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# A Petition to Amend the Australia New Zealand Food Standards Code with a Polygalacturonase Enzyme Preparation produced by *Aspergillus Oryzae*

**AB Enzymes GmbH**

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## II. EXECUTIVE SUMMARY

The present application seeks to schedule 18 - Processing Aids of the Australia New Zealand Food Standards Code (the Code) to approve a polygalacturonase[1] enzyme preparation from *Aspergillus Oryzae* produced by AB Enzymes GmbH for use as a processing aid in:

- Fruit juices/products
- Vegetable juices/products
- Coffee processing
- Flavouring production
- Wine production

### Proposed change to Standard 1.3.3 - Processing Aids

The table schedule 18—9(3), Permitted processing aids various purposes, is proposed to be amended to include a genetically modified strain of *Aspergillus Oryzae* as permitted source for polygalacturonase EC 3.2.1.15.

This application is submitted under a general assessment procedure.

### Description of Enzyme Preparation

The food enzyme is a biological isolate of variable composition, containing the enzyme protein, as well as organic and inorganic material derived from the microorganism and fermentation process.

The main activity of the food enzyme is polygalacturonase

| Property | Requirement | |
|---|---|---|
| Activity | min. | 75,000 PGU/mg |
| Appearance | Brown liquid | |

[1] AB Enzymes has also submitted to FSANZ the Pectin esterase enzyme preparation from *Aspergillus Oryzae* which is used in conjunction with the polygalacturonase described in this dossier.

| | |
|---|---|
| Density | 1.1 g/ml |

**Use of the Enzyme and Benefits**

The main activity of the *Aspergillus oryzae* AR-183 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.

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, and oil extraction, etc.

**Safety Evaluation**

The food enzyme object of the present dossier was subjected to several toxicological studies to confirm its safety for consumers. The mutagenicity studies showed that the food enzyme does not have the potential to damage the genetic material of living organisms, including mammals. The oral toxicity study showed that the food enzyme does not exhibit signs of toxicity, up to doses that are several thousand times higher than those which are consumed via food.

The product complies with the recommended purity specifications (microbiological and chemical requirements) of the FAO/WHO's Joint Expert Committee on Food Additives (JECFA) and the Food Chemicals Codex (FCC) for food-grade enzymes.

The product is free of production strain and recombinant DNA.

The safety of the polygalacturonase preparation was confirmed or is under consideration by external expert groups, as follows:

**Approved evaluation**

- Denmark – appendix 1a
- France – appendix 1b

**Under evaluation:**

- EFSA
- USA
- Brazil

**Conclusion**

Based on the safety evaluation, AB Enzymes GmbH respectfully request the inclusion of polygalacturonase from *Aspergillus tubigiensis* expressed in *Aspergillus oryzae* in the table – 18-9(3) of schedule 18 - Permitted processing aids various purposes.

## III. INTRODUCTION

The dossier herein describes *Aspergillus oryzae* produced polygalacturonase expressing a gene from *Aspergillus tubigiensis* produced by submerged fermentation.

Polygalacturonase from *Aspergillus tubingensis* expressed in *Aspergillus oryzae* is mainly intended to be used in fruit and vegetable, coffee, flavouring and wine production

The following sections describe the genetic modifications implemented in the development of the production microorganism to create a safe standard host strain resulting in a genetically well-characterized production strain, free from harmful sequences.

Further sections show the enzymatic activity of the enzyme, along with comparison to other similar enzymes. The safety of the materials used in manufacturing, and the manufacturing process itself is described. The hygienic measurements, composition and specifications as well as the self-limiting levels of use for polygalacturonase are described. Information on the mode of action, applications, and use levels and enzyme residues in final food products are described. The safety studies outlined herein indicate that the polygalacturonase preparation from *Aspergillus oryzae* shows no evidence of pathogenic or toxic effects. Estimates of human consumption and an evaluation of dietary exposure are also included.

## IV. Section 3.1, GENERAL REQUIREMENTS

### 3.1.1. Executive Summary

An Executive Summary is provided as a separate copy together with this application.

### 3.1.2. Applicant Details

**Applicant's name**

[redacted]

### 3.1.3. Purpose of the Application

The table (section 1.3.3—11) 18—9(3), Permitted processing aids various purposes, is proposed to be amended to include a genetically modified strain of *Aspergillus oryzae* as permitted source for polygalacturonase.

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

### 3.1.4. Justification for the Application

**The need for the proposed change:**

*Aspergillus oyrzae* expressing a polygalacturonase gene from *Aspergillus tubingensis* is not present as an approved source in the table to schedule 18 of standard 1.3.3.; Permitted Enzymes of Microbial Enzymes. AB Enzymes GmbH is requesting that this source organism be added. See 3.1.5 for details regarding the advantages of the proposed change.

### 3.1.5. The Advantages of the Proposed Change over the Status Quo:

The polygalacturonase enzyme is one of AB Enzymes latest achievements and has showed great potential in food manufacturing as detailed in this customer support letter, Appendix #1.1.

Based on performance and market benchmarking we have proven that our products made with the two pectinases combined have excellent technical characteristics in targeted applications. The specific degradation of water-soluble, highly esterified "smooth region" pectin is of particular importance in fruit processing. Particularly polygalacturonase with a high content of pectin esterase are suitable for this purpose. This enzyme combination is an ideal mash enzyme for core fruit or grapes, because the breakdown of the soluble pectin reduces its viscosity and thus causes a faster flow of the juice. On the other hand, the mash structure is preserved because the scaffolding-forming protopectin is less attacked. Another advantage of the selective reaction is that less "hairy region" pectins are solved, which can cause filtration problems in further processing. Overall, applying specific enzymes has proven to deliver higher yields, produce less waste, reduced energy consumption and stable end products. There is also a cost benefit associated with the use of *Aspergillus oryzae* as superior producer of enzymes resulting in a cost benefit that is passed on to the final user of the enzyme. It will increase the choice to local manufacturers and help in reducing production costs as compared to the currently known and marketed products of the same enzyme class used for the same type of applications.

Furthermore, there are no public health or safety issues related to the proposed change.

### 3.1.6. Regulatory Impact Statement:

The addition of the enzyme to Schedule 18-9(3) is not intended to place any costs or regulatory restrictions on industry or consumers. Inclusion of the enzyme will provide food manufacturers with an alternative. For government, the burden is limited to necessary activities for a variation of Standard 1.3.3.

### 3.1.7. Impact on International Trade:

There will be a positive impact on Australia / New Zealand manufacturers of fruit, vegetable, and wine products. Many of these companies are active in export markets of Southeast Asia or the Middle East and are facing local competition and competitors from Europe or North America. Many of the competitors have already access to these new tools and their beneficial cost/performance. The approval of the enzyme could therefore have a positive impact to keep Australia / New Zealand manufacturers competitive in international trade.

### 3.1.8. Information to Support the Application

**Public Health and Safety Issues related to the Proposed Change:**

No public health and safety issues are expected from the proposed changes.

