October 31, 2022

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 (IUBMB 3.2.1.15) from a Genetically Modified *Trichoderma reesei* produced by submerged fed-batch fermentation is Generally Recognized as Safe; therefore, they are exempt from statutory premarket approval requirements.

The following information is provided in accordance with the proposed regulation:
Proposed 21C.F.R. § 170.36 (c)(i) *The name and address of notifier.*

AB Enzymes Inc.[1]
8211 W. Broward Blvd. Suite 420
Plantation, FL 33324 USA

Proposed 21C.F.R. § 170.36 (c)(ii) *The common or usual name of notified substance:*
Polygalacturonase (IUBMB 3.2.1.15) from a Genetically modified *Trichoderma reesei*

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

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

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

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

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

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

Sincerely,

AB Enzymes GmbH

i.V. Candice Cryne

Senior Global Regulatory Affairs Manager

31-Oct-2022 | 21:33 GMT

Joab Trujillo

Regulatory Affairs Specialist

31-Oct-2022 | 21:36 GMT

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

AB Enzymes GmbH – Feldbergstrasse 78 , D-6412 Darmstadt

GRAS Notification of a Polygalacturonase from a Genetically Modified *Trichoderma reesei*

AB ENZYMES GmbH

October 31, 2022

Contents

1 PART 1 §170.225 – SIGNED STATEMENTS AND CERTIFICATIONS

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

In conformity with the established regulation 21 C.F.R. Section 170, subsection E, AB Enzymes GmbH hereby claims that **Polygalacturonase** (IUBMB 3.2.1.15) from a Genetically Modified *Trichoderma reesei* produced by fed-batch submerged fermentation is Generally Recognized as Safe; therefore, they are exempt from statutory premarket approval requirements.

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

AB Enzymes Inc.[1]

8211 W. Broward Blvd. Suite 420

Plantation, FL 33324 USA

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

Polygalacturonase (IUBMB 3.2.1.15) from a Genetically modified *Trichoderma reesei*

§170.225(b) – Trade secret or confidential:

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

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

The polygalacturonase enzyme is to be used as a processing aid in fruit and vegetable processing, coffee processing, flavoring production, and wine production. The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements under current Good Manufacturing Practices. There are no maximal limits set, just suggested dosages.

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

This GRAS determination is based upon scientific procedures.

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

§170.225(c)(6) – Premarket approval:

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

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

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

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

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

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

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

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

2.1 Identity of the notified substance

The dossier concerns a **polygalacturonase from a genetically modified *Trichoderma reesei*.**

2.1.1 Common name of the enzyme

Name of the enzyme protein: Polygalacturonase

Synonyms: (1→4)-alpha-D-galacturonan glucanohydrolase, pectin depolymerase, pectolase, pectin hydrolase

2.1.2 Classification of the enzyme

IUBMB #	3.2.1.15
CAS #	9032-75-1

EC 3. is for hydrolyases;

EC 3.2. is for glycosylases;

EC 3.2.1. is for glycosidases; i.e. enzymes that hydrolyze *O*- and *S*-glycosyl compounds

EC3.2.1.15 is for endo-polygalacturonase.

2.2 Strain Lineage Information

2.2.1 Production Strain

Production strain	*Trichoderma reesei* AR-414

Synopsis

Polygalacturonase from *Aspergillus kawachii* is produced from a genetically modified *Trichoderma reesei* production strain (AR-414). The genetic modifications conducted to develop the production strain are described in section 2.3 of the GRAS narrative along with confirmation on integration of expression cassettes in the *T. reesei* genome, the stability of the production strain, absence of DNA, antibiotic genes and toxic compounds. Information on the safety of the *Trichoderma reesei* production strain is provided in section 6 of the GRAS narrative. In this notice, we provide information on that the *T. reesei* production organism is non-pathogenic and non-toxigenic. In short, safety of the production strain is substantiated by the safety of the genetic modifications, history of safe use for *Trichoderma reesei* as a food enzyme producer and the use of the safe strain linage concept described in Pariza and Johnson (2001).

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

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

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

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

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

The *Trichoderma reesei* production strain AR-414 is deposited in the Westerdijk Fungal Biodiversity Institute, formerly known as the "Centraalbureau voor Schimmelcultures" (CBS) in the Netherlands with the deposit number CBS 148292.

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

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

Kingdom:	Fungi
Division:	*Ascomycota*
Class:	Sordariomycetes
Order:	Hypocreales
Family:	Hypocreaceae
Genus:	Trichoderma
Species:	*Trichoderma reesei*
Strain:	*Trichoderma reesei* AR-414

2.2.2. Recipient Strain

The recipient strain used in the genetic modification for the construction of the production strain is *Trichoderma reesei* AR-329. The recipient strain was created from parental strain AR-256 through a series of native enzyme gene deletions to limit enzyme side activity expression in *Trichoderma reesei*. The recipient is negative in e.g. the genes encoding the four major *T. reesei* cellulases (cellobiohydrolases I and 2 and endoglucanases 1 and 2) and the major xylanases, (xylanase I, xylanase II and xylanase III). The gene deletions have been done using a *T. reesei* gene counter selection method. The gene used was deriving from *T. reesei*, meaning that no heterologous marker genes were used in these gene deletions.

Therefore, the recipient can be described as followed:

Kingdom:	Fungi
Division:	*Ascomycota*
Class:	*Sordariomycetes*
Order:	*Hypocreales*
Family:	*Hypocreaceae*
Genus:	*Trichoderma*

Species: *Trichoderma reesei*

Strain: *Trichoderma reesei* AR-329

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

2.2.3 Donor

The polygalacturonase gene described in this application derives from *Aspergillus* kawachii. The fungi in the *Aspergillus* species, also known as koji molds are recognized by the color, black (ex. *Aspergillus niger),* white (ex. *Aspergillus kawachii*), and yellow (*Aspergillus oryzae) (Futagami et al. 2015)*. Aspergillus *kawachii* is known as the white koji fungus, a member of the *Aspergillus niger clade* (Hong et al. 2013). The white color of the mold is due to a mutation,causing the white phenotype; *Aspergillus kawachii* is actually an albino mutant version of the black koji mold *Aspergillus luchensis* (Futagami 2022; Hong et al. 2013). From the perspective of the food industry, similar to *Aspergillus niger* and others in the clade, *Aspergillus kawachii* is used for the production of a Japanese traditional beverage, shochu in this case (Futagami et al. 2015; Futagami 2022; Hong et al. 2013; Rojas et al. 2011). One of the most notable safety characteristics of *Aspergillus kawachii* is that the fungus does not produce mycotoxins (Futagami et al. 2015; Futagami 2022).

Aspergillus kawachii is known to produce acidic enzymes such as polygalacturonase, alpha amylase and glucoamylase (Futagami 2022; Rojas et al. 2011). This fungus can be considered as a safe producer of industrial food enzymes due to its history of use and safety profile in the food industry.

Genus: *Aspergillus*

Species: *Aspergillus kawachii*

Subspecies (if appropriate): Not applicable

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

2.3 Genetic modification

T. reesei was constructed for polygalacturonase production. The production strain differs from the recipient strain in its high polygalacturonase production capacity due to expression of the polygalacturonase gene from the expression cassette integrated into the genome of the production strain. Besides the heterologous polygalacturonase production, no other changes in phenotype are made.

T. reesei AR-414 secretes high amounts of polygalacturonase into its culture supernatant, resulting in high polygalacturonase activity in the cultivation broth. The secreted polygalacturonase is the main component of the enzyme mix produced by AR-414. In addition, the strain AR-414 produces endogenous *Trichoderma* enzymes in small amounts. These activities are not relevant from an application or safety point of view, due to the small amount and the fact that such activities are included in products which have been approved for decades in food processing.

The production strain AR-414 was constructed from AR-329 in one genetic modification. The inserted expression cassette contains the polygalacturonase gene under a *T. reesei* promoter and terminator. The inserted sequence also contains an *Aspergillus nidulans amd*S selection marker gene. The expression cassette used for transformation was cleaved from the pUC19 vector plasmid by restriction enzyme digestion followed by isolation of the expression cassette from agarose gel. A Southern blot hybridization experiment was performed on the genomic DNA of the production strain AR-414 to confirm that no pUC19 vector DNA is included in the genome of AR-414.

Expression Cassette Components Table

Component	Description
***Aspergillus kawachii polygalacturonase* gene**	The polygalacturonase gene encodes the *A. kawachii* polygalacturonase enzyme
***Synthetic amdS* gene and promoter**	The marker gene has been isolated from *A. nidulans* VH1-TRSX6 (Kelly and Hynes 1985;

	Hynes et al. 1983). *A. nidulans* is closely related to *Aspergillus tubingensis*, which is used in industrial production of food enzymes. The gene codes for an acetamidase that enables the strain to grow on acetamide as a sole nitrogen source (Kelly and Hynes 1985). This characteristic has been used for selecting transformants. The product of the *amdS* gene, acetamidase, can degrade acetamide and is not harmful or dangerous. The *amdS* marker gene has been widely used as a selection marker in fungal transformations without any disadvantage for more than 30 years.
***Trichoderma reesei* promoter and terminator**	The polygalacturonase gene is fused to *T. reesei* native promoter. This promoter is strong and is used to overexpress polygalacturonase gene transcription, to obtain high yields of polygalacturonase. The transcription is terminated by the native terminator from *T. reesei*

Information relating to the genetic modification process

Standard DNA techniques were used in the construction and transformation of the plasmids. The transformation of AR-329 recipient strain with the polygalacturonase expression cassette was performed as described by Penttilä et al. 1987 with the modifications. The transformants were selected according to their ability to grow on acetamide selection plates (*amd*S marker gene).

Genes of concern

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

Confirmation of Insertion

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

2.3.1 Genetic stability of the production strain

The fermentation process always starts from identical replicas of the AR-414 (production strain) seed ampoule. Production preserves from the "Working Cell Bank" are used to start the fermentation process. A Working Cell Bank is a collection of ampoules containing a pure culture. The cell line history and the production of a Cell Bank, propagation, preservation and storage is monitored and controlled. Ampoules in a WCB ampoule Bank are only accepted for production runs if their quality meets the required standards. This is determined by checking identity, viability, microbial purity and productivity of the WCB ampoules. The accepted WCB ampoules are used as seed material for the inoculum.

Testimony to the stability of the strain is given by monitoring the growth behavior and by production of comparable levels of polygalacturonase activity in number of fermentation batches performed for the AR-414 strain. The activity measurements from parallel fermentations show that the productivity of the AR-414 strain remains similar. This clearly indicates that the strain is stable. In addition, to confirm the genetic stability of AR-414, a Southern blot was prepared using the genomic DNAs isolated from the mycelia collected from the end of three independent fermentations. Three different restriction digestions were performed, and the expression cassette transformed was used as a probe in the hybridization. The hybridization patterns were identical in all samples. The data of the analysis of enzyme activities from preparations deriving from different fermentation batches of the recombinant AR-414 strain is presented in Appendix #1.

Another conformational method was used to determine genetic stability of the production strain. The production strain was cultivated in liquid medium for three subsequent cultivation cycles. The cultivation started from the original strain deposit at -80 ºC being cultivated on solid agar. The spores were collected and inoculated to liquid medium. After finalizing the cultivation, a sample from the liquid culture was transferred to fresh cultivation medium and liquid cultivation was repeated. Still another transfer to fresh cultivation medium was done similarly, leading to three subsequent cultivation cycles in liquid medium. After the cultivations the polygalacturonase activities were analyzed from the culture supernatants and samples were run on SDS-PAGE gels. The genomic DNAs were isolated from mycelia (representing different cultivation cycles) and were analyzed by using Southern blot. The results from the analysis showed that the production strain remained stable: the enzyme activities and protein patterns remained similar in all culture supernatants and there were no changes in the strain genome. It is expected that in case the strain is unstable, the enzyme activities would decrease from cultivation cycle to the next and, also, differences in the banding patterns (in SDS-PAGE and Southern blot) would be detected.

