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A Petition to Amend the Australia New Zealand Food Standards Code with a Glucose Oxidase Enzyme Preparation produced by *Trichoderma reesei*

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 Glucose Oxidase enzyme preparation from *Trichoderma reesei* produced by AB Enzymes GmbH for use as a processing aid in the manufacturing of cereal based products (baking) and egg processing.

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 *Trichoderma reesei* as permitted source for glucose oxidase (EC 1.1.3.4).

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 glucose oxidase (EC 1.1.3.4).

Composition	
Constituent	**%**
Glucose oxidase	32.8%
Sunflower oil	0.4
Wheat flour	remainder

Use of the Enzyme and Benefits

The glucose oxidase from *T. reesei* object of this dossier is specifically intended to be used in baking (e.g. bread, biscuits, tortillas, cakes, steamed bread and croissants) and other cereal-based processes (e.g. pastas, noodles and snacks), and in egg processing. In these processes, the glucose oxidase is used as a processing aid in food manufacturing and is not added directly to final foodstuffs.

The benefits of the use of industrial glucose oxidase 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.

Glucose oxidase has been used e.g. in baking and other cereal based processes for over 20 years and their use in the bakery industry is continuously increasing. Some of these applications have been specifically approved for a number of years in Denmark and France (baking, egg processing), which together with the extensive use for decades in a number of EU countries justifies the technological need of glucose oxidase in these food processes.

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 glucose oxidase preparation was confirmed or is under consideration by external expert groups, as follows:

- **France**: The enzyme preparation was safety assessed according to the Guidelines for the evaluation of food enzymes. This resulted in the authorisation of the enzyme product by the French authorities in 2017.
- **Denmark:** The enzyme preparation was safety assessed according to the Guidelines for the evaluation of food enzymes. This resulted in the authorisation of the enzyme product by the authorities in 2017.
- **USA**: The enzyme preparation was safety assessed according to the Guidelines for the evaluation of food enzymes under GRAS. A GRAS no objection letter determined that the xylanase enzyme reparation is GRAS for its intended use GRAS #707
- **Mexico:** The enzyme preparation was safety assessed according to the Guidelines for the evaluation of food additives. This resulted in the authorisation of the enzyme product by the authorities in 2019.

- **Canada:** The enzyme preparation was safety assessed according to the Guidelines for the evaluation of food additives. Approved and listed for use in bread, flour, whole wheat flour, liquid egg white, pasta and unstandardized bakery products[1]
- **EFSA/ EU Commission**: A dossier was submitted in 2016 in compliance with Regulation (EC) 1332/2008 and is currently being reviewed by EFSA.

Conclusion

Based on the safety evaluation, AB Enzymes GmbH respectfully request the inclusion of glucose oxidase from *Penicillium amagasakiense* expressed in *T. reesei* in the table – 18-9(3) of schedule 18 - Permitted processing aids various purposes.

[1] https://www.canada.ca/en/health-canada/services/food-nutrition/food-safety/food-additives/lists-permitted/5-enzymes.html

III. INTRODUCTION

The dossier herein describes a *Trichoderma reesei* produced glucose oxidase expressing a gene from *Penicillium amagasakiense* produced by submerged fermentation.

Glucose oxidase from *Penicillium amagasakiense* expressed in *T. reesei* is mainly intended to be used in baking processes, (e.g. bread, biscuits, tortillas, cakes, steamed bread and croissants) and other cereal based processes (e.g. pastas, noodles and snacks), as well as egg processing.

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 glucose oxidase 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 glucose oxidase preparation from *T. reesei* 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]

Company

[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 *Trichoderma reesei* as permitted source for glucose oxidase (EC 1.1.3.4).

3.1.4. Justification for the Application

The need for the proposed change:

Trichoderma reesei expressing a glucose oxidase gene from *Penicillium amagasakiense* 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 glucose oxidase 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.

The enzymes known in the art and listed in standard 3.1.1 as current status quo derived from other sources have technical limitations, especially with regards to processing (tolerance to withstand mechanical shock during process). Based on market benchmarking we have found that our product has superior technical characteristics resulting in improved quality for bakery product manufacturers. This is a characteristic that is strongly preferred by manufacturers. There is also a cost benefit associated with the use of *Trichoderma reseei* as superior producer of enzymes resulting in a cost benefit that is passed on to the final user of the enzyme. Increased competition on the market is also a desired characteristic in the context of competition laws. 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 bread making and egg processing applications.

