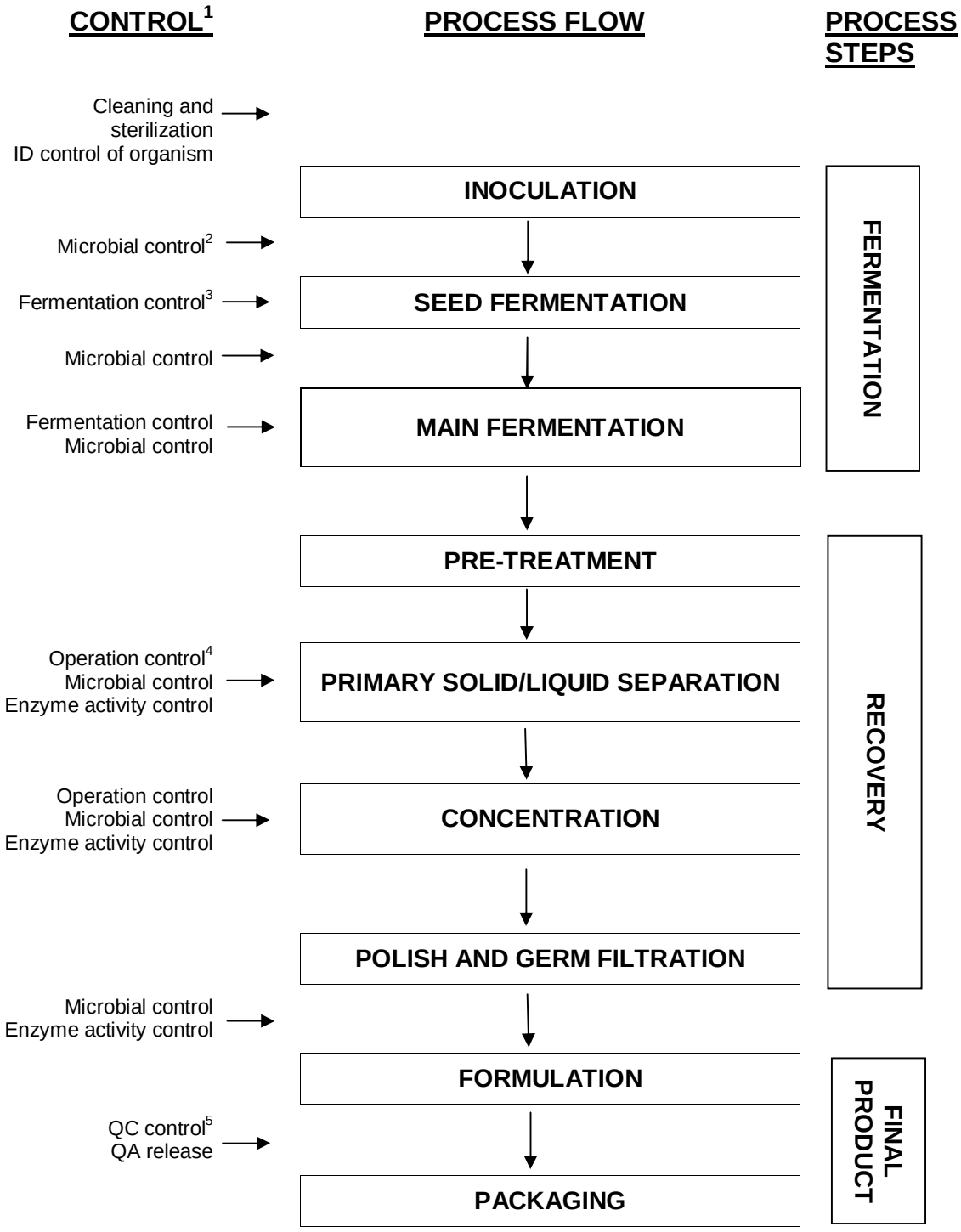
[1] Pariza M.W. and Johnson E.A. Reg. Toxicol. Pharmacol. Vol. **33** (2001) 173-186

[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, CO_2, sterile air overflow are monitored / controlled.

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

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

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
Washington DC 20204

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-trimethyl-ammonium 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