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

No vector sequences were integrated.

A Southern blot hybridization experiment using plasmid backbone as a labelled probe and genomic DNA of the production strain AR-414 was performed to confirm that no vector DNA is integrated in the genome of AR-414. It produced negative result (no hybridization), demonstrating that no part of the plasmid vector removed to generate the linear transforming DNA fragments was introduced into the *Trichoderma* production host.

2.3.3 Demonstration of the absence of the GMM in the product

The down-stream process following the fermentation includes unit operations to separate the production strain. The procedures are executed by trained staff according to documented standard operating procedures complying with the requirements of the quality system.

The 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[13] method. This method has been validated in-house. The sensitivity of the method is 1 cfu/20 ml in liquid and 1 cfu/0.2 gram in dried semifinals.

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

The AR-414 enzyme preparation is free from detectable, viable production organism as demonstrated in Appendix #1. As the absence of the production strain is confirmed for every production batch, no additional information regarding the inactivation of the GMM cells is required.

2.3.5 Information on the possible presence of DNA

The polygalacturonase enzyme preparation is produced by an aerobic fed-batch submerged microbial fermentation using a genetically modified *Trichoderma reesei* strain. All viable cells of the production strain, AR-414, are removed during the down-stream processing: the fermentation broth is filtered with pressure filters and subsequent sheet filters, concentrated with ultra-filtration, and optionally followed by sheet filtration(s).

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

2.3.6 Absence of Antibiotic Genes and Toxic Compounds

As noted above, the transformed DNA does not contain any antibiotic resistance genes. Further, the production of known mycotoxins according to the specifications elaborated by the General Specifications for Enzyme Preparations Used in Food Processing Joint FAO/WHO Expert

[13] Roal Oy is the sole manufacturer of AB Enzymes' enzyme preparations. Roal Oy is based in Finland.

Committee on Food Additives, Compendium of Food Additive Specifications, FAO Food and Nutrition Paper[14] (FAO/WHO 2006) has been also tested from the fermentation product of the *Trichoderma reesei* strain AR-414. The Food Chemicals Codex ("FCC", 13th edition 2022), 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 (FAO/WHO 2006), was also confirmed from three AR-414 enzyme production batches in Appendix #1 and no antibiotic or toxic compounds were detected.

We confirm that the *T. reesei* AR-414 production strain is non-toxigenic. Regarding mycotoxins produced by *Trichoderma reesei*, the composition report provided as Appendix #1 demonstrates mycotoxin values below the levels of quantification (LoQs) for the enzyme concentrate batches tested.

2.4 ENZYME PRODUCTION PROCESS

2.4.1 Overview

The food enzyme is produced by ROAL Oy[15] by submerged fermentation of *Trichoderma reesei* AR-414 in accordance with current Good Manufacturing Practices for Food (GMP) and the principles of Hazard Analysis of Critical Control Points (HACCP). As it is run in the EU, it is also subject to the Food Hygiene Regulation (852/2004).

The enzyme preparation described herein is produced by controlled fed-batch submerged fermentation. The production process involves the fermentation process, recovery (downstream

[14] 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.

[15] See footnote 13

processing) and formulation and packaging. Finally, measures are taken to comply with cGMPs and HACCP. The manufacturing flow-chart is presented in Appendix #2.

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

2.4.2 Fermentation

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

- Inoculum
- Seed fermentation
- Main fermentation

2.4.3 Raw Materials

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

AB Enzymes expects no major food allergens to be in the final enzyme preparation. A rigorous allergen risk assessment is routinely conducted during the manufacturing of the final ingredient (i.e. enzyme preparation) for the purpose of determining and avoiding cross-contamination of food allergens into the final enzyme concentrate (before formulation). AB Enzymes uses a wheat-

based fermentation ingredient for production of the polygalacturonase enzyme preparation from AR-414 production strain. We routinely test our enzyme products for gluten traces at an external testing partner using an R5 antibody-based ELISA (Codex Alimentarius specifies in Codex Standard 118-1979 (2008)) and recent analysis has detected gluten traces in the polygalacturonase enzyme preparation below the limit of quantification (LoQ) <5ppm.

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

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

2.4.5 Inoculum

A suspension of a pure culture of AR-414 is aseptically transferred to shake flasks containing fermentation medium.

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

2.4.6 Seed fermentation

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

2.4.7 Main Fermentation

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

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

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

2.4.8 Recovery

The purpose of the recovery process is:

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

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

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

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

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

2.4.9 Materials

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

- Flocculants
- Filter aids
- pH adjustment agents

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

2.4.10 Pre-Treatment

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

2.4.11 Primary solid/liquid separation

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

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

2.4.12 Concentration

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

2.4.13 Polish and germ filtration

After concentration, for removal of residual cells of the production strain and as a general precaution against microbial contamination, filtration on dedicated germ filters is applied at

various stages during the recovery process. Pre-filtration (polish filtration) is included if needed to remove insoluble substances and facilitate the germ filtration. The final polish and germ filtration at the end of the recovery process results in a concentrated enzyme solution free of the production strain and insoluble substances.

2.4.14 General Production Controls and Specifications

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

Identity and purity of the producing microorganism:

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

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

Microbiological hygiene:

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

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

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

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

- Training of staff:
    - all the procedures are executed by trained staff according to documented procedures complying with the requirements of the quality system.

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

Chemical contaminants:

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

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

These in-process controls comprise:

Microbial controls:

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

Monitoring of fermentation parameters may include:

- pH
- Temperature
- Aeration conditions

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

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

2.4.15 Formulation and Packaging

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

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

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

2.4.16 Stability of the enzyme during storage and prior to use

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

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

2.5 Composition and specifications

2.5.1 Characteristics of the enzyme preparation

The characteristics of the enzyme preparation are:

Property	**Requirement**	
Activity	min.	4,000 PGX/g
Appearance	brown liquid	
Density	1.15-1.25 g/mL	

2.5.2 Formulation of a typical enzyme preparation

Composition	
Constituent	**%**
Enzyme concentrate	15-20
Glycerol	50
Potassium chloride	1.5
Tri-sodium citrate	1.2
Citric acid	1.1
Water	Remainder

2.5.3 Molecular mass and amino acid sequence of the enzyme

The polygalacturonase protein subject for this dossier consists of 338 amino acid residues with a calculated molecular mass of 35 kDa (or 35,000 Da).

2.5.4 Purity and identity specifications of the enzyme preparation

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

Lead:	Not more than 5 mg/kg[16]
Salmonella sp.:	Absent in 25 g sample
Total coliforms:	Not more than 30 per gram
Escherichia coli:	Absent in 25 g of sample
Antimicrobial activity:	Not detected
Mycotoxins:	No significant levels[17]

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

Other enzymatic activities: the food enzyme is standardized on enzyme activity. Apart from it, the production organism *Trichoderma reesei* produces other endogenous proteins, e.g. **beta-glucanase and cellulase**. As mentioned in section 2.2.2, the four major cellulase genes and three major xylanase genes have been deleted from the *T. reesei* genome. The enzymatic side activities

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

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

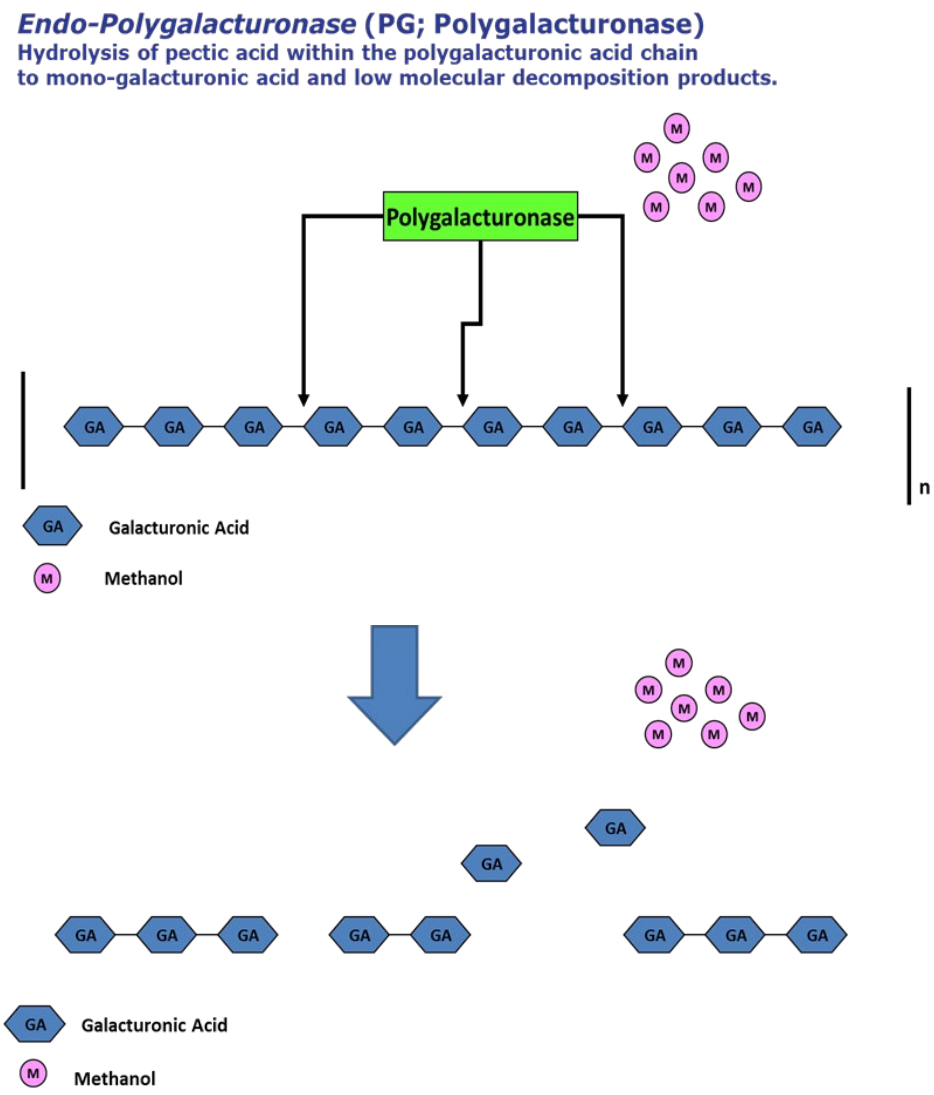
are present in a small amount and those enzyme activities are already present in the human diet and are not relevant from a safety point of view.

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

2.6 Enzymatic Activity

The main activity of the *Trichoderma reesei* AR-414 enzyme preparation is polygalacturonase (IUBMB 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 pathogenesis, symbiosis and decomposition of plant deposits. Pectic enzymes have two classes namely pectin esterases and pectin depolymerases. Pectin esterases have the ability to de-esterify pectin by the removal of methoxy residues. Pectin depolymerases readily split the main chain and have been further classified as polygalacturonases (PG) and pectin lyases (PL).

This food enzyme catalyzes the hydrolysis of "smooth" region- pectin within the polygalacturonic acid chain (depolymerization) to give oligosaccharides (mainly mono-galacturonic acid), see figure below:



The method to analyze 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 PGX/g. 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.

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

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

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

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

Adverse effects from side activities are not expected from the **polygalacturonase** enzyme preparation. *Trichoderma reesei* has a long history of safe use in the food industry can described in the dosser (section 6.1.). The side activities that would arise in the enzyme preparation comes from the production microorganism *Trichoderma reesei*. The main enzyme activities produced by *T. reesei*, the four major cellulases, and the three major xylanases, are not present in the polygalacturonase enzyme preparation as the encoding genes have been deleted from the recipient strain. This recombinant microorganism is known to produce enzymatic side activities of **beta-glucanase and cellulase** in low amounts. These side activities are considered to be normal and of no adverse consequence to human health. Both beta-glucanase and cellulase are considered globally as safe food enzymes and are part of the human diet.