Due to the effectiveness of this enzyme in the above-mentioned food processes, AB Enzymes has received authorization to sell in USA, Denmark, Mexico, Canada and France. An application has been submitted in the EU and is currently under review.

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 bakery products and bakery mixes. 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 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. Glucose oxidase 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 glucose oxidase 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 [redacted] A summary of this information is given in section E of section 3.2.2. The formal request for treatment of [redacted] as confidential commercial information (CCI) is included as [redacted].

3.1.11. Other Confidential Information

Information related to the methods used to analyze enzymatic activity is company specific and this information is not publically available and known only to AB Enzymes GmbH, as such we respectfully ask that this information is kept confidential as presented in Appendix #2. The formal request for treatment of Appendix #2 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 in cereal and cereal-based products and egg processing is not restricted by any Codex Alimentarius Commission (Codex) Standards or any other known regulations.

National Standards:

Use of enzymes as processing aids in food applications (baking, brewing, starch processing, etc.) has specific standard in France (arrêté du 19 octobre 2006[2]), and the use of this enzyme has been approved for the accordant food applications in this dossier (please see **Section C.1**).

In the USA, under CFR, Code of Federal Regulations, title 21, [21CFR170.3], Food for Human Consumption, Food additives - *Enzymes* : Enzymes used to improve food processing and the quality of the finished food.

Also, this food enzyme, glucose oxidase, complies with the internationally accepted JECFA specifications for chemical and microbiological purity of food enzymes (FAO/WHO, 2006).

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 March 1, 2016, are the following sections:

• SECTION 3.1 – GENERAL REQUIREMENTS

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

[2] https://www.legifrance.gouv.fr/affichTexte.do?cidTexte=LEGITEXT000020667468

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 glucose oxidase enzyme, produced with the help of *Trichoderma reesei* strain RF11400. The representative current commercial product is Veron® Oxibake ST.

Glucose oxidase 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 glucose oxidase used in baking processes and other cereal-based processes, as well as egg processing. 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	Glucose oxidase
Common names	β-D-glucose oxidase, β-D-glucose: quinone oxidoreductase, D-glucose oxidase, D-glucose-1-oxidase, glucose oxyhydrase; deoxin-1, glucose aerodehydrogenase, aero-glucose dehydrogenase, glucose oxyhydrase, Notatin , corylophyline; penatin
Enzyme Commission No.	EC 1.1.3.4
CAS number	9001-37-0
Host	*Trichoderma reesei*
Donor	*Penicillium amagasakiense*

A.2.2.Enzyme Preparation

The commercial names representative of the enzyme preparation, formulated with the enzyme produced with RF11400 *T. reesei*, is Veron® Oxibake ST. The product data sheet is provided in Appendix #1.

A.2.3.Enzyme preparation composition:

Composition Veron® Oxibake ST	
Glucose oxidase	32.8%
Sunflower oil	0.4
Wheat flour	remainder

The main activity of the enzyme preparation is glucose oxidase (IUB 1.1.3.4), which has been identified in many sources, including plants, microorganisms and animals.

Glucose oxidase catalyses the oxidation of β-D-glucose to D-glucono-1,5-lactone and the reduction of oxygen to hydrogen peroxide. In the presence of water, D-glucono-1,5 -lactone is hydrolysed to gluconic acid.