The food enzyme object of the present dossier was subjected to several toxicological studies to confirm its safety for consumers. The genotoxicity studies showed that the food enzyme does not have the potential to damage the genetic material of living organisms, including mammals. The oral toxicity study showed that the food enzyme does not exhibit signs of toxicity, up to doses that are several thousand times higher than those which are consumed via food.

The product complies with the recommended purity specifications (microbiological and chemical requirements) of the FAO/WHO's Joint Expert Committee on Food Additives (JECFA) and the most recent Food Chemicals Codex (FCC) for food-grade enzymes.

The product is free of production strain and recombinant DNA.

**Consumer choice related to the Proposed Change:**

Consumer choice is not expected to be changed directly as the enzyme is used as a processing aid and is not purchased by consumers. Polygalacturonase does not perform any technological function in the final foods containing ingredients prepared with the help of this enzyme. Moreover, the food products prepared with the help of polygalacturonase do not have other characteristics than what is expected by the consumer. Consumers could be impacted indirectly by companies able to pass cost savings from utilizing enzymes in food processing on to their customers.

### 3.1.9. Assessment Procedure

Because the application is for a new source organism for an existing enzyme in the Code, it is considered appropriate that the assessment procedure is characterized as "General Procedure, Level 1".

### 3.1.10. Confidential Commercial Information (CCI)

Detailed information on the construction and characteristics of the genetically modified production strain is provided in the confidential Appendix CCI. A summary of this information is given in section E of section 3.2.2. The formal request for treatment of Appendix CCI as confidential commercial information (CCI) is included as Appendix #1.2.

### 3.1.11. Other Confidential Information

Information related to the approval letters from government authorities is company specific and this information is not publicly available and known only to AB Enzymes GmbH, as such we respectfully ask that this information is kept confidential as presented in Appendix #1a,b. The formal request for treatment of Appendix #1a,b as other confidential information is included as Appendix #1.3.

### 3.1.12. Exclusive Capturable Commercial Benefit (ECCB)

This application is not expected to confer an Exclusive Capturable Commercial Benefit, as once the enzyme and source organism is listed publically on FSANZ website, any company can benefit from the use of the enzyme.

### 3.1.13. International and other National Standards

**International Standards:**

Use of enzymes as processing aids fruit and vegetable, wine and coffee is not restricted by any Codex Alimentarius Commission (Codex) Standards or any other known regulations.

**National Standards:**

Not applicable

### 3.1.14. Statutory Declaration

The Statutory Declaration is included as Appendix #1.4.

This application concerns an enzyme product intended to be used as a processing aid for food manufacturing.

Therefore, the relevant documentation according to the Application Handbook from Food Standards Australia New Zealand as of July 1, 2019, are the following sections:

• SECTION 3.1 – GENERAL REQUIREMENTS

• SECTION 3.3.2 – PROCESSING AIDS, subsections A, C, D, E, F

Accordingly, the checklist for General Requirements as well as the Processing Aids part of the checklist for Standards related to Substances added to Food was used and is included as Appendix #1.5.

## V. Section 3.3.2. STANDARDS RELATED TO SUBSTANCES ADDED TO FOOD PROCESSING AID

### A. Technical Information of the Processing aid

#### A.1. Information on the type of processing aid

This dossier includes a polygalacturonase enzyme, produced with the help of *Aspergillus oryzae* strain AR-183. The representative current commercial product is ROHAPECT MA Plus.[3]

Polygalacturonase is a microbial produced enzyme and already belongs to the table to Schedule 18 of standard 1.3.3.; Permitted enzymes of Microbial Enzymes.

Enzyme preparations are generally used *quantum satis*. The average dosage of the enzyme depends on the application, the type and quality of the raw materials used, and the process conditions. This dossier is specifically submitted for use of polygalacturonase in fruit/vegetable processing, coffee, wine and flavouring production. A further description of the enzyme in these food technology applications will be given in subsequent sections.

#### A.2. Information on the identity of the processing aid

A.2.1. **Enzyme**

| | |
|---|---|
| **Systematic name** | Polygalacturonase |
| **Common names** | Pectin depolymerase; pectinase; endopolygalacturonase; pectolase; pectin hydrolase; pectin polygalacturonase; endo-polygalacturonase; poly-α-1,4-galacturonide glycanohydrolase; endogalacturonase; endo-D-galacturonase; poly(1,4-α-D-galacturonide) glycanohydrolase; (1→4)-α-D-galacturonan glycanohydrolase |
| **Enzyme Commission No.** | **3.2.1.15** |
| **Host** | *Aspergillus oryzae* |
| **Donor** | *Aspergillus tubingensis* |

[3] This is a combined product with pectin esterase AR-962, both have been submitted to FSANZ

### A.2.2. **Enzyme Preparation**

The enzyme is a polygalacturonase and is sold as a liquid enzyme preparation as ROHAPECT® MA Plus (Appendix #2).

### A.2.3. **Enzyme preparation composition:**

| **Composition** | |
|---|---|
| **Constituent** | **%** |
| Pectinase Concentrate | 25-30 |
| Glycerol | 45 |
| Sodium chloride | 6 |
| Water | Remainder |

In the case of the final products for the AR-183 *Aspergillus oryzae* polygalacturonase strain, the presence of the following food additives in the final product are present:

- Glycerol (Function: Stabilizer)
- Sodium Chloride (Function: Stabilizer)