2.7 Allergenicity

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

- The allergenic potential of enzymes was studied by (Bindslev-Jensen et al. 2006) and reported in the publication: "*Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry*". The investigation conducted involved enzymes produced by wild-type and genetically modified strains as well as wild-type enzymes and protein engineered variants. To add on, the investigation comprised 400 patients with a diagnosed allergy to inhalation allergens, food allergens, bee or wasp. The conclusion from the study was that ingestion of food enzymes in general is not likely to be a concern regarding food allergy.

- Previously, the AMFEP Working Group on Consumer Allergy Risk from Enzyme Residues in Food performed an in-depth analysis of the allergenicity of enzyme food products (Daurvin et al. 1998). The overall conclusion is that exposure to enzyme proteins by ingestion, as opposed to exposure by inhalation, are not potent allergens and that sensitization to ingested enzymes is rare.
- Enzymes when used as digestive (Abad et al. 2010) aids are ingested daily, over many years, at much higher amounts when compared to enzymes present in food (up to 1 million times more).

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

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

- The majority of proteins are not food allergens and based on previous experience, the enzyme industry is not aware of any enzyme proteins used in food that are homologous to known food allergens[18].
- Only a small amount of the food enzyme is used during food processing, which leads to very small amount of enzyme protein present in the final food. A high concentration generally equals a higher risk of sensitization, whereas a low level in final food equals a lower risk (Goodman et al. 2008).
- For cases where the proteins are denatured which is the case for this enzyme due to the food process conditions, the tertiary conformation of the enzyme molecule is destroyed. These types of alterations to the conformation in general, are associated with a decrease in the antigenic reactivity in humans. In the clear majority of investigated human cases, denatured proteins are much less immunogenic than the corresponding native proteins (Valenta and Kraft 2002; Valenta 2002; Takai et al. 2000; Nakazawa et al. 2005; Kikuchi et al. 2006).

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

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

2.7.1 Allergenicity Search

Alignments of the **polygalacturonase** mature amino acid sequence to the sequences in the allergen database were performed and results obtained were used to estimate the level of potential allergenicity of this enzyme. Similarity searches were performed to the sequences available in chosen public Allergen Online (FARRP) allergen database.

The alignment methods used in the searches are:

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

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

The identity limit set for the protein having an allergenic cross-reactivity is 35 % when alignment is performed using a full-length query sequence or an 80-mer sliding window. According to EFSA (2010) even the set above 35 % identity is regarded conservative and above 50 % identity cut-off has been suggested.

Results of Allergenicity searches:

Type of Search	Outcome
Alignment of the Polygalacturonase mature amino acid sequence to sequences in allergen online databases	No matches having greater than 35 % identity were found from the AllergenOnline database using the full-length search
Alignment of sliding 80-amino acid window of the query protein to known protein allergens	Multiple matches were found between 35-50% identity from the AllergenOnline database using the 80-mer sliding window search. Matches found are linked to pollen allergens.
Search of 8 amino acid exact matches	No sequences detected with at least 1 8mer match.

In the 80-mer sliding window analysis some of the 80mers of 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*, the common olive *olea europaea*, the easter lily *lilium lomgiflorum*, and papaya. As recommendation by the FAO/WHO, a possible cross-reactivity has to be considered, when there is more than 35% identity in the amino acid sequence of the expressed protein using an 80 amino acids window and a suitable gap penalty (FAO/WHO 2001). This recommendation has however been challenged. According to Ladics et al. (2007) comparing the predictive value of a full-length (conventional) FASTA search to the 80-mer analysis, "*a conventional FASTA search provides more relevant identity to the query protein and better reflects the functional similarities between proteins. It is recommended that the conventional*

FASTA analysis be conducted to compare identities of proteins to allergens". This judgement on the predictive inferiority of the 80-mer (35% threshold) approach was supported recently by Goodman and Tetteh (2011) who suggested: "*Because the purpose of the bioinformatics search is to identify matches that may require further evaluation by IgE binding, full-length sequence evaluation or an increase in the threshold from 35% identity toward 50% for the 80 amino acid alignment should be considered*" (Goodman and Tetteh, 2011). Using the latter recommendation, the polygalacturonase in question would be below threshold even using the 80-mer sliding window approach.

To summarize, the bioinformatics approach to estimate potential allergenicity and cross-reactivity based on relatedness to known allergens and taking into account the most recent scientific recommendations on the interpretation of such data leads us to conclude that the **polygalacturonase** produced by *Trichoderma reesei* AR-414 is of no concern.

Narrative on Allergenic Potential

To demonstrate the unlikelihood of any significant risk for allergic reaction from its intended use in spite of potential cross-reactivity to pollen allergens, a narrative was constructed with the following points:

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

Polygalacturonases (PGs) catalyze the degradation of highly polymeric galacturonate, a major component of pectin in plant cell walls, into individual galacturonic acid residues. PG belongs to family 28 of glycosyl hydrolases and has been identified in ripening fruit, abscission, dehiscence, pollen maturation, and rapidly expanding tissue and has been implicated to be a pollen allergen (Ibarrola et al. 2004). The polygalacturonase from AR-414 *Trichoderma reesei* is used as a

processing aid in very small quantities (ppm) in the food applications described in this notice. The enzyme will either be denatured or removed (by filtration processes, bentonite etc.) which will further decrease any negligible amounts of enzyme residues in the final food, for more details please refer to section 2.10 of the narrative (pages 49-50). Given the conditions in which the polygalacturonase is exposed to in the manufacturer of final foods, there is a low chance of active enzyme protein carryover into the final food. In the case where a final food containing polygalacturonase were to be ingested, it has been shown by (Bindslev-Jensen et al. 2006) that ingestion of food enzymes in general is not likely to be a concern with regard to food allergy. Moreover, (Dauvrin et al. 1998) showed there are no scientific indications that the small amounts of enzymes in food can sensitize or induce allergy reactions in consumers. We expect the enzyme to be digested when reaching the gastro-intestinal track due to the harsh pH conditions. The pH level of gastric juice is known to be 2, the polygalacturonase notified cannot survive at a pH level of 2.

Furthermore, we have conducted a comprehensive literature search for data on possible adverse reactions or allergenicity after oral exposure to polygalacturonase, particularly with relation to pollen. PCM database by the US National library of medicine and National institutes of health (Home - PMC - NCBI (nih.gov)), containing global scientific literature (more than 6 million records) as well as the Pubmed database from the National Library of Medicine, National Center for Biotechnology (PubMed (nih.gov); more than 32 million records) were searched with the following terms:

"polygalacturonase*" AND "allergen*" AND "oral"

"polygalacturonase *" AND "adverse" AND "oral"

"polygalacturonase *" AND "exposure" AND "oral"

"polygalacturonase *" AND "pollen" AND "oral"

The searches were performed on June 6th, 2022 for references up to this date.

After removal of duplicates, 219 publications were retrieved. The publications were assessed for their relevance by stepwise means:

First, the title and abstract, and

Second, the whole text was assessed.

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

After assessment of title and abstract, 3 publications remained possibly relevant and were further assessed.

(Ghiani et al. 2016) describe a polygalacturonase from tomato as being one of the putative allergens present in tomato. The rest of the article mainly focusses on a possible difference in IgE recognition between peel and pulp and subsequently differences between tomato lines. According to the authors, 27 potential tomato allergens, including different isoforms, have been reported and only six tomato allergens were recognized by the International Union of Immunological Society, polygalacturonase not being one of them. The article does not show clear evidence of polygalacturonase actually being an allergen in humans. The results from Ghiani et al. (2016) do not challenge the initial risk assessment as presented in the dossier and after consideration of the full article is not considered relevant.

(Sarkar et al. 2018) use sera from patients who already clearly suffered from a respiratory and food allergy to papaya and tried to find a putative allergen present both in papaya pollen and fruit. One of the proteins found is a polygalacturonase, which has a known function in both tissues. A challenge experiment in mice shows the possibility to elicit a respiratory reaction and a food allergen reaction with this protein after a subcutaneously sensitization. It is good to mention that the experiments do not show sensitisation via one pathway can lead to a challenge reaction in the other pathway. Nor does the data show that it is the polygalacturonase which is the reason for allergenicity in humans. The data therefore does not show that a respiratory sensitization can lead to a food allergy or vice versa.

In contrast, Bindslev-Jensen et al. (2006) showed that ingestion of food enzymes in general is not likely to be a concern with regard to food allergy. Moreover, Dauvrin et al., 1998 showed there are no scientific indications that the small amounts of enzymes in food can sensitize or induce allergy reactions in consumers. The results from Sakar *et al.* (2018) do not challenge the initial risk assessment as presented in the dossier and after consideration of the full article is not considered relevant for this specific risk assessment.

Finally, the last reference was an EFSA opinion (EFSA 2022)[19], showing no additional risks for allergenicity.

In conclusion, no indication of allergenic risk was identified after an extensive literature search. Two articles with information regarding the putative allergenicity of polygalacturonase were considered not relevant for risk assessment.

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

The polygalacturonase from *Trichoderma reesei* AR-414 object of this dossier is specifically intended to be used in fruit and vegetable processing. Furthermore, polygalacturonase AR-414 is intended to be used in wine production, coffee processing and flavoring production.

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

[19] Safety evaluation of the food enzyme endo-polygalacturonase from the genetically modified Aspergillus luchuensis strain FLYSC | EFSA (europa.eu)

galacturonic acid residues and poly methyl esterases act on pectin methyl esters releasing methanol.

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.

The substrate of polygalacturonase is pectin. Pectin consists of a complex set of polysaccharides (with different molecular weights and degrees of esterification) that are present in most primary cell walls and are particularly abundant in the non-woody parts of terrestrial plants (it can be found in various plant materials including the cell walls and endosperm of cereals, such as wheat and barley and fruits, such as apple, pear, etc). Pectin contains long galacturonic acid chains with residues of carboxyl groups and with varying degree of methyl esters (Voragen et al. 2009). A relatively large proportion, some 60 - 90 %, consists of the so-called "smooth"-region pectin. Their main components are non-esterified galacturonic acid units (pectinic acid) or such units esterified with methanol. These are "smooth regions" or blocks of alpha-1,4-galacturonic acid with polymer linkages. In addition, a smaller proportion of pectin (10 to 40%) consists of the so-called "hairy"-region pectin, which is mainly constituted of galacturonic acid units and rhamnose (with arabinan chains). A scheme of the pectin molecule is presented below.

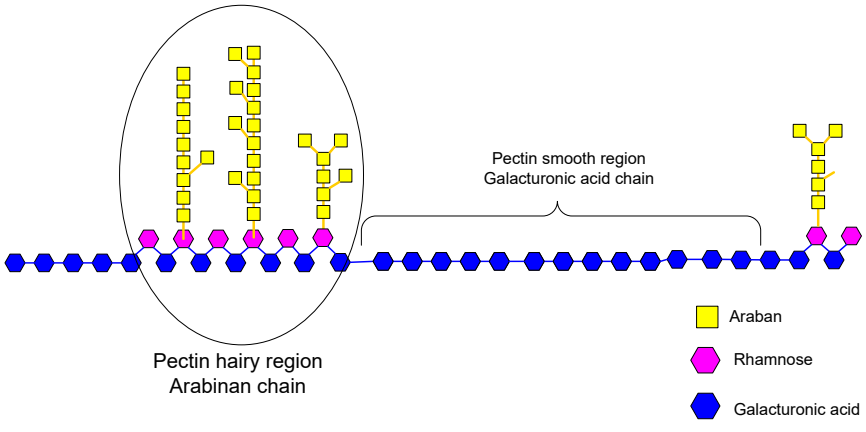


Figure #1: Pectin schematic

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

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

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

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

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

to give desired process advantages. Therefore, industrial polygalacturonase is used during food processing.