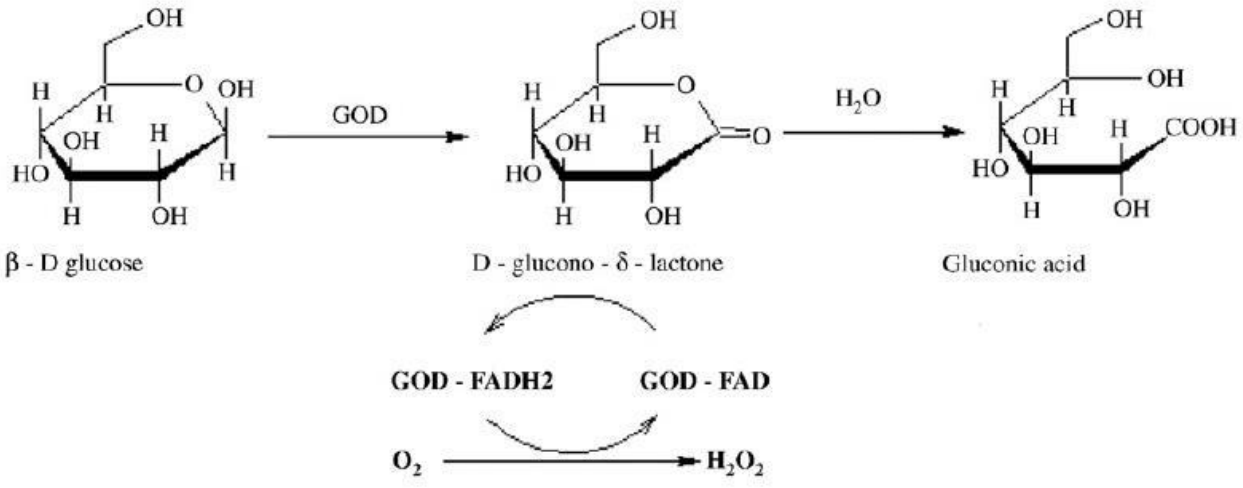


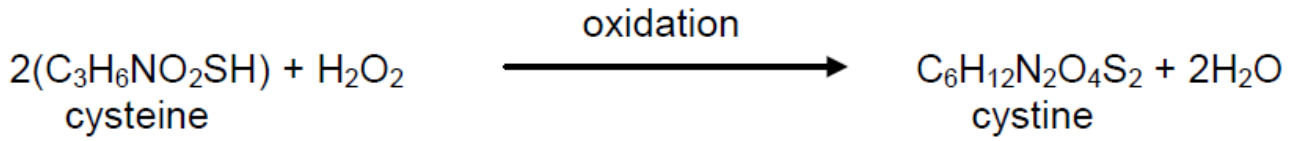
Fig 1: Representation of the GOX reaction

D-glucose is widespread in sweet fruits. But by far, the greatest part of glucose serves as monomer for the formation of oligo- and polysaccharides such as sucrose, dextrines, starch or cellulose (Online Encyclopedia ROEMPP Georg Thieme Verlag KG 2015). D-glucose can be found in various plant materials including the endosperm of cereals, such as wheat and barley, as monomer in small amount and in form of starch in large amount. During dough preparation endogenous amylases are able to release D-glucose from starch (Poutanen 1997; Rosell et al. 2001).

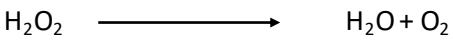
Oxygen is naturally present in the dough making environment (air) and therefore incorporated into the dough as air bubbles during the process.

Consequently, the substrates for glucose oxidase occur naturally and are therefore a natural part of the human diet.

Reaction products: as a result of the catalytic activity of glucose oxidase, glucon lacton / gluconic acid and hydrogen peroxide are formed. Gluconic acid can be found in plants, fruits and other foodstuffs and is used as food additive for several applications (Ramachandran et al. 2006). Hydrogen peroxide disappears either due to its oxidising reaction with the cysteine residues of proteins to form cystine and thus converting the sulfhydryl groups to a disulfide bond (see reaction below) or as substrate for endogeneous peroxidases for phenolic crosslink formation (Rasiah et al. 2005).



When glucose oxidase is used in egg processing, hydrogen peroxide is consumed as to produce oxygen with the help of added catalase.



The method to analyse the activity of the enzyme is company specific and is capable of quantifying glucose oxidase activity as defined by its IUBMB classification. The enzyme activity is usually reported in *GOX* g^{-1} ([redacted]).

A.2.4.Enzyme genetic modification

The enzyme is from a *Trichoderma reesei* host strain genetically modified with a glucose oxidase gene deriving from *Penicillium amagasakiense*. The enzyme is not considered protein engineered.

Name of the enzyme protein:	**Glucose oxidase**
Donor:	*Penicillium amagasakiense*
Host:	*Trichoderma reesei*
Production strain:	*Trichoderma reesei* RF11400

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 –Veron® Oxibake ST

Properties	
Activity	Activity min. 11500 GOX g^{-1}
Appearance	Solid, light beige
Density	1.0-1.1 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, glucose oxidase 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 glucose oxidase under such conditions, it can be concluded that the enzyme glucose oxidase does not exert any (unintentional) enzymatic activity in final bakery products.

Please refer to product data sheets for shelf-life and storage conditions.

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

A.4. Information on the technological need and mechanism of action of the enzyme in food

In principle, the enzymatic oxidation of glucose with the help of glucose oxidase can be of benefit in processing of all foods and food ingredients which naturally contain glucose.

When used in food applications glucose oxidase's function is to remove either glucose or oxygen (as reviewed by Wong et al. (2008) and Bankar et al. (2009). Typically, glucose oxidase is used to remove glucose, preventing product browning caused by Mailliard reaction which may occur during food processing (e.g. pateurisation of powdered eggs and fruit processing) and other food processes involving high temperatures. Glucose oxidase is also used to lower alcohol content in wine production (through the removal of some of the glucose which would otherwise be converted into alcohol).