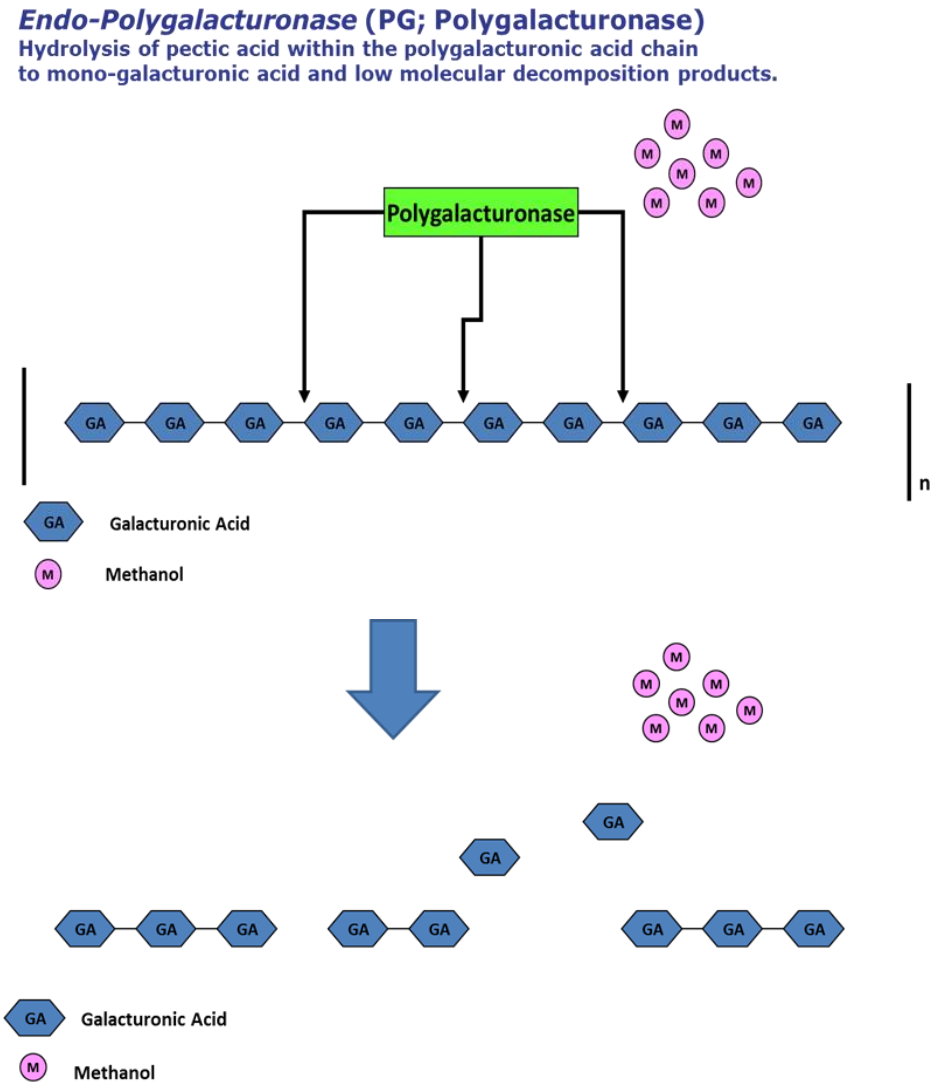
### A.2.4. **Enzyme activity**

The enzyme preparation is a blend of pectin esterase (3.1.1.11) and polygalacturonase and the product is standardized on polygalacturonase activity only.

The main activity of the *Aspergillus oryzae* AR-183 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 esterase has the ability to de-esterify pectin by the

removal of methoxy residues. Pectin depolymerases readily split the main chain and have been further classified as polygalacturonases (PG) and pectin lyases (PL).

This food enzyme catalyses 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 analyse the activity of the enzyme is company specific and is capable of quantifying polygalacturonase activity as defined by its IUBMB classification. The enzyme activity is usually reported in PGU/mg. Polygalacturonase activity is determined using in-house validated methods.

Polygalacturonase causes a reduction of viscosity of a pectin substrate. The activity is calculated based on an enzymatic activity value of a known standards sample.

### A.2.5. **Enzyme genetic modification**

The enzyme is from *Aspergillus oryzae* host strain genetically modified with a polygalacturonase gene deriving from *Aspergillus tubingensis* . The enzyme is not protein engineered.

| | |
|---|---|
| Name of the enzyme protein: | Polygalacturonase |
| Donor: | *Aspergillus tubingensis* |
| Host: | *Aspergillus oryzae* |
| Production strain: | AR-183 *Aspergillus oryzae* |

For more detailed information on the genetic modification, please see **Section E**.

## A.3. Information on the chemical and physical properties of the processing aid

**Product –Rohapect® MA Plus**

| **Property** | **Requirement** | |
|---|---|---|
| Activity | declared | 75,000 PGU/g |
| Appearance | Brown liquid | |
| Density | 1.1 g/ml | |

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

Like most of the enzymes, polygalacturonase performs its technological function during food processing and does not perform any technological function in the final food. The reasons why the enzyme does not exert any (unintentional) enzymatic activity in the final food can be due to a combination of various

factors, depending on the application and the process conditions used by the individual food producer. These factors include depletion of the substrate, denaturation of the enzyme during processing (which is clearly the case during baking process), lack of water activity, wrong pH, etc. In some cases (e.g. after alcohol distillation, products resulting from starch processing), the enzyme may no longer be present in the final food.

Based on the conditions of use described in **Section F** and the activity of polygalacturonase under such conditions, it can be concluded that the enzyme polygalacturonase does not exert any (unintentional) enzymatic activity in final food products.

Recommended storage: Store cool below 10 °C. The best before date is displayed on the certificate of analysis and product label. The product should be used within 36 months of the production date.

For the Chemical properties – see **Section A.5**.

Technological need:

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

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.

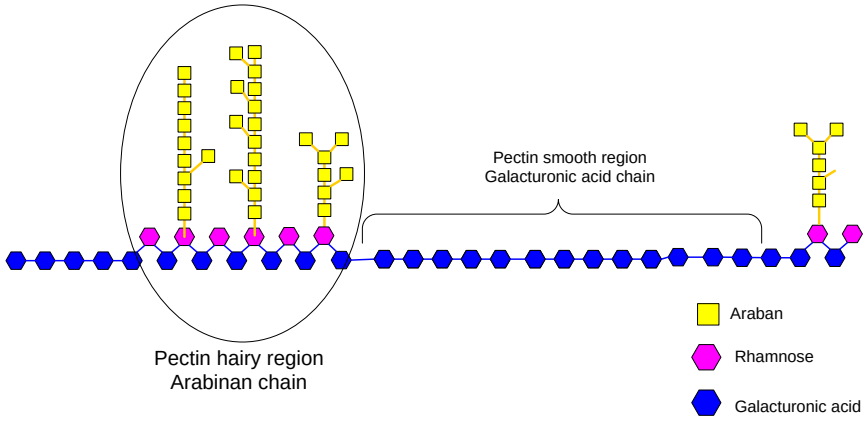



**Figure #1: Pectin schematic**

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

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.

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[4] and USA[5] 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 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[6] in fruits and vegetable processing/purees are:

- Efficient peel removal

[4] 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

[5] GRN 89: http://www.fda.gov/downloads/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/UCM266873

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

- Faster viscosity reduction leading to increased press/centrifugation capacity and filtration efficiency
- Increased concentrate of juice
- Higher juice extract yield, due to efficient solubilisation of pectin
- Increased cloud stability (reduced turbidity) of the clear concentrate
- Less use of raw materials
- Energy savings and production of less waste products
- Improve juice extraction yield (facilitation of the press capacity due to rapid viscosity reduction and better pectin solubilisation) and extraction of valuable substances contained in fruits and vegetables (e.g. enhanced nutrients levels –polyphenols, anthocyans, antioxydants...)
- Achieve appearance and taste of juices, meeting consumers' expectations (by e.g. reducing post-processing hazes leading to clearer product), increasing colour extraction, reducing turbidity and preventing sedimentation)
- Among the fruit and vegetable processing applications, polygalacturonase can be used in pomace treatment i.e. treatment of solid remains of fruit after pressing for juice

**Wine Production**

Enzymes are used at various stages of winemaking, depending on the variety of grape and processing technology. Enzyme preparations may be used to facilitate wine clarification, decolouration, dealcoholisation, enhance flavour development, or augment anthocyanin liberation. Pectinases have been used since the 1960's in wine production (Kashyap 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 colour (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).

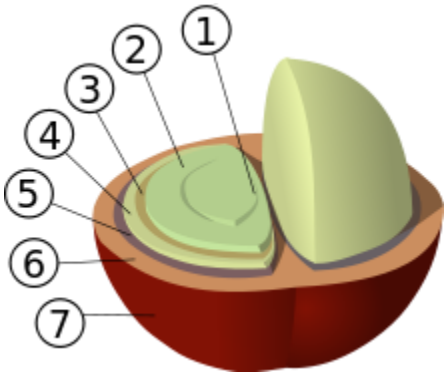
The advantages of the addition of pectinases during winemaking are:

- 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.