This dossier is specifically submitted for the use of polygalacturonase in fruit and vegetable processing, coffee processing, flavoring production, and wine production. Below, the benefits of the use of industrial polygalacturonase in those processes are described. The beneficial effects are of value to the food chain because they lead to better and/or more consistent product quality. Moreover, the applications lead to more effective production processes, resulting in better production economy and environmental benefits such as the use of less raw materials and the production of less waste. The use of pectinases, including polygalacturonase, has been specifically approved for a number of years, which together with the extensive use since the 1930s (Godfrey and West; Sharma et al. 2013) in a number of countries including the EU[20] and USA[21] and in the rest of the world demonstrates the technological need of such food enzymes in food processes.

Fruit and Vegetable Processing:

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 and Bennett 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. This

[20] 1 The use of pectinolytic enzymes is allowed in fruit juices processing and wine making, according to the Council Directive 2001/112/EC relating to fruit juices and certain similar products intended for human consumption and the Regulation (EC) No 606/2009 laying down certain detailed rules for implementing Council Regulation (EC) No 479/2008 as regards the categories of grapevine products, oenological practices and the applicable restrictions

[21] GRN 89: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=89

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.

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 depolymerization of pectin with the help of polygalacturonase[22] 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
- Increased cloud stability (reduced turbidity) of the clear concentrate
- Less use of raw materials
- Energy savings and production of less waste products
- Improve juice extraction yield due to efficient solubilization of pectin

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

Please refer to figure #2 below, for process flow diagram of polygalacturonase used in fruit and vegetable processing.

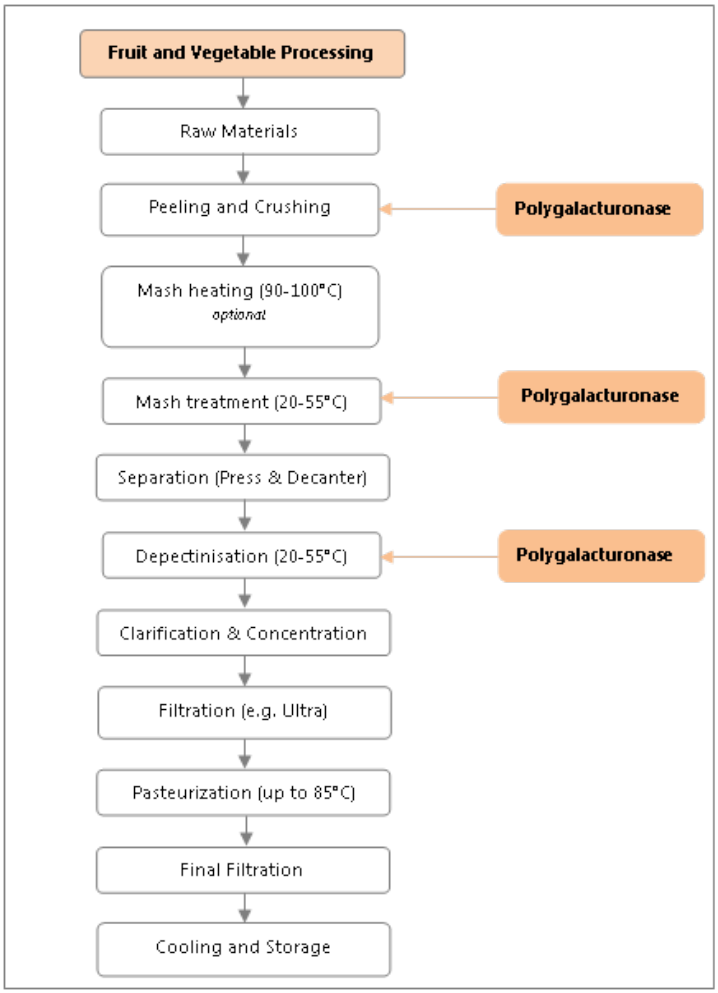


Figure #2 Enzymatic Fruit and Vegetable Processing

Please refer to figure #3 below, for process flow diagram of polygalacturonase used in puree processing.

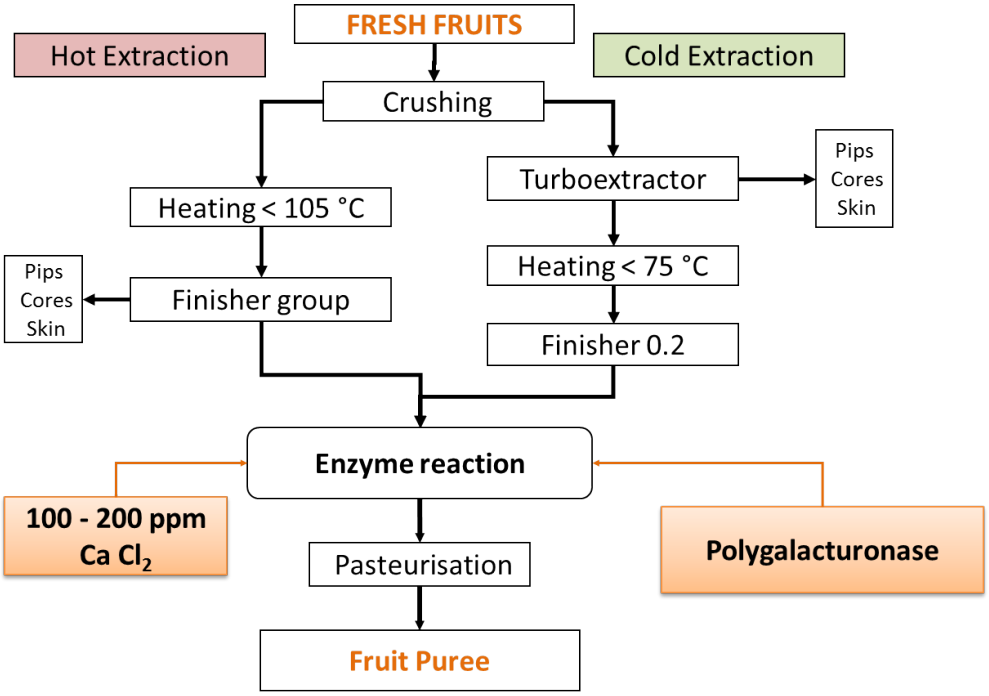
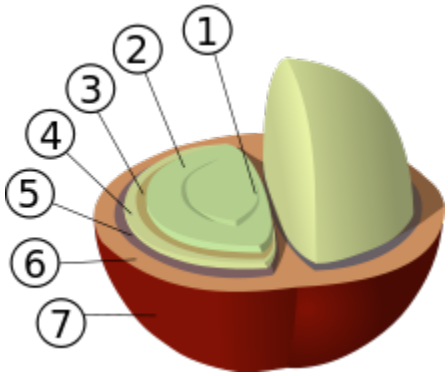


Figure #3 Enzymatic Fruit Puree Processing

Coffee Production

A Coffee bean is a seed of the coffee plant, and the pit inside the red/purple fruit is commonly referred to as a cherry. During green coffee production from harvested coffee cherries, the fruit covering the coffee beans need to be removed before the coffee beans can be dried.



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" flavor.
- Improve environmental impact and sustainability of the entire milling process:
- No water is added during fermentation and less washing during post fermentation.

- Simultaneously less polluted wastewater is achieved.
- 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 figure #4:

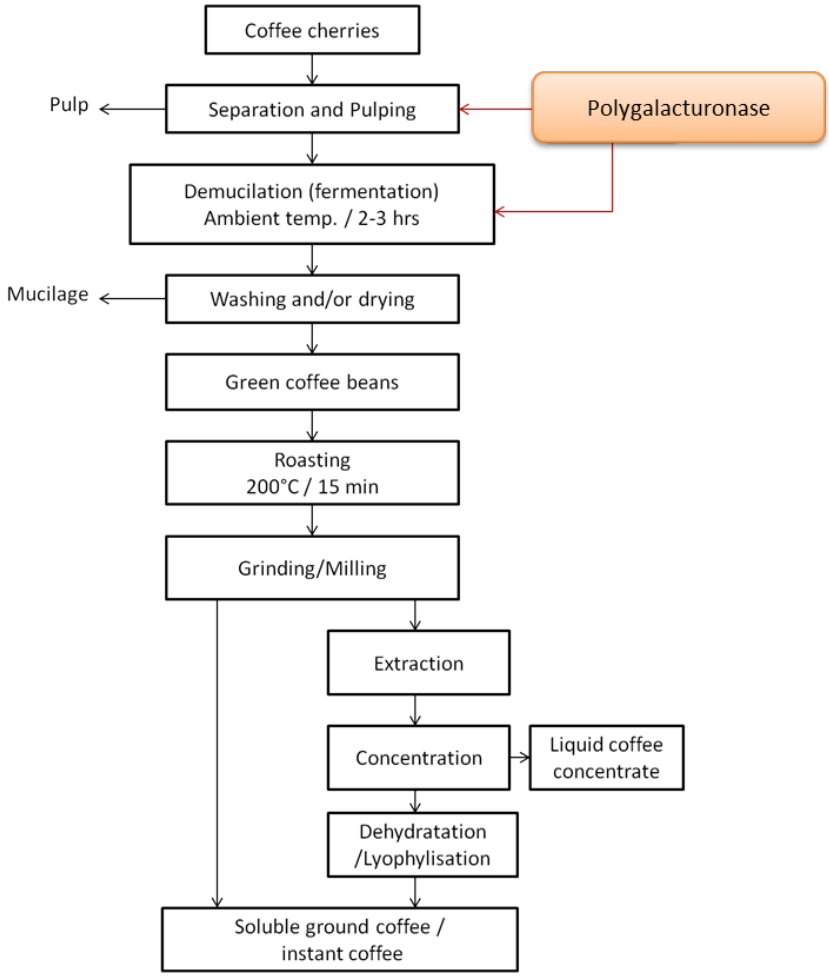


Figure #4 Coffee Production flow

Flavoring Production

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

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

Wine Production

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

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

The advantages of the addition of pectinases during winemaking are:

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

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.

Please refer to figure #5 for process flow chart of polygalacturonase use in wine production.