Other examples are the oxygen removal from the top of bottled beverages before sealing, or mayonnaise production and gluconic acid production (Bankar et al. 2009; Wong et al. 2008).

Finally, glucose oxidase is also currently used to strengthen the protein complexes contained in starch-based products through increased formation of cross-links between proteins, thereby improving the quality of such foods, as breads and other baked products (Birgbauer and Chun 2006; Vemulapalli and Hoseney 1998; Bonet et al. 2006; Eugenia Steffolani et al. 2012).

The glucose oxidase from *T. reesei* RF11400 object of this dossier is specifically intended to be used in baking (e.g. bread, biscuits, tortillas, cakes, steamed bread and croissants) and other cereal-based processes (e.g. pastas, noodles and snacks), and in egg processing. In these processes, the glucose oxidase is used as a processing aid in food manufacturing and is not added directly to final foodstuffs. Below, the benefits of the use of industrial glucose oxidase 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.

Glucose oxidase has been used e.g. in baking and other cereal based processes for over 20 years and their use in the bakery industry is continuously increasing. Some of these applications have been specifically approved for a number of years in Denmark and France (baking, egg processing), which together with the extensive use for decades in a number of EU countries justifies the technological need of glucose oxidase in these food processes.

BAKING PROCESS

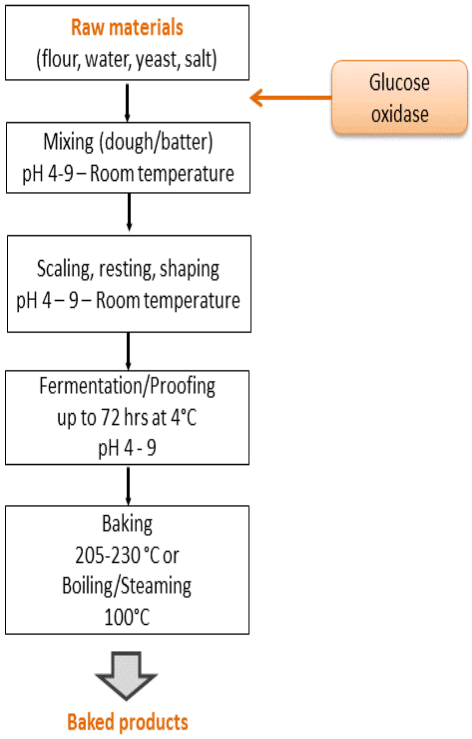
Glucose oxidase can be used in the manufacturing of bakery products such as, but not limited to, bread, biscuits, steamed bread, cakes, pancakes, tortillas, wafers and waffles.

Functional properties of bread dough greatly depend on the protein forming the gluten network. Protein crosslinking or the formation of covalent bonds between protein chains is a way of modifying the protein functionality and simultaneously increasing its applications in different processes.

The hydrogen peroxide produced during glucose oxidase reaction causes the oxidation of thiol groups of gluten proteins forming disulphide linkages It also produces dityrosine cross-linking and the gelation of water soluble pentosans (Steffolani et al. 2012 and references therein). The oxidation of bread dough induces therefore an important modification on the gluten proteins through the formation of both disulfide and non-disulfide crosslinks which results in significant improvement in the functional properties of the dough and leads to better bread making performance (Vemulapalli and Hoseney 1998; Eugenia Steffolani et al. 2012; Bonet et al. 2006).

Glucose oxidase is normally not present in vegetable raw materials. This means that the enzyme has to be added to a food system in order to benefit from its functionality. Glucose oxidase is often used together with other enzymes (enzyme systems) which modify other components of the raw materials. In particular glucose oxidase is often applied together with endo-amylase, xylanase and cellulase (Primo-Martín et al. 2005; Steffolani et al. 2012).

The process flow chart is presented below and shows the conditions under which the food enzyme is used.



The benefits of the conversion of glucose and oxygen with the help of glucose oxidase in baking can be summarised as follows:

- Facilitate the handling of the dough
- Reduce dough stickiness which results in processing tolerance
- Improve dough stability and behaviour during the shaping or moulding step
- Improve dough machinability

Due to the better processing described above, the beneficial effects of the use glucose oxidase in the production of baked products may be the following:

- Ensure a uniform volume and an improved/uniformed crumb structure of the bakery product, which might otherwise be impaired by fluctuating processing of the dough;
- Support the creation of a more appealing crust surface of cutted bread types such as e.g. rolls or baguettes
- Possible effects are less product variation, ensuring uniform/standardised quality products.