**Coffee Production**

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



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

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

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

- Improvement of environmental impact and sustainability of the entire milling process
- Processing improvement (better demucilation, shorter fermentation time, etc) ensuring better and consistent coffee beans characteristics.

**Flavouring Production**

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

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

## Effect of the Food Enzyme on the Final 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 favourable

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 pasteurisation step. In addition, during fruit puree production, the substrate is depleted (due to calcium pectate formation) rendering the enzyme non-functional anymore;
- in (rare) case of non-pasteurised 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 stabilisation); filtration processes (removal of proteins in general); inactivation by some natural wine ingredients like alcohol, polyphenols, metals, sulphur 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).

- During flavouring production, the enzyme protein is denatured or removed.

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

### A.4. Manufacturing Process

Like all food enzymes, polygalacturonase described in this dossier is manufactured in accordance with current Good Manufacturing Practices for Food (cGMPs) and the principals of Hazard Analysis of Critical Control Points (HACCP). Compliance to Food Hygiene Regulation is regularly controlled by relevant food inspection services in Finland. Quality certificates are provided in Appendix #3.

The enzyme described herein is produced by controlled submerged fermentation. The production process involves the fermentation process, recovery (downstream processing) and formulation and packaging. A manufacturing flow-chart is given in Appendix #4.

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

#### A.4.1. **Fermentation**

The polygalacturonase enzyme is produced by submerged fermentation of the genetically modified strain of *Aspergillus oryzae*. Please see **Section E** for a more detailed description of the genetic modification.

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

### A.4.2. **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 (See **Section C**). The raw materials conform to either specifications set out in the Food Chemical Codex, 12th edition, 2020 or The Council Regulation 93/315/EEC, setting the basic principles of EU legislation on contaminants and food, and Commission Regulation (EC) No 1881/2006 setting maximum limits for certain contaminants in food.

The raw materials used for the formulation are of food grade quality.

### A.4.3. **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

For details regarding fermentation ingredients please refer to Appendix CCI.

### A.4.4. **Inoculum**

A suspension of a pure culture of AR-183 is aseptically transferred to a shake flask containing fermentation medium.

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

### A.4.5. **Seed fermentation**

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

### A.4.6. **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.

### A.4.7. **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 secreted 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.

### A.4.8. **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.

### A.4.9. **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.

### A.4.10. **Primary solid/liquid separation**

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

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

### A.4.11. **Concentration**

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

### A.4.12. **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.

### A.4.13. **Formulation and Packaging**

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

Polygalacturonase enzyme preparation from *Aspergillus oryzae* AR-183 is sold mainly as a liquid product.

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

### A.5. Specification for the purity and identity

The final enzyme product complies with the recommended General Specifications for Enzyme Preparations Used in Food Processing Joint FAO/WHO Expert Committee on Food Additives, Compendium of Food Additive Specifications, FAO Food and Nutrition Paper *(FAO/WHO 2006)* and the Monograph "Enzyme Preparations" Food Chemicals Codex (FCC) 12th edition (2020) for food-grade enzymes. Specifications for the food enzyme preparation have been defined as follows:

| | |
|---|---|
| Lead: | Not more than 5 mg/kg |
| *Salmonella* sp.: | Absent in 25 g sample |
| Total coliforms: | Not more than 30 per gram |
| *Escherichia coli*: | Absent in 25 g of sample |
| Antimicrobial activity: | Not detected |
| Mycotoxins: | No significant levels[7] |

Analytical data is provided in Appendix #5.

The methods used are provided in Appendix #6.

See **Section A.3** for more information regarding physical properties.

### A.6. Analytical method for detection

This information is not required in the case of an enzymatic processing aid.

## B. Information Related to the Safety of a Chemical Processing Aid

Not applicable - this application does not concern a chemical processing aid.

[7] 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 suchfungal species should not contain toxicologically significant levels of mycotoxins that could be produced by these species.

## C. Information related to the safety of an enzyme processing aid

### C.1. General information on the use of the enzyme as a food processing aid in other countries

The safety of the polygalacturonase preparation was confirmed or is under consideration by external expert groups, as follows:

**Approved:**

- Denmark
- France

**Under evaluation**:

- Denmark
- USA
- Brazil

### C.2. Information on the Potential Toxicity of the Enzyme Processing Aid

#### C.2.1. Information on the enzyme's prior history of human consumption and its similarity to proteins with a history of safe human consumption

The enzyme preparation from *Aspergillus oryzae* produced polygalacturonase expressing a gene from *Aspergillus tubingensis* was evaluated according to the Pariza and Johnson Decision Tree. The decision tree is based on the safety evaluation published by Pariza and Foster in 2001, adapted from their original evaluation in 1983. Based on the Pariza and Johnson decision tree analysis, AB Enzymes concludes that the polygalacturonase enzyme preparation is safe, see Appendix #7.

Pectinases[8] from various micro-organisms (including genetically modified ones) are widely accepted and *A. oryzae* – whether or not genetically modified - is widely accepted as safe production organism for a broad range of enzymes. Similar food enzymes and/or food enzymes from similar production organisms

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

have been evaluated by EFSA and internationally, food enzymes similar to the one described in this dossier have already been evaluated.

| Non-exhaustive list of authorised pectinases from production organisms other than *Aspergillus oryzae* | | |
|---|---|---|
| **Authority** | **Production Organism** | **Reference** |
| **Australia/NZ** | *Aspergillus tubingensis* (pectin lyase, polygalacturonase, pectin esterase), pectin esterase from *A. aculeatus* expressed in *A. tubingensis* , pectinase / polygalacturonase from *Trichoderma reesei* | Schedule 18 Processing Aids |
| **France** | *Aspergillus tubingensis* (pectinase, pectinmethylesterase, pectinmetylesterase from *A. aculeatus* in *A. tubingensis* , or from self-cloned *A. tubingensis* , polygalacturonase from *A. tubingensis* ), *Aspergillus wentii* (pectinase), *Trichoderma reesei* (pectin methylesterase and polygalacturonase from *A. tubingensis* in *Trichoderma reesei*) | Arrêté du 19 octobre 2006 |
| **USA**[9] | *Aspergillus tubingensis , Trichoderma reesei* | GRAS Notice Inventory No. 89, GRAS Notice Inventory No. 557 & GRAS Notice Inventory No. 558 |
| **Canada** | *Aspergillus tubingensis , Bacillus amyloliquefaciens, Bacillus subtilis, Trichoderma reesei* | List of Permitted Food Enzymes Health Canada |
| **JECFA** | *Aspergillus alliaceus* | FAS 22-JECFA 31 and TRS 789-JECFA 35/18.pdf |

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

| | | |
|---|---|---|
| | *Aspergillus tubingensis* | FAS 22-JECFA 31/21 and JECFA Monograph 305 |

For information regarding sales globally, please refer to Appendix CCI.