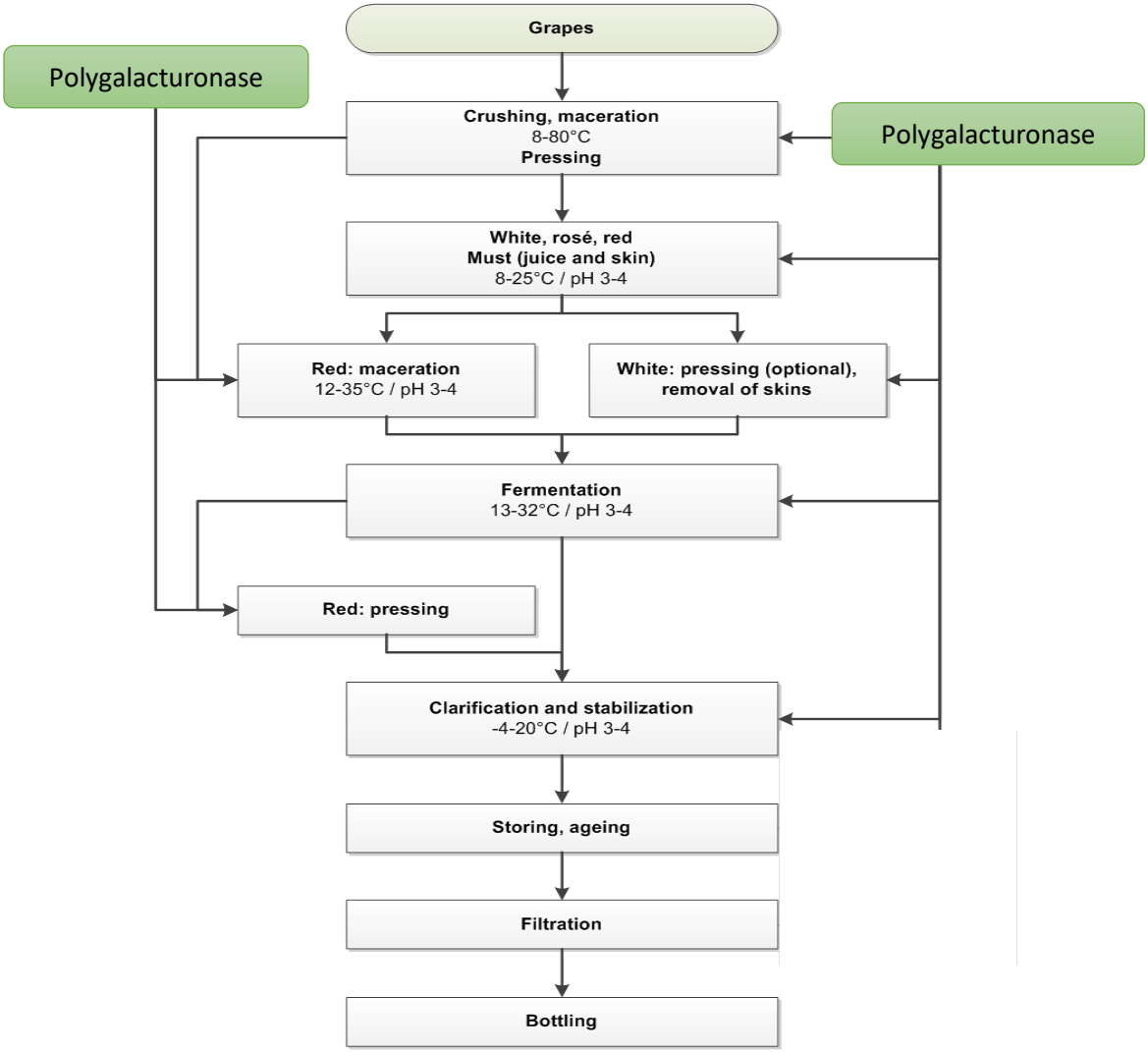


Figure #5 Enzymatic wine processing

2.9 Use Levels

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

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

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

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

The table below shows the range of recommended use levels for each application where the **polygalacturonase** from *Trichoderma reesei* AR-414 may be used:

Food Application		Raw material (RM)	Suggested recommended use levels (mg TOS/kg RM)
Coffee processing		Coffee cherries	0.25
Flavoring production		Fruits/Vegetables	0.5
Fruit and vegetable processing	**Fruit juices**	Fruits/Vegetables	5
	Fruit purees	Fruits/Vegetables	2
Wine production		Grapes	1

2.10 Fate in food

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

Polygalacturonase performs its technological function during food processing. In some cases, the enzyme may no longer be present in the final food. In other cases, where the enzyme protein is still present in the final food, it does not perform any technological function in the final food, just like the endogenous polygalacturonase present in the fruit and vegetable raw materials and ingredients. In order to be able to perform a technological function in the final food, a number of conditions have to be fulfilled at the same time:

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

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

- in fruit and vegetable processing, the polygalacturonase is denatured by heat pasteurization step. In addition, during fruit puree production, the substrate is depleted (due to calcium pectate formation) rendering the enzyme non-functional anymore;
- Inactivation conditions in pasteurized products:
    - Fruit-own polygalacturonase: > 80°C / >2min
    - Polygalacturonase: >75°C / >2min
- in (rare) case of non-pasteurized juices, as well as in wine production, polygalacturonase can be removed by one of the following procedures: precipitation by bentonite (which is added prior to filtration to absorb and therefore remove proteins for wine stabilization); filtration processes (removal of proteins in general); inactivation by some natural wine ingredients like alcohol, polyphenols, metals, sulfur in form of SO_2 (forming the so-called tannin-protein cloudiness), etc.
- during coffee processing, the enzyme is denatured by heat during roasting (typically run at temperatures between 240-275°C) for a period of time ranging from 3 to 30 minutes
- during flavoring production, the enzyme protein is denatured during a heating step (at around 100°C) and subsequently removed during a purification step (i.e. distillation)

Due to the above-mentioned reasons, it can be concluded that polygalacturonase enzyme from *Trichoderma reesei* AR-414 has no technological function in the final food products.

3 Part 3 § 170.325- Dietary Exposure

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

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

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

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

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

Applications		Raw material (RM)	Suggested recommended use level (mg TOS/kg RM)	Final food (FF)	Ratio RM/FF*	Suggested level in final food (mg TOS/kg food)
Liquid foods	Fruit and vegetable processing	Fruit/Vegetable	5	Juices	1.3	6.5
	Coffee processing	Coffee cherries	0.25	Coffee	0.4	0.1
	Flavoring production	Various	0.5	Various beverages	0.01	0.01
	Wine production	Grape	1	Wine	1.6	1.6
Solid foods	Fruit and vegetable processing	Fruit/Vegetable	2		1	2

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

<u>Fruit juices:</u>

- *For fruit juices, we assume that a RM/FF ratio of 1.3 kg fruit per L of fruit juice will be used (typically 0.75-0.9 L juice is produced per kg of fruit thus the range for RM/FF will be 1.1-1.3 kg fruit per L of fruit juice).*
- *For fruit purees, we assume a RR/FF of 1 (1 kg of fruits/ kg of puree).*

<u>Wine:</u>

- *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).*

Flavoring Production

- *Flavorings are generally used in small amounts in final foods. Depending on the composition of the flavoring and the final food application, the typical use levels / dosages range from 0.1 to 1%. Therefore, the corresponding RM/FF ratio is 0.01 kg flavoring per kg of final food.*

Coffee processing:

- *For coffee processing, we assume that a RM/FF of 0.4 will be used (1kg de-pulped coffee cherries lead to 330 g green coffee (ratio: 3) and 1kg green coffee leads to the production of 380 g ground coffee (ratio 2.6), typically 50g ground coffee makes 1 L coffee beverage (ratio 0.05)).*
- *For wine production, we assume that a RM/FF ratio of 1.60 kg grapes per liter of wine will be used (corresponding to a yield of 100 L of wine per 160 kg of grapes).*

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

TMDI in food (mg TOS/kg body weight/day)	TDMI in beverage (mg TOS/kg body weight/day)	Total TMDI (mg TOS/kg body weight/day)
0.025	0.1625	**0.188**

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

- It is assumed that ALL producers of the above-mentioned foodstuffs (and beverages) use specific polygalacturonase from *Trichoderma reesei* AR-414;
- It is assumed that ALL producers apply the HIGHEST use level per application;
- For the calculation of the TMDI's in food, only the above foodstuffs were selected containing the highest theoretical amount of TOS. Therefore, foodstuffs containing lower theoretical amounts were not included;
- It is assumed that the final food containing the calculated theoretical amount of TOS is consumed DAILY over the course of a lifetime;
- Assumptions regarding food and beverage intake of the general population are overestimates of the actual average levels (Douglass et al. 1997).

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

The Margin of Exposure (MoE)[23] for human consumption can be calculated through the division of the NOAEL (no-observed adverse effect) value by the TMDI (Total Theoretical Maximal Daily Intake). Total TMDI of the food enzyme 0.093 mg TOS/kg body weight/day.

As a result, the MoE is:

MoE =1000/0.188 =**5333**

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

Conclusion:

To conclude, the use of the food enzyme polygalacturonase from *Trichoderma reesei* AR-414 in the production of food is safe. Considering the high safety value determined by the MoS, even when calculating using means of overestimation of intake via the Budget method, there is no need to restrict the use of the enzyme in food. The suggested dosage for food manufacturers is not a restrictive value and could be higher or lower depending on usage within cGMPs.

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

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

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

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

This part is not applicable to this notified substance.

6 Part 6 § 170.250- GRAS Notice- Narrative

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

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

6.1 Safety Risk Assessment for Production Strain

6.1.1 History of Production Microorganism in Food

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

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

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

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

Authority	Food enzyme	Reference
JECFA	Cellulase Beta-glucanase Glucoamylase	FAS 30-JECFA 39/15 and FAS 22-JECFA 31/31 FAS 22-JECFA 31/25, JECFA monograph gluco amylase
Australia/NZ	Cellulase Glucan 1-3 beta-glucosidase Beta-glucanase Hemicellulase complex Gluco-amylase Endo 1,4-beta xylanase	Australia New Zealand Food Standards Code – Schedule 18 – Processing aids (legislation.gov.au)
Canada	Cellulase Glucanase Pentosanase Xylanase Protease	5. List of Permitted Food Enzymes (Lists of Permitted Food Additives)
France	Alpha-amylase (GM) Amyloglucosidase (GM) Beta-glucanase (GM) Xylanase Cellulase Lysophospholipase (GM)	Arrêté du 19 octobre 2006
USA[24]	Pectin lyase Transglucosidase Glucoamylase Phospholipase A2	GRAS Notice Inventory, GRN 32 GRAS Notice Inventory, GRN 315 GRAS Notice Inventory, GRN 372

[24] GRAS affirmations and GRAS notifications

	Pectin esterase Mannanase Endo-1,4-beta xylanase Serine endopeptidase	GRAS Notice Inventory, GRN 524 GRAS Notice Inventory, GRN 558 GRAS Notice Inventory, GRN 566 GRAS Notice Inventory, GRN 628

Non-exhaustive list of authorized polygalacturonase from production organisms other than *Trichoderma reesei*		
Authority	**Production Organism**	**Reference**
Australia/NZ	*Aspergillus niger* *Aspergillus oryzae*	Australia New Zealand Food Standards Code – Schedule 18 – Processing aids (legislation.gov.au)
France	*Aspergillus niger*	Arrêté du 19 octobre 2006
USA[25]	*Aspergillus oryzae*	GRAS Notice Inventory, GRN 982
Canada[26]	*Aspergillus niger var.* *Rhizopus oryzae var.*	5. List of Permitted Food Enzymes (Lists of Permitted Food Additives)

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

[26] Entries for pectinases

JECFA	*Aspergillus niger*	JECFA Evaluations-PECTINASES FROM ASPERGILLUS NIGER- (inchem.org)

6.1.2 Safety of the genetic modification

The recipient strain used for the genetic modification is a *Trichoderma reesei* mutant strain, which originates from QM6a. This mutant strain has been shown to be genetically stable for industrial production. The production strain only differs from its recipient strain (AR-329) by the production of polygalacturonase from *Aspergillus kawachii*. As explained in section 2.2.3 of this notice, *Aspergillus kawachii* is a safe source for industrial food enzymes and is taxonomically related to *Aspergillus niger* which has decades of safe use in the food industry. AB Enzymes limits the possibilities of mutations in the product enzyme, as well as in the production strain, through the inoculation of the seed culture for the fermentation with controlled spore stocks that have been stored at -80°C. AB Enzymes has conducted an internal risk assessment to verify the strain is stable and safe, for more information please refer to the sections discussing safety, toxicity and stability of the production strain found in this dossier.

Our evaluation of the genetically modified *T. reesei* strain is comparable to that of the recipient strain and the produced food enzyme is non-pathogenic for healthy humans and animals.

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

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

No additional growth/mutagenesis cycles have been performed after the AR-414 strain has been constructed and thereafter deposited to the culture collection (Master Cell Bank, MCB).

Therefore, it can be concluded that the *Trichoderma reesei* strain AR-414 can be regarded as safe as the recipient or the parental organism to be used for production of enzymes for food processing.

Enzyme Safety Narrative:

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

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

Polygalacturonases are not harmful, and they have a long history of use in food (Pariza M.W. and Foster E.M. 1983; Pariza and Johnson 2001; Sharma et al. 2013). Polygalacturonases have been evaluated worldwide, with multiple national (US GRAS, DK, France…) and international (JECFA) bodies concluding that these food enzymes do not constitute a toxicological hazard. In the USA, AB Enzymes has been able to demonstrate the GRAS status of polygalacturonase sourced from the *Aspergillus* species (*Aspergillus tubingensis*) for the same food applications as in this notice

through scientific procedures[27, 28] with 'No Questions' letters from FDA. Several different types of polygalacturonases are also naturally produced by organisms in nature, even though in much lower relative amounts compared to the production of the recombinant polygalacturonase by *Trichoderma reesei* AR-414 production strain.