### C.2.2. **Toxicological Studies**

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

It is generally accepted that known commercial enzyme preparations from *Aspergillus oryzae* are not toxic and since *Aspergillus oryzae* is a natural constituent in the environment, it is concluded that the polygalacturonase enzyme from *Aspergillus oryzae* is safe as for use as a food processing aid in various applications.

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

- The US EPA has exempted *A. oryzae* from review by the Agency, due to its extensive history of safe use (EPA 1997);
- In Europe, *A. oryzae* is classified as a low-risk-class microorganism, as exemplified in the listing as Risk Group 1 in the microorganism classification lists of the German Federal Institute for Occupational Safety and Health (BauA, 2002[10]) and the Federal Office of Consumer Protection and Food Safety (BVL, 2010[11]).

[10] http://www.baua.de/de/Themen-von-A-Z/Biologische-Arbeitsstoffe/TRBA/TRBA-460.html

[11] http://www.bvl.bund.de/SharedDocs/Downloads/06_Gentechnik/register_datenbanken/organismenliste_2010.pdf?__blob=publicationFile&v=6

- It is not mentioned on the list of pathogens in Belgium (Belgian Biosafety Server, 2010[12]).

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

To further confirm that the polygalacturonase enzyme preparation AR-183 does not have any toxic properties and to ensure the toxicological safety of the use of the enzyme preparation from *Aspergillus oryzae* the following studies were conducted:

- Ames test
- Micronucleus test, in vitro
- 90 Day Oral Toxicity Study (Rodents)

#### C.2.2.1. Bacterial Reverse Mutation Test

The assay, based on OECD Guidelines No. 471, was run at Eurofins BioPharma Product Testing Munich GmbH Behringstraße 6/8 82152 Planegg Germany during January 14, 2019 - February 5, 2019.

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

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

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

[12] http://www.biosafety.be/RA/Class/ClassBEL.html

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 Polygalacturonase produced with *Aspergillus oryzae* at any concentration level, neither in the presence nor absence of metabolic activation in experiment I and II.

Conclusion:

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

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

### C.2.2.2. Mammalian Micronucleus Assay in Human Lymphocytes

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

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

| | **Without S9 mix** | | **With S9 mix** |
|---|---|---|---|
| | **Experiment I** | **Experiment II** | **Experiment I** |
| **Exposure period** | 4 h | 44 h | 4 h |

| | | | |
|---|---|---|---|
| **Cytochalasin B exposure** | 40 h | 43 h | 40 h |
| **Preparation interval** | 44 h | 44 h | 44 h |
| **Total culture period*** | 92 h | 92 h | 92 hrs |

*Exposure started 48 h after culture initiation

The selection of the concentrations was based on data from the pre-experiment. In the main experiment I **without** metabolic activation 600 µg/mL and **with** metabolic activation 750 µg/mL test item, respectively, and in experiment II **without** metabolic activation 100 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: 400, 500 and 600 µg/mL

**with** metabolic activation: 250, 500 and 750 µg/mL

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

**without** metabolic activation: 25, 50 and 100 µg/mL

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

If cytotoxicity is observed the highest concentration evaluated should not exceed the limit of 55% ± 5% cytotoxicity according to the OECD Guideline 487 [4][13]. 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

[13] Test No. 487 2016

is a value of the relative cell growth of 70% compared to the negative/solvent control which corresponds to 30% of cytostasis.

In both experiments an increase of the cytostasis above 30% was noted. In experiment I an increase of the cytostasis was noted at 600 µg/mL (**without** metabolic activation) and at 750 µg/mL (**with** metabolic activation). In experiment II an increase of the cytostasis was seen at 50 µg/mL and higher (**without** metabolic activation).

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

The nonparametric $\chi^2$ Test was performed to verify the results in both experiments. No statistically significant enhancement ($p<0.05$) of cells with micronuclei was noted in the dose groups of the test item evaluated in experiment I and II **without** metabolic activation. In experiment I **with** metabolic activation a statistically significant increase ($p = 0.0273$) of cells with micronuclei was noted at a concentration of 500 µg/mL. Since the corresponding number of micronucleated cells was within the historical control limits for the negative control and no concentration-related increase was observed, this effect was regarded as not biologically relevant.

The $\chi^2$ Test for trend was performed to test whether there is a concentration-related increase in the micronucleated cells frequency in the experimental conditions. No statistically significant increase in the frequency of micronucleated cells under the experimental conditions of the study was observed in experiment I and II.

Methylmethanesulfonate (MMS, 50 and 65 µg/mL) and cyclophosphamide (CPA, 15 µg/mL) were used as clastogenic controls. Colchicine (0.02 and 0.8 µg/mL) was used as aneugenic control. All induced distinct and statistically significant increases of the micronucleus frequency. This demonstrates the validity of the assay.

Conclusion:

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

Therefore, Polygalacturonase produced with *Aspergillus oryzae* is considered to be non-mutagenic with respect to clastogenicity and/or aneugenicity in the *in vitro* Mammalian Cell Micronucleus Test.

### C.2.2.3. 90-Day Sub-Chronic Toxicity Study

The assay, based on OECD Guidelines No. 408, was run at BSL BioPharma BIOSERVICE Scientific Laboratories Munich GmbH Behringstraße 6/8 82152 Planegg Germany during September 25, 2019 – June 6, 2020.

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

The test item was administered daily in graduated doses to 3 groups of test animals, one dose level per group for a treatment period of 90 days. Animals of an additional control group were handled identically as the dose groups but received *aqua ad injectionem* (sterile water), the vehicle used in this study. The 4 groups comprised of 10 male and 10 female Wistar rats. The control group C was shared with Eurofins Munich / BSL Munich Study No. 191880.

The following doses were evaluated:

Control: 0 mg/kg body weight

Low Dose: 100 mg/kg body weight

Medium Dose: 300 mg/kg body weight

High Dose: 1000 mg/kg body weight

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

The clinical sign salivation, which was noted in single male HD and female MD animals on several days during the treatment period, was observed immediately after the dose administration and therefore considered to be a sign of discomfort after oral administration without toxicological relevance. Other clinical findings, such as hairless area seen at several female MD animals or female control animals, anophthalmia/closed left eye for one female MD animal, lacrimation seen for one male control animal were considered to be incidental and no test item-related effect.

No test item-related abnormalities occurred during weekly detailed clinical observation for all parameters in males and females. In absence of test item-related findings during daily clinical observation the statistical significances in single weeks for animal is sleeping, decrease of moving in the cage or changes in skin or response to handling in the female LD, MD and/or HD groups were considered to be of no toxicological relevance and not test item-related. Furthermore, the findings were seen in single weeks throughout the observation period and without consistency within the dose groups.

No test item-related findings were found in the functional observation battery for all parameters in the male and female dose groups. The statistical significances in males before the first treatment (decrease of the body temperature in the MD group), in the last week of treatment in males (increase of moving in the cage in all dose groups) and females (fear decreased in the LD and MD group) were considered to be not related to the treatment with test item as no test item-related findings were noted during daily clinical observation or they were seen before start of treatment.

The test item had no effect on body weight development in this study. Overall the mean body weight increased during the observation period in the control and in all male and female dose groups and no statistical significances were found.

Conclusion:

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

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

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

## C.3. Information on any Significant Similarity between the Amino Acid Sequence of the Enzyme and that of Known Protein Toxins.

A homology search was performed from the NCBI Identical Protein Groups (IPG) database using the BLAST-P. The amino acid sequence of the polygalacturonase (as shown in Appendix CCI) was used as the query sequence in the searches.

BLAST-P is a basic local alignment search tool. By using this tool identities between two protein sequences can be found if the proteins contain similar sequence stretches (domains) even though the overall sequence homology between the sequences might be very low.

According to the results obtained from the searches performed found in Appendix CCI, it can be concluded that the polygalacturonase protein does not show significant homology to any protein sequence identified or known to be a toxin.

## C.4. Information on the Potential Allergenicity of the Enzyme Processing Aid

### C.4.1. The source of the Enzyme Processing Aid

The dossier concerns a polygalacturonase gene from *Aspergillus tubingensis* expressed in *Aspergillus oryzae*

Name of the enzyme protein: **Polygalacturonase**

Production strain: *Aspergillus oryzae* AR-183

### C.4.2. Donor

Name of the Donor: *Aspergillus tubingensis*

### C.4.3. **An Analysis of Similarity between the Amino Acid Sequence of the Enzyme and that of known Allergens.**

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 a possibility of causing such occupation allergy in sensitive individuals. However, the possibility of an allergic reaction to the polygalacturonase residues in food seems remove. 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.
- In the past, the AMFEP Working Group on Consumer Allergy Risk from Enzyme Residues in Food performed an in-depth analysis of the allergenicity of enzyme food products (Dauvrin et al. 1998). The overall conclusion is that exposure to enzyme proteins by ingestion, as opposed to exposure by inhalation, are not potent allergens and that sensitization to ingested enzymes is rare.
- Enzymes when used as digestive (Abad et al. 2010) aids are ingested daily, over many years, at much higher amounts when compared to enzymes present in food (up to 1 million times more).

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

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

- The majority of proteins are not food allergens and based on previous experience, the enzyme industry is not aware of any enzyme proteins used in food that are homologous to known food allergens[14].
- 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 x due to the food process conditions. During the food process conditions, the tertiary conformation of the enzyme molecule is destroyed. These types of alterations to the conformation in general, are associated with a decrease in the antigenic reactivity in humans. In the clear majority of investigated human cases, denatured proteins are much less immunogenic than the corresponding native proteins (Valenta and Kraft 2002; Valenta 2002; Takai et al. 2000; Nakazawa et al. 2005; Kikuchi et al. 2006).
- To add on, residual enzyme still present in the final food will be subjected to digestion in the gastro-intestinal system, that reduces further the risk of enzyme allergenicity. While stability to digestion is considered as a potential risk factor of allergenicity, it is believed that small protein fragments resulting from digestion are less likely to be allergenic (FAO/WHO 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.
- To specifically evaluate the risk of the polygalacturonase enzyme cross reacting with known allergens and induce a reaction, the sequence homology testing to known allergens was performed. The testing involved using an 80-amino acid (aa) sliding window search and conventional FASTA alignment (overall homology), with the threshold of 35% homology as recommended in the most recent literature (FAO/WHO 2001; Ladics et al. 2007; Goodman et al. 2008).

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

- All documents related to the allergenicity searches for polygalacturonase AR-183 can be found in Appendix CCI.

For the results of the allergenicity search, the following allergen databases were used, "AllergenOnline" database also known as FARRP and the Structural Database of Allergenic Proteins (SDAP). AB Enzymes followed the recommendations for bioinformatics searches proposed in EFSA (2010). Two databases out of the 13 databases listed in the above publication were used in the searches (Table 1), since other databases are no longer maintained; of these one has been updated this year (2020) and also contains risk assessment tools, namely AllergenOnline (FARRP). The comparison of query sequence with sequences of known allergens using the sliding 80-mer window was recommended by the FAO/WHO Expert panel in 2001 (FAO/WHO 2001) and by the Codex Alimentarius Commission in 2003 (Codex Alimentarius Commission 2003) as a method to evaluate the extent of which a protein is similar in structure to known allergens. The alignments methods used in the searches are as following, alignment of the entire amino acid sequence to sequences in allergen databases and alignment of sliding 80-amino acid windows of the query protein to known protein allergens. The results of the two allergenicity searches conducted are summarized below.

The identity percentages of all the hits from both FARRP and SDAP were below the set 35 % identity limit and the three hits having the best E-values were all different in the different databases. Aalberse suggested that "cross-reactivity is rare below 50% amino acid identity and, in most situations requires more than 70% identity" (Aalberse 2000), making unlikely that the polygalacturonase in question can be presumed to be allergenic based on full-length sequence relatedness to known allergens.

In the 80-mer sliding window analysis the polygalacturonase protein sequence did show degrees of identity from 35.8 % to 46.3 % with pollen allergens of different species such as maize pollen allergen, pollen allergen of the subtropical *Bahia grass*, *Japanese cedar* pollen, pollen allergen of conifer *Cryptomeria japonica* (Enclosure 2). 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.

Conclusion:

Based on the results obtained from the bioinformatics approach to estimate potential allergenicity on relatedness to known allergens and taking into account the most recent scientific recommendations on the interpretation of such data, and based on the fact that the enzyme is typically denatured during the food manufacturing process and that any residual enzyme still present in the final food will be subject to digestion in the gastro-intestinal system, it is not likely that polygalacturonase produced by *Aspergillus oryzae* AR-183 under evaluation will cause allergic reactions after ingestion of food containing the residues of these enzymes.

### C.5. Safety assessment reports prepared by international agencies or other national government agencies, if available

Please see section C.1.

## D. Additional information related to the safety of an enzyme processing aid derived from a microorganism

### D.1. Information on the source organism

The microorganism that is used for the production of Polygalacturonase is the fungus *Aspergillus oryzae*.

**Scientific name:**

Genus: *Aspergillus*

Species: *Aspergillus oryzae*

**Taxonomy:**

*Aspergillus oryzae* is a fungus which is characterized by producing conidia from phialids (a bottle-shaped structure within or from which conidia (conidiospores) are formed). Conidiophores are hyaline and mostly rough-walled. Conidia are large and smooth to finely roughened. The fungus is a saprophyte which can grow on a wide variety of complex substrates. The optimal growth temperature for *Aspergillus oryzae* is 20-40 °C. It can grow at a wide pH range, though acidic conditions are more favourable. *Aspergillus oryzae* favours high oxygen concentrations and therefore grows best on the surface of organic substrates. It uses asexual reproduction via formation of conidiospores. Conidiospore formation is strongly inhibited during submerged fermentation.

*Aspergillus oryzae* is the domesticated form of *A. flavus*. Both species are difficult to distinguish. The domestication took place at least 2000 years ago. *Aspergillus oryzae* is principally found in some locations in China and Japan. Outside this area the fungus may be sporadically found in soil or on decaying plant material. *A. oryzae* is used in Chinese and other East Asian cuisines to ferment soybeans for making soy sauce and fermented bean paste, and also to saccharify rice, other grains, and potatoes in the making of alcoholic beverages such as *huangjiu*, *sake*, *makgeolli*, and *shōchū*. *A. oryzae* is also used for the production of rice vinegars.