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

Furthermore, the allergenic potential of the enzyme is not of significant concern. As explained in section 2.7.1. the allergen searches did not detect matches over 50% homology for any of the searches and the narrative on the allergenic potential of the enzyme reinforce the unlikely chance of the enzyme acting as an allergen.

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

[27] GRAS Notice Inventory, GRN 557
[28] GRAS Notice Inventory, GRN 982

6.1.3 Toxicological testing

The safety of the polygalacturonase produced by the genetically modified *Trichoderma reesei* AR-414 from a toxicological perspective is supported by the historical safety of strain lineage. Toxicological studies were performed on a strain (AR-852) which derives from the same intermediate strain within the strain lineage of AR-414.

The following studies were performed for strain AR-852:

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

All tests were performed according to the principles of Good Laboratory Practices (GLP) and the current OECD and EU guidelines.
Please refer to below to the summary of each of the toxicological studies in Appendix #3.

As mentioned above both the AR-414 and AR-852 have been developed from the same intermediate strain. Expression constructs are very similar, differing by the expression cassette/enzyme gene of interest. As both production strains are free of any harmful sequences or any potential hazards, the expression cassettes are very similar and are stably integrated into the genome of the strains without any additional growth/mutagenesis cycles thereafter, differences in the genetic modification of AR-414 and AR-852 are not a safety concern.

Furthermore, the manufacturing conditions between the two production strains are very similar. The slight changes in pH levels and fermentation medium (food-grade) have been thoroughly

assessed. They are considered minor (common industry practice) and do not trigger any additional safety issue.

To add on, enzyme product from AR-414 production strain complies with JECFA specifications for chemical and microbiological purity of food enzymes (Food and Agriculture Organization of the United Nations 2006) which confirms the safety of the production strain AR-414.

Safety of the Production strain (SSL):

For more details on the safety of the *Trichoderma reesei* AR-414 production strain, we refer to the following appendices:

- **Pariza and Johnson Decision Tree** (Appendix #3)
- **JECFA Safe Progeny Strain statement** (Appendix #4)
- **Differences between tox tested strain and AR-414 production strain** (Appendix #4)
- **Diagram on Strain Lineage** (Appendix #4)

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

6.1.4 Pathogenicity and Toxigenicity

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

Trichoderma reesei is globally regarded as a safe microorganism:

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

Administration (FDA) for numerous enzyme preparations from *T. reesei* for human and animal consumption demonstrate that the enzymes are nontoxic. The Environmental Protection Institute (EPA) completed a risk assessment on *T. reesei* in 2011 resulting in a Proposed Rule in 2012, concluding that it is appropriate to consider *T. reesei* as a recipient microorganism eligible for exemptions from full reporting requirements[29], if this fungus was to be used in submerged standard industrial fermentation for enzyme production. To add on in March 2020, the EPA issued a final rule on **Microorganisms; General Exemptions From Reporting Requirements; Revisions to Recipient Organisms Eligible for Tier I and Tier II Exemptions**[30] as part of the 40 Code of Federal Regulations Part 725 where *Trichoderma reesei* is classified as a Tier I organism.

As a result, AR-414 can be used under the lowest containment level at large scale, GILSP, as defined by OECD (OECD 1992).

The genus *Trichoderma* contains filamentous fungi which are frequently found on decaying wood and in soil. Industrial *T. reesei* strains have a long history of safe use and several of the *Trichoderma* based products have been approved for food and feed applications[31]. *T. reesei* is listed as a "Risk Group 1" organism according to German TRBA classification (Federal Institute for Occupational Safety and Health, www.baua.de) and as "Biosafety Level 1" organism by the American Type Culture Collection (www.atcc.org). *Trichoderma reesei* strains are non-pathogenic for healthy humans and animals. The DNA based identification methods have shown that *T. reesei* is taxonomically different from the other *Trichoderma* species of the section *Longibrachiatum* (Druzhinina et al. 2005).

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

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

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

Some species belonging to *Trichoderma* genus are able to secrete various types of antibiotics in laboratory cultures. However, strains of *T. reesei* used in industrial applications are proven to be devoid of antibiotic activities (Coenen et al. 1995; Hjortkjaer et al. 1986). The absence of antibiotic activities, according to the specifications recommended by JECFA (FAO/WHO 2006), was also confirmed for AR-414. The analyzed data are presented in Appendix #1.

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

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

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

With the use of safe strain lineage, we have substantiated the safety of the AR-414 *Trichoderma reesei* production strain via three toxicological studies on the *Trichoderma reesei* AR-852 production strain to demonstrate non-toxigenicity of the strain lineage. The toxicological studies conducted include, a reverse mutation assay using bacteria, a Micronucleus Assay in Bone Marrow Cells of the Rat, and a 90-day repeated dose oral toxicity study in Wister rats. All three toxicological studies showed negative findings demonstrating the AR-852 production strain to be non-mutagenic, to not induce structural and/or numerical chromosomal damage, and to not cause toxigenic effects on the Wister rats tested in the 90-day oral toxicity study. For more details on the results of the toxicological studies conducted on the production strain, please refer to Appendix #3.

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

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

Conclusion: Based on the above-mentioned available data, it is concluded that the organism *T. reesei*, has a long history of safe use in industrial-scale enzyme production and can be considered as a safe production organism for enzymes for food as well as feed processing and numerous other industrial applications. As an example, *T. reesei* strains have been cultivated in the production plant of Alko Oy/Roal Oy since 1987. During this time, genetic engineering techniques have been used to improve the industrial production strains of *Trichoderma reesei* and considerable experience on the safe use of recombinant *Trichoderma reesei* strains at industrial scale has accumulated. From above, secondary metabolites are of no safety concern in fermentation products derived from *Trichoderma reesei*. Thus, *Trichoderma reesei* and its derivatives can be considered generally safe not only as a production organism of its natural enzymes, but also as a safe host for heterologous gene products. In section 6.1.2 we also provided a short narrative on the safety of the polygalacturonase enzyme.

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

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

Appendices

1. AR-414 Composition Report
2. Flow Chart of the manufacturing process with control steps
3. Summary of Toxicological Studies and Decision Tree Polygalacturonase 2022
4. Safe Strain Lineage Narrative AR-414

Publication bibliography

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

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

Blumenthal, Cynthia Z. (2004): Production of toxic metabolites in Aspergillus niger, Aspergillus oryzae, and Trichoderma reesei: justification of mycotoxin testing in food grade enzyme preparations derived from the three fungi. In *Regulatory toxicology and pharmacology : RTP* 39 (2), pp. 214–228. DOI: 10.1016/j.yrtph.2003.09.002.

Coenen, T. M.; Schoenmakers, A. C.; Verhagen, H. (1995): Safety evaluation of beta-glucanase derived from Trichoderma reesei: summary of toxicological data. In *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association* 33 (10), pp. 859–866.

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

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

Druzhinina, Irina S.; Kopchinskiy, Alexei G.; Komoń, Monika; Bissett, John; Szakacs, George; Kubicek, Christian P. (2005): An oligonucleotide barcode for species identification in Trichoderma and Hypocrea. In *Fungal genetics and biology : FG & B* 42 (10), pp. 813–828. DOI: 10.1016/j.fgb.2005.06.007.

FAO/WHO (2006): Compendium of food additive specifications. Joint FAO/WHO Expert Committee on Food Additives : 67th Meeting 2006. Rome: FAO (FAO JECFA monographs, 1817-7077, 3). Available online at http://www.fao.org/documents/card/en/c/a6fe72dc-82fb-437c-81cc-bc4d739043a5/.

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

Futagami, Taiki (2022): The white koji fungus Aspergillus luchuensis mut. kawachii. In *Biosci. Biotechnol. Biochem.* 86 (5), pp. 574–584. DOI: 10.1093/bbb/zbac033.

Futagami, Taiki; Mori, Kazuki; Wada, Shotaro; Ida, Hiroko; Kajiwara, Yasuhiro; Takashita, Hideharu et al. (2015): Transcriptomic analysis of temperature responses of Aspergillus kawachii during barley koji production. In *Applied and environmental microbiology* 81 (4), pp. 1353–1363. DOI: 10.1128/AEM.03483-14.

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

Godfrey, T.; West, S.: Industrial Enzymology. Second.

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

Hadfield, K. A.; Bennett, A. B. (1998): Polygalacturonases: many genes in search of a function. In *Plant physiology* 117 (2), pp. 337–343.

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

Hjortkjaer, R. K.; Bille-Hansen, V.; Hazelden, K. P.; McConville, M.; McGregor, D. B.; Cuthbert, J. A. et al. (1986): Safety evaluation of Celluclast, an acid cellulase derived from Trichoderma reesei. In *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association* 24 (1), pp. 55–63.

Hong, Seung-Beom; Lee, Mina; Kim, Dae-Ho; Varga, Janos; Frisvad, Jens C.; Perrone, Giancarlo et al. (2013): Aspergillus luchuensis, an industrially important black Aspergillus in East Asia. In *PLOS ONE* 8 (5), e63769. DOI: 10.1371/journal.pone.0063769.

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

Ibarrola, Ignacio; Arilla, M. Carmen; Martínez, Alberto; Asturias, Juan A. (2004): Identification of a polygalacturonase as a major allergen (Pla a 2) from Platanus acerifolia pollen. In *The Journal of allergy and clinical immunology* 113 (6), pp. 1185–1191. DOI: 10.1016/j.jaci.2004.02.031.

Joint FAO/WHO Codex Alimentarius Commission; World Health Organization; Food and Agriculture Organization of the United Nations (2009): Foods derived from modern biotechnology. 2nd edition. Rome: World Health Organization : Food and Agriculture Organization of the United Nations (Codex alimentarius, 0259-2916).

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

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

Kikuchi, Yuko; Takai, Toshiro; Kuhara, Takatoshi; Ota, Mikiko; Kato, Takeshi; Hatanaka, Hideki et al. (2006): Crucial commitment of proteolytic activity of a purified recombinant major house dust mite allergen Der p1 to sensitization toward IgE and IgG responses. In *Journal of immunology (Baltimore, Md. : 1950)* 177 (3), pp. 1609–1617. Available online at https://pubmed.ncbi.nlm.nih.gov/16849469/.

Nakazawa, Takuya; Takai, Toshiro; Hatanaka, Hideki; Mizuuchi, Eri; Nagamune, Teruyuki; Okumura, Ko; Ogawa, Hideoki (2005): Multiple-mutation at a potential ligand-binding region decreased allergenicity of a mite allergen Der f 2 without disrupting global structure. In *FEBS letters* 579 (9), pp. 1988–1994. DOI: 10.1016/j.febslet.2005.01.088.

Nevalainen, H.; Suominen, P.; Taimisto, K. (1994): On the safety of Trichoderma reesei. In *Journal of biotechnology* 37 (3), pp. 193–200.

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

Olempska-Beer, Zofia S.; Merker, Robert I.; Ditto, Mary D.; DiNovi, Michael J. (2006): Food-processing enzymes from recombinant microorganisms--a review. In *Regulatory toxicology and pharmacology : RTP* 45 (2), pp. 144–158. DOI: 10.1016/j.yrtph.2006.05.001.

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

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

Penttilä, M.; Nevalainen, H.; Rättö, M.; Salminen, E.; Knowles, J. (1987): A versatile transformation system for the cellulolytic filamentous fungus Trichoderma reesei. In *Gene* 61 (2), pp. 155–164.