Synonyms[15]: *Aspergillus flavus*

### D.2. Information on the pathogenicity and toxicity of the source microorganism

*Aspergillus oryzae* strains are non-pathogenic for healthy humans and animals. As mentioned above, *Aspergillus oryzae* is not present on the list of pathogens in the EU (Directive Council Directive

[15] Mycobank taxonomic database (see: http://www.mycobank.org/Biolomics.aspx?Table=Mycobank&Page=200&ViewMode=Basic).

2000/54/EC) and is present in major culture collections worldwide, as it is globally regarded as a safe microorganism:

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

- In the USA, has exempted *Aspergillus oryzae* from review by the EP Agency, due to its extensive history of safe use (EPA 1997);
- In Europe, *Aspergillus oryzae* is classified as a low-risk-class microorganism, as exemplified in the listing as Risk Group 1 in the microorganism classification lists of the German Federal Institute for Occupational Safety and Health (BAuA) (BauA, 2002) and the Federal Office of Consumer Protection and Food Safety (BVL) (BVL, 2010). It is not mentioned on the list of pathogens in Belgium (Belgian Biosafety Server, 2010[16]).

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

**Secondary Metabolites:**

Metabolites of human toxicological concern are usually produced by microorganisms for their own protection. Microbes in natural environments are affected by several and highly variable abiotic (e.g. availability of nutrients, temperature and moisture) and biotic factors (e.g. competitors and predators). Their everchanging environments put a constant pressure on microbes as they are prompted by various environmental signals of different amplitude over time. In nature, this results in continuous adaptation of the microbes through inducing different biochemical systems; e.g. adjusting metabolic activity to current availability of nutrients and carbon source(s), or activation of stress or defense mechanisms to produce secondary metabolites as 'counter stimuli' to external signals (Earl et al. 2008; Klein and Paschke 2004). On the contrary, culture conditions of microbial production strains during industrial scale fermentation have been optimized and 'customized' to the biological requirements of the strain in question (see e.g. review by Parekh et al. 2000). Thus, the metabolic activity and growth of a particular microbial production strain during the fermentation process (primarily the 'exponential growth phase') will focus on efficiently

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

building cell biomass which in turn produces the molecule of interest. Industrial fermentations are run as monocultures (i.e. no external competitors or predators) with optimal abiotic conditions; and the fermentation process is terminated before or when the production strain enters the 'stationary growth phase'. Hence, there are no strong environmental signals that would induce stress (e.g. lack of nutrient or low/high temperature) or defense mechanisms (e.g. production of antibiotic, antiviral or neurotoxic molecules). Biosynthesis of stress and/or defense secondary metabolites of toxicological relevance by industrial microbial production organisms during the fermentation process is thus highly unexpected (Sanchez and Demain 2002) and is furthermore avoided from an economical perspective to optimize production.

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

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

Furthermore, it should be noted that the toxicological tests (C.2.2.) performed on the polygalacturonase produced by *Aspergillus oryzae* AR-183 confirm the absence of toxic secondary metabolites.

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

Invasive growth or systemic infections by *A. oryzae* in healthy humans have never been reported. In a few cases, however, isolates identified as *A. oryzae* have been recovered from debilitated patients which are immunocompromised. Factors that may lead to immunosuppression include an underlying debilitating disease (e.g., chronic granulomatous diseases of childhood), chemotherapy, and the use of supraphysiological doses of adrenal corticosteroids (Bennett 1980; EPA 1997). *A. oryzae* has therefore low

pathogenic potential but may, like many other harmless microorganisms, grow in human tissue under exceptional circumstances (Barbesgaard et al. 1992).

### D.3. Information on the genetic stability of the source organism

The genetic stability of the strain over the fermentation time was analyzed by southern blotting and no instability of the strain was detected. For more detailed description of the strain construction and characteristics, please see **Section E** below.

## E. Additional information related to the safety of an enzyme processing aid derived from a genetically-modified microorganism

### E.1. Information on the methods used in the genetic modification of the source organism

This section contains summarized information. The detailed information is provided in the Appendix CCI.

### E.2. Host/recipient organism

The recipient strain used in the genetic modification for the construction of the production strain is a genetically modified spontaneous mutant of the *Aspergillus oryzae* parental strain. *Aspergillus oryzae* parental strain from South America was deposited to the RÖHM[17] strain collection in May 1984 and its taxonomy identification was reconducted in 2018 and confirmation granted that the strain is *A. oryzae* (Ahlburg) Cohn. The parental strain was identified by the Westerdijk Fungal Biodiversity Institute in the Netherlands and deposited with the accession number CBS 146745.

Therefore, the recipient can be described as followed:

Kingdom: Fungi

Division: *Ascomycota*

Class: *Eurotiomycetes*

Order: *Eurotiales*

Family: *Trichocomaceae*

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

Genus: *Aspergillus*

Species: *Aspergillus oryzae*

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

### E.3. Donor

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

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

Genus: *Aspergillus*

Species: *Aspergillus tubingensis*

Subspecies (if appropriate): not applicable

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

### E.4. Genetic modification

*A. oryzae* AR-183 was constructed for specific polygalacturonase production. The production strain differs from its recipient strain in its high polygalacturonase production capacity due to expression of the *Aspergillus tubigiensis* polygalacturonase gene from the expression cassette into the recipient strain's genome. Besides the high polygalacturonase production, no other significant changes in phenotype are made.

*A. oryzae* AR-183 secretes high amounts of polygalacturonase into its culture supernatant, resulting in high polygalacturonase activity in the cultivation broth. The heterologous polygalacturonase is the main

component of the enzyme mix produced by AR-183. In addition to the heterologous polygalacturonase, strain AR-183 produces endogenous *Aspergillus* enzymes in small amounts. These activities are not relevant from an application/safety point of view, due to the small amount and the fact that such activities have been approved for decades in food processing.

Standard DNA techniques were used in the construction and transformation of the plasmids. The constructs were characterized by restriction endonuclease digestion and verified by DNA sequencing. Standard transformation techniques using protoplasts were used to integrate the expression cassette into the genome of the *Aspergillus oryzae* production strain.

The production strain was constructed from the parental strain in two modification steps. The first step was the creation of the spontaneous mutant recipient strain. The second step was the insertion of polygalacturonase gene expression cassette into the recipient strain using an acetamidase gene from *Aspergillus nidulans* ( (Hynes et al. 1983; Kelly and Hynes 1985).

The plasmid vector pUC18 was only used in constructing the expression cassette and but was not introduced into the recipient strain in fungal transformation.

### E.5. Stability of the transformed genetic sequence

When implemented, the fermentation process always starts from identical replicas of the AR-183 (production strain) seed ampoule. Production preserves from the "Working Cell Bank" are used to start the fermentation process. A Working Cell Bank is a collection of ampoules containing a pure culture. The cell line history and the production of a Cell Bank, propagation, preservation and storage is monitored and controlled. The WCB is prepared from a selected strain. A WCB ampoule is only accepted for production runs if its quality meets the required standards. This is determined by checking identity, viability, microbial purity and productivity of the WCB ampoule. The accepted WCB ampoule is used as seed material for the inoculum.

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

Testimony to the stability of the strain is given by monitoring the growth behaviour and by comparable levels of polygalacturonase activity in number of fermentation batches performed for the AR-183 strain. The activity measurements from parallel fermentations showed that the productivity of the AR-183 strain remains similar. This clearly indicates that the strain is stable.

For more details, please see Appendix CCI.

## F. Information Related to the Dietary Exposure to the Processing Aid

### F.1. A list of foods or food groups likely to contain the processing aid or its metabolites

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

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 or products. 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.

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 has to be determined case by case, based on the desired effect and process conditions. Therefore, the enzyme manufacturer can only issue a recommended enzyme dosage range. Such a dosage range is the starting point for the individual food producer to fine-tune his process and determine

the amount of enzyme that will provide the desired effect and nothing more. Consequently, from a technological point of view, there are no 'normal or maximal use levels' and polygalacturonase from *Aspergillus oryzae* AR-183 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.

Microbial food enzymes contain – apart from the enzyme protein in question – also some substances derived from the producing micro-organism and the fermentation medium. The presence of all organic materials is expressed as Total Organic Solids[18] (TOS, FAO/WHO, 2006). Whereas the dosage of a food enzyme depends on the enzyme activity present in the final food enzyme preparation, the dosage on basis of TOS is more relevant from a safety point of view. Therefore, the use levels are expressed in TOS. The Table below shows the range of recommended use levels for each application where the enzyme is to be used.

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

| Foods Uses for Polygalacturonase | |
|---|---|
| **Food Grouping** | **Proposed Food Uses** |
| Fruit and Vegetable Products and dishes - Fruit and Vegetable processing | Including but not limited to Processed fruits/vegetables (canned fruits/vegetables, jams) + pomace treatment |
| Wines | Including but not limited to Wine, port, sherry, reduced alcohol wine, sparkling grape juice |
| Miscellanous - Flavouring | including soups, sauces, bouillons, dressings, condiments, processed foods, snack foods, meat-derived foods, breads/crackers, etc |
| Non-alcoholic beverages - Coffee | Black coffee, white coffee, coffee substitutes |

| Application | | Raw material (RM) | Maximal recommended use levels (mg TOS/kg RM) |
|---|---|---|---|
| Coffee production | | Coffee cherries | 0.5 |
| Flavouring production | | Fruits/Vegetables | 1.5 |
| Fruit and vegetable processing | Fruit juices | Fruit/Vegetable | 2 |
| | Fruit purees | Fruit/Vegetable | 1 |
| Wine production | | Grapes | 2 |

**F.2. The levels of residues of the processing aid or its metabolites for each food or food group**

The most appropriate way to estimate the human consumption in the case of food enzymes is using the so-called Budget Method, originally known as the Danish Budget Method *(Douglass et al. 1997; Hansen 1966)*. This method enables one to calculate a Theoretical Maximum Daily Intake (TMDI) based on conservative assumptions regarding physiological requirements for energy from food and the energy density of food rather than on food consumption survey data.

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

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

Consumption of food patterns:

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

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

| **Applications** | | **Raw material (RM)** | **Maximal recommended use level (mg TOS/kg RM)** | **Final food (FF)** | **Ratio RM/FF*** | **Suggested level in final food (mg TOS/kg food)** |
|---|---|---|---|---|---|---|
| **Liquid foods** | Coffee processing | Coffee cherries | 0.5 | Coffee | 0.4 | 0.2 |
| | Flavouring production | Fruit/vegetable | 1.5 | Various beverages | 0.01 | 0.015 |
| | Fruit and vegetable processing | Fruit/vegetable | 2 | Juices | 1.3 | 2.6 |
| | Wine making process | Grape | 2 | Wine | 1.6 | **3.2** |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Solid foods** | Fruit and vegetable processing | Fruit/vegetable | 1 | Processed fruits (e.g. canned fruits, jams, …) | 1 | **1** |

* Assumptions behind ratios of raw material to final food:

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

The Total Theoretical Maximum Daily Intake (TMDI) can be calculated on basis of the maximal values found in food and beverage, multiplied by the average consumption of food and beverage/kg body weight/day.

| The Total TMDI will consequently be: TMDI in food (mg TOS/kg body weight/day) | TMDI in beverage (mg TOS/kg body weight/day) | Total TMDI (mg TOS/kg body weight/day) |
|---|---|---|
| 1 x 0.0125 = 0.013 | 3.2 x 0,025 = 0.08 | 0.093 |

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

- It is assumed that ALL producers of the above-mentioned foodstuffs use polygalacturonase
- It is assumed that ALL producers apply the HIGHEST use level per application; For the calculation of the TMDI's in food, only THOSE foodstuffs were selected containing the highest theoretical

amount of TOS. Thus, foodstuffs containing lower theoretical amounts were not taken into account;

- It is assumed that the amount of TOS does not decrease as a result of the food production process;
- It is assumed that the final food 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).

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

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

The Margin of Safety (MoS) for human consumption can be calculated by dividing the NOAEL by the Total Theoretical Maximal Daily Intake (TMDI). Total TMDI of the food enzyme 0.093 mg TOS/kg body weight/day. Consequently, the MoS is:

- MoS is 1000 / 0.093 = **10,753**

As is explained above, the Total TMDI is highly exaggerated. Moreover, the NOAEL was based on the highest dose administered, and is therefore to be considered as a minimum value. Therefore, the actual MoS in practice will be some magnitudes higher.

The overall conclusion is that the use of the food polygalacturonase from *Aspergillus oryzae* AR-183 in the production of food is absolutely safe. Considering the high safety factor – even when calculated by means of an overestimation of the intake via the Budget method – there is no need to restrict the use of the enzyme in food processing.

Consequently, it is concluded that polygalacturonase can be used *Quantum Satis* in fruit/vegetable processing, wine, flavouring and coffee production.

### F.3. For foods or food groups not currently listed in the most recent Australian or New Zealand National Nutrition Surveys (NNSs), information on the likely level of consumption

Not applicable.

### F.4. The percentage of the food group in which the processing aid is likely to be found or the percentage of the market likely to use the processing aid

Since we used the Budget Method to quantity the potential of residues in the final food consumed by individuals, it is assumed that all products containing the substrate are produced using the polygalacturonase enzyme as a processing aid at the recommended dose.

### F.5. Information relating to the levels of residues in foods in other countries

The Budget Method assumes a worst-case scenario, and as such it is predicted that all countries would have the same level of residues in the processed food product.

### F.6. For foods where consumption has changed in recent years, information on likely current food consumption

Not applicable.

## VI. List of appendices

**Section 3.1**

1.1 Customer Support Letter
1.2 Formal Request for Confidential Information (CCI)
1.3 Formal Request for other Confidential Information
1.4 Statutory Declaration
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**Section 3.2**

1. France and Denmark Approval Letters – Other Confidential Information
2. Product Data Sheet
3. Quality Certificates
4. Manufacturing Flow Chart
5. Analytical Data
6. Methodology
7. Pariza and Johnson Decision Tree Analysis

CCI Appendix Confidential

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