Rojas, N. L.; Ortiz, G. E.; Baruque, D. J.; Cavalitto, S. F.; Ghiringhelli, P. D. (2011): Production of heterologous polygalacturonase I from Aspergillus kawachii in Saccharomyces cerevisiae in batch and fed-batch cultures. In *Journal of industrial microbiology & biotechnology* 38 (9), pp. 1437–1447. DOI: 10.1007/s10295-010-0929-9.

Sarkar, Moumita B.; Sircar, Gaurab; Ghosh, Nandini; Das, Abhishek K.; Jana, Kuladip; Dasgupta, Angira; Bhattacharya, Swati G. (2018): Cari p 1, a Novel Polygalacturonase Allergen From Papaya Acting as Respiratory and Food Sensitizer. In *Frontiers in plant science* 9, p. 823. DOI: 10.3389/fpls.2018.00823.

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

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

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

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

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

Voragen, Alphons G. J.; Coenen, Gerd-Jan; Verhoef, René P.; Schols, Henk A. (2009): Pectin, a versatile polysaccharide present in plant cell walls. In *Struct Chem* 20 (2), pp. 263–275. DOI: 10.1007/s11224-009-9442-z.

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

FDA USE ONLY

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

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

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

SECTION A INTRODUCTORY INFORMATION ABOUT THE SUBMISSION

1. Type of Submission *(Check one)*

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

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

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

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

1a. Notifier	Name of Contact Person Joab Trujillo		Position or Title Regulatory Affairs Specialist	
	Organization *(if applicable)* AB Enzymes Inc.			
	Mailing Address *(number and street)* 8211 W. Broward Blvd. Suite 420			
City Plantation	State or Province Florida	Zip Code/Postal Code 33324	Country United States of America	
Telephone Number +1 954 800 8606	Fax Number	E-Mail Address joab.trujillo@abenzymes.com		
1b. Agent or Attorney *(if applicable)*	Name of Contact Person		Position or Title	
	Organization *(if applicable)*			
	Mailing Address *(number and street)*			
City	State or Province	Zip Code/Postal Code	Country	
Telephone Number	Fax Number	E-Mail Address		

SECTION C GENERAL ADMINISTRATIVE INFORMATION

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

Polygalacturonase enzyme preparation from a genetically modified Trichoderma reesei

2. Submission Format: *(Check appropriate box(es))*

☒ Electronic Submission Gateway ☐ Electronic files on physical media

☐ Paper

If applicable give number and type of physical media

3. For paper submissions only:

Number of volumes _____

Total number of pages _____

4. Does this submission incorporate any information in CFSAN's files? *(Check one)*

☒ Yes *(Proceed to Item 5)* ☐ No *(Proceed to Item 6)*

5. The submission incorporates information from a previous submission to FDA as indicated below *(Check all that apply)*

☒ a) GRAS Notice No. GRN 557

☐ b) GRAS Affirmation Petition No. GRP _____

☐ c) Food Additive Petition No. FAP _____

☐ d) Food Master File No. FMF _____

☒ e) Other or Additional *(describe or enter information as above)* GRAS Notice No. GRN 982

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

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

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

☐ Yes *(Proceed to Item 8*

☒ No *(Proceed to Section D)*

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

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

☐ No

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

☐ Yes, a redacted copy of the complete submission

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

☐ No

SECTION D INTENDED USE

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

The polygalacturonase enzyme is to be used as a processing aid in fruit and vegetable processing, coffee processing, flavoring production, and wine production. The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements under current Good Manufacturing Practice.

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

(Check one)

☐ Yes ☒ No

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

(Check one)

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

SECTION E PARTS 2 7 OF YOUR GRAS NOTICE

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

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

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

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

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

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

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

Other Information

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

☒ Yes ☐ No

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

☒ Yes ☐ No

SECTION F SIGNATURE AND CERTIFICATION STATEMENTS

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

has concluded that the intended use*(s)* of Polygalacturonase enzyme preparation from a genetically modified Trichoderma reesei
(name of notified substance)

described on this form, as discussed in the attached notice, is (are) not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on your conclusion that the substance is generally recognized as safe recognized as safe under the conditions of its intended use in accordance with § 170.30.

2. AB Enzymes Inc. agrees to make the data and information that are the basis for the conclusion of GRAS status available to FDA if FDA asks to see them; agrees to allow FDA to review and copy these data and information during customary business hours at the following location if FDA asks to do so; agrees to send these data and information to FDA if FDA asks to do so.
(name of notifier)

8211 W. Broward Blvd. Suite 420 Plantation Florida 33324 USA
(address of notifier or other location)

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

3. Signature of Responsible Official, Agent, or Attorney	Printed Name and Title	Date (mm/dd/yyyy)
Joab Trujillo Digitally signed by Joab Trujillo Date: 2022.10.31 11:45:48 -04'00'	Joab Trujillo Regulatory Affairs Specialist	10/31/2022

SECTION G LIST OF ATTACHMENTS

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

Attachment Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)
	Form3667 AB Enzymes Polygalacturonase 2022	Administrative
	AB Enzymes' Cover Letter for Polygalacturonase GRAS Notice 2022	Submission
	AB Enzymes Polygalacturonase GRAS Notice 2022	Submission
	1_AR-414 Composition Report	Submission
	2_Flow Chart of the manufacturing process with control steps	Submission
	3_Summary of Toxicological Studies and Decision Tree Polygalacturonase	Submission
	4_Safe Strain Lineage Narrative AR-414	Submission
	References for AB Enzymes' Polygalacturonase GRAS Notice.zip	Submission

Objective: **Chemical Composition Analysis of Polygalacturonase from *Trichoderma reesei*, Strain AR-414**

Samples:	**Batch**	**LIMS ID**
Liquid enzyme concentrate	**P210024**	**1-21-02360-003**
Liquid enzyme concentrate	**P210026**	**1-21-02666-002**
Liquid enzyme concentrate	**P220003**	**1-22-00296-001**

Table 1. Main activity

	Batch		
	P210024	**P210026**	**P220003**
Polygalacturonase activity (PGX/g)	56 500	54 400	52 500

PGX: Assay of polygalacturonase activity, Roal internal method B501

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

	Batch		
	P210024	**P210026**	**P220003**
Antimicrobial activity	not detected	not detected	not detected
Presence of production strain	not detected	not detected	not detected
Escherichia coli (/25 g)	not detected	not detected	not detected
Salmonella (/25 g)	not detected	not detected	not detected
Total coliforms cfu*/g)	<30	<30	<30
Lead (mg/kg)	<0,05	<0,05	<0,05

Antimicrobial activity: Specifications for Identity and Purity of Certain Food Additives, FAO Food and Nutrition Paper 65 (2006), Rome, Vol.4, p. 122.
Production strain: Detection of production strain (*Trichoderma*, *Aspergillus*), Roal internal method M001.
E. coli: ISO 16649-3:2015, mod.
Salmonella: NMKL 71:1999, mod.
B. cereus: NMKL 67:2021
Total coliforms: ISO 4832:2006, mod.
*cfu: colony forming units
Lead: ISO 17294-2

Table 3. Mycotoxins (µg/kg)

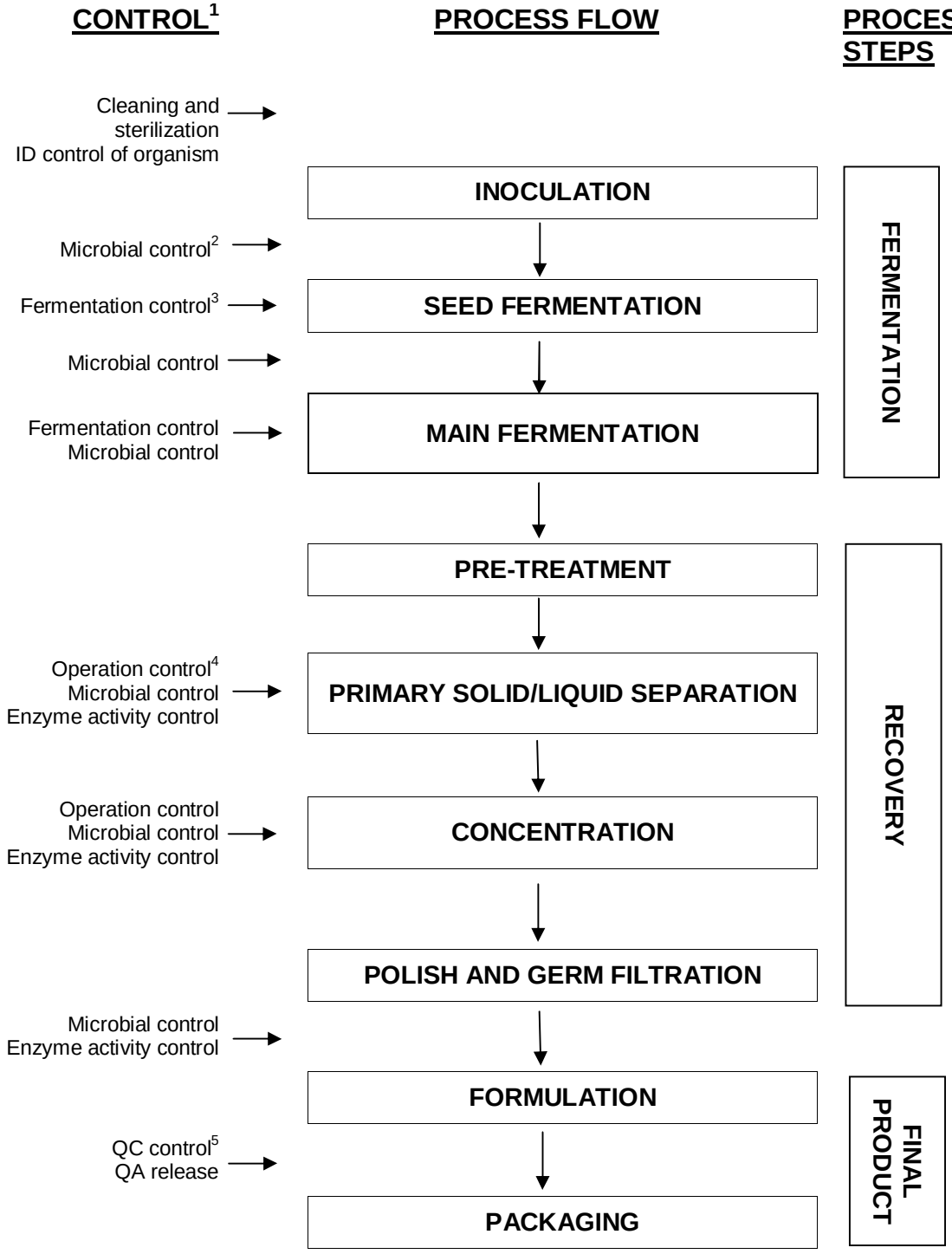
	Batch		
	P210024	**P210026**	**P220003**
HT-2 Toxin	<10	<10	<10
T-2 Toxin	<10	<10	<10

Fusarium toxins: Eurofins Internal Method, LC-MS/MS

Rajamäki 18/10/2022

Anna He
Quality Information Specialist
Roal Oy

Production Process of Food Enzymes from Fermentation



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

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

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

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

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

TOXICOLOGICAL STUDIES SUMMARY

Reverse Mutation Assay using Bacteria (*Salmonella typhimurium*)

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Control: 0 mg TOS/kg body weight

Low Dose: 100 mg TOS/kg body weight

Medium Dose: 300 mg TOS/kg body weight

High Dose: 1000 mg TOS/kg body weight

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

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

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

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

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

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

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

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

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

No test item related alterations were reported during histopathological examination.

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

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

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

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

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

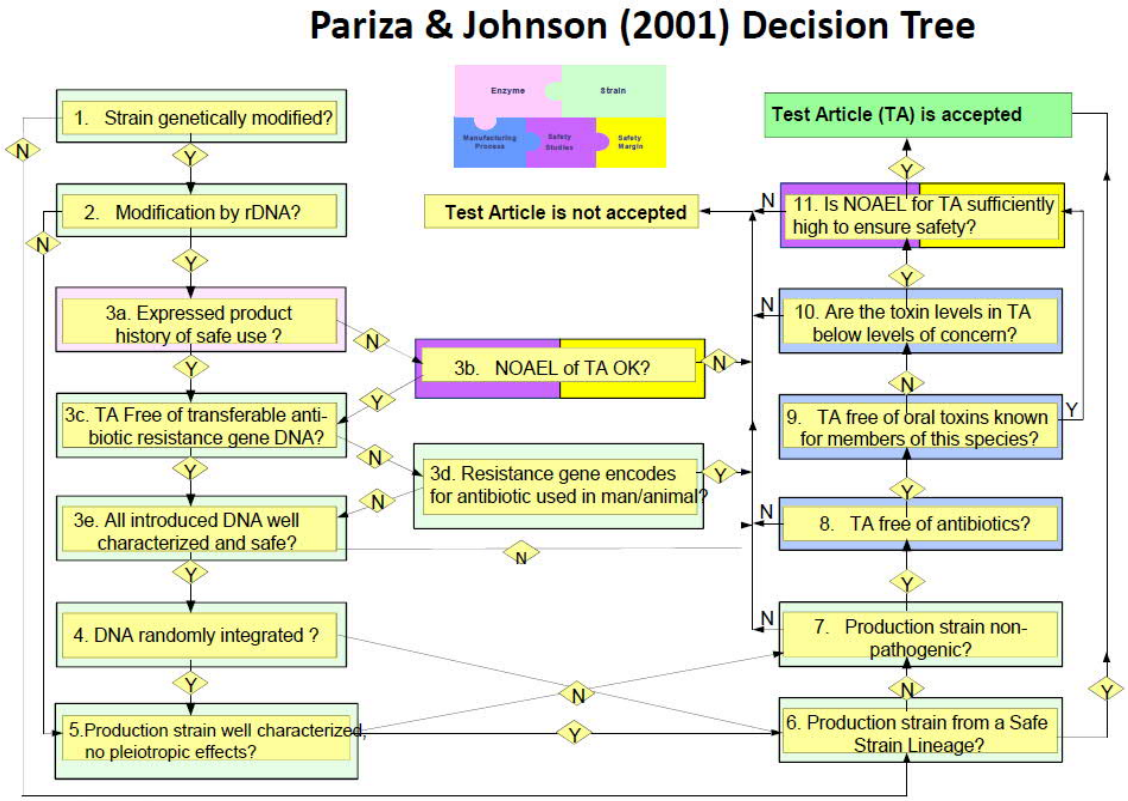
ANALYSIS OF SAFETY BASED ON PARIZA AND JOHNSON DECISION TREE

Pariza and Johnson have published updated guidelines for the safety assessment of microbial enzyme preparations (2001)[1] from the IFBC Decision Tree[2]. The safety assessment of a given enzyme preparation is based upon an evaluation of the toxigenic potential of the production organism. The responses below follow the pathway indicated in the decision tree as outlined in Pariza and Johnson, 2001. The outcome of this inquiry is that *Trichoderma reesei* AR-414 production strain producing a polygalacturonase is "ACCEPTED" as safe for its intended use.

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

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

Decision Tree:



1. Strain genetically modified? (Yes, AR-414 strain is genetically modified, see section 2.3 for genetic modification description). Go to #2

2. Modification by rDNA? (Yes, AR-414 strain is modified by rDNA) Go to #3a

3.

   3a. Expressed product history of safe use (Yes, please refer to section 6.1 on the safety of the production strain and section 2.8 technological purpose for evidence) Go to #3c

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

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

4. DNA randomly integrated? (Yes, the introduced DNA is randomly integrated into the chromosome) Go to #5

5. Production strain well characterized, no pleiotropic effects? (Yes, as demonstrated in section 6.1.10, the production strain is well characterized, 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, *Trichoderma reesei* has been demonstrated as a safe production host and methods of modification have been well documented. Safety of this organism has been evaluated and confirmed through toxicological testing as described herein). If yes, the test article is **ACCEPTED**.

Thus, AB Enzymes concludes that the decision tree shows that the *Trichoderma reesei* production strain AR-414 is **ACCEPTED.**

Safe Strain Lineage:

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

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

As of 2020, JECFA has evaluated over 80 food enzyme preparations from a variety of microorganisms and has never recorded a positive result in any toxicity study, suggesting either that toxins were not present or that toxins were present at levels that were below the limit of detection of the bioassays. JECFA concluded provided that the genetic modification (either recombinant DNA or chemical mutagenesis) is well characterized, additional toxicological testing would not be required.

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

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

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

The polygalacturonase is produced by *Trichoderma reesei* AR-414 production strain. The transformation of the recipient strain AR-329 *Trichoderma reesei* with the expression cassette results in the recombinant strain AR-414 (see Table 1 below).

The production organism *Trichoderma reesei* has been genetically engineered by deleting genes from the genome and by transformation of the strain with the expression plasmid to promote polygalacturonase production. All genetic modifications are well characterized and as such the safe strain lineage concept was employed as the *Trichoderma* reesei intermediate strain, AR-288, was similarly used for the AR-852 strain which has been assessed in several toxicological studies as presented below in **Table 1 and Table 2**. AR-288 is the last common intermediate strain after which the lineage separates. Both AR-414 and AR-852 derive from AR-288. The recipient strain for AR-414, AR-329 contains one additional deletion than the recipient strain for AR-852, AR-555. The additional deletion is minor and done to further reduce the already low side activities. The safe strain lineage flow chart is present in **Figure 1** illustrating the relationship between the different strains in the lineage. The differences between the two strains (AR-414 and AR-852) are the inserted expression cassettes containing the enzyme genes of interest and the additional deletion applicable to AR-414.

Table 1: Comparison of the AR-414 and Toxicological tested strain AR-852 Expression Cassettes

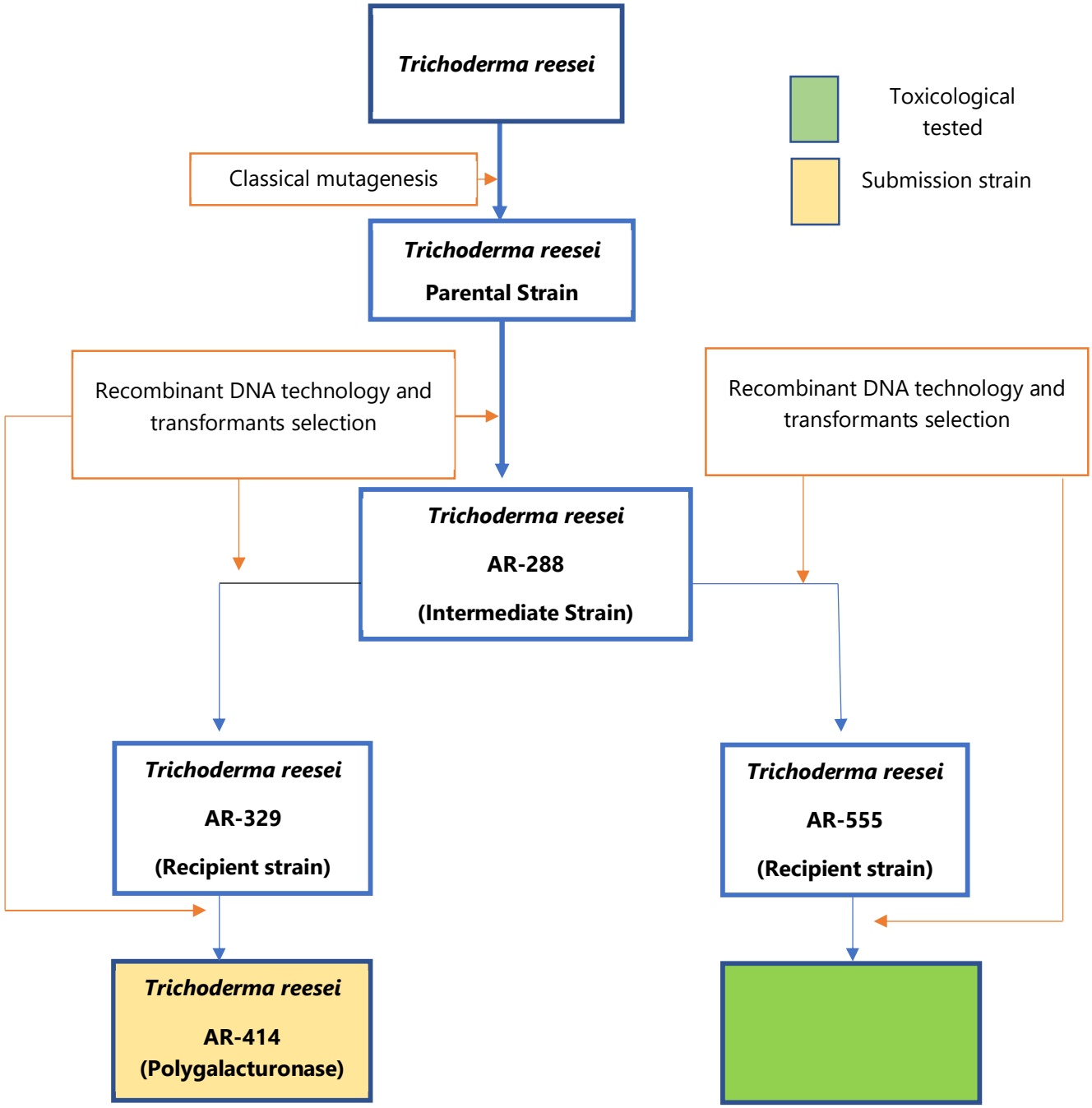
Production Strain	Promoter[3]	Signal Sequence	Enzyme	Terminator[4]	Selection marker
AR-414 *T. reesei*	*T. reesei* promoter	Native polygalacturonase signal sequence	Polygalacturonase	*T. reesei* terminator	*A. nidulans* amdS
AR-852 *T. reesei*	*T. reesei* promoter	Native cellulase signal sequence	Cellulase	*T. reesei* terminator	*A. nidulans* amdS

[3] Expression cassettes for AR-414 and AR-852 use different *T. reesei* promoters
[4] Expression cassettes for AR-414 and AR-852 use different *T. reesei* terminators

Table 2: Toxicological Test Summaries

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

Figure 1: *Trichoderma reesei* Safe Strain Lineage of AR-414



Safety Narrative:

As described above the AR-414 and the safe strain AR-852 come from the same parental strain and intermediate strain **(Fig. 1)**. Both strains are very similar. This is also true for the expression cassette, which differs by the enzyme of interest as described in Table 1.

As the production strains (including the expression cassettes) are free of any harmful sequences or any potential hazards, differences in the genetic modification of AR-414 and AR-852 are not a safety concern.

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

To add on, enzyme product from AR-414 production strain complies with JECFA specifications for chemical and microbiological purity of food enzymes (Food and Agriculture Organization of the United Nations 2006) which confirms the safety of the production strain AR-414.

Based on the rationale provided above as per JECFA, 2020, as well as on the review of the strains meeting the requirements of Pariza and Johnson Decision Tree (Appendix #3 of GRAS narrative), AB Enzymes concludes polygalacturonase produced by *Trichoderma reesei* AR-414 to be safe and does not pose a significant risk to human health.

Margin of Exposure:

According to the Safe Strain Lineage concept, the Margin of Exposure (MoE) for human consumption can be calculated by dividing the NOAEL by the Total Theoretical Maximal Daily Intake (TMDI). In the case of the safe strain lineage concept for AR-414, there is no NOAEL. However, the NOAEL from the 90-day toxicological study from the closely related production strain *Trichoderma reesei* AR-852 is used to calculate the MoE and support the safety of the *Trichoderma reesei* AR-414.