Those beneficial effects may be associated with effects on the final food, which are however not exclusively obtainable by means of enzyme treatment: they can be achieved without the use of enzymes through e.g. modified, maybe more expensive, production processes, the use of chemicals or recipe changes.

The fate of the enzyme protein during baking process: In baking, glucose oxidase performs its technological function during dough or batter handling in order to contribute to an improved and consistent baking process. The glucose oxidase is denatured by heat during the baking or steaming step.

OTHER CEREAL-BASED FOOD PROCESSES

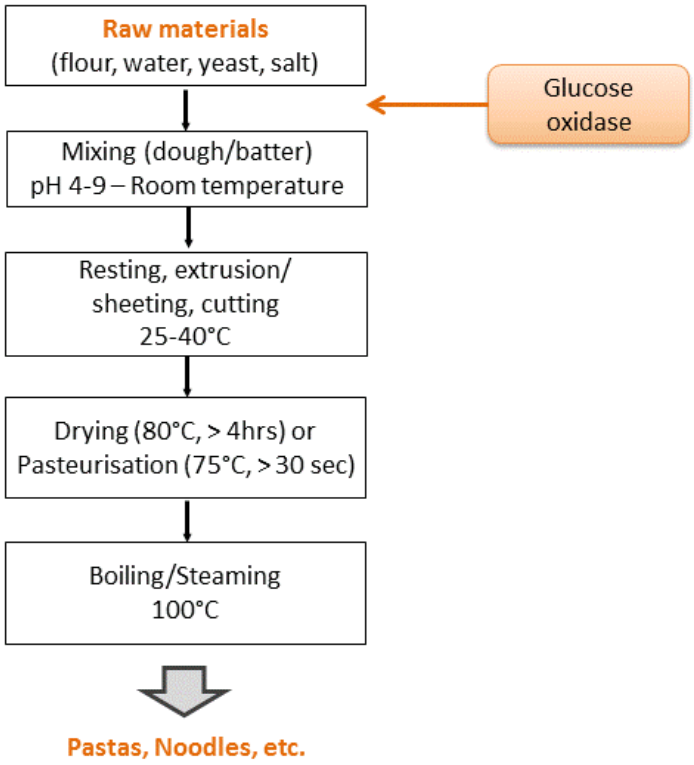
Glucose oxidase can also be used in the processing of other cereal based products such as, but not limited to, pasta, noodles and snacks.

As mentioned above, the reaction products resulting from the conversion of the glucose with the help of glucose oxydase interact with the gluten proteins. Gluten proteins provide functional properties during pasta, noodle and snack making due to their ability to determine dough viscosity and dough stability. Modification of the gluten protein structure can improve the functional properties of the flour endogenous proteins as explained below.

Dried pasta has, among cereal derived foodstuffs, a very distinct microscopic structure. Starch granules are entrapped in an amorphous protein matrix. While cooking in hot water, physical competition between starch swelling and properties of polymerised and polymerising proteins determines whether the final cooked pasta is firm and elastic or rather sticky and soft. The first is the case when a strong gluten network is formed and starch particles are entrapped in this network. The second is the case when the gluten network is too weak resulting in a significant starch swelling (Resmini P and Pagani M.A 1983; Bruneel et al. 2010).

Because gluten has a predominant role in the pasta structure the use of glucose oxidase increases the gluten protein network resistance to cooking and decrease surface stickiness (Täufel 1988; Fuad and Prabhasankar 2010).

The process flow of other cereal based processes is presented below:



The benefits of the conversion of glucose and oxygen with the help of glucose oxidase in other cereal based processes can be summarised as follows:

- Facilitate the handling of the dough
- Reduce dough stickiness which results in processing tolerance
- Strengthen dough structure
- Improve cooking tolerance of pasta or noodle

Due to the better processing described above, the beneficial effects of the use glucose oxidase in the production of other cereal-based products may be the following:

- Firmer bite and better texture of pasta or noodle
- Possible effects are less product variation, ensuring uniform/standardised quality products.

The fate of the enzyme protein during the pasta and noodles production process: In other cereal based processes such as pasta and noodles, the glucose oxidase performs its function during dough handling. The enzyme is denatured by heat during the drying, boiling or steaming step.

EGG PROCESSING

Glucose oxidase can also be used to treat whole egg, yolk or whites, prior to drying, to prevent these unwanted changes occurring in the drying operation. These can be problematic for dried egg whites if the products are traditionally pasteurized after drying in a hot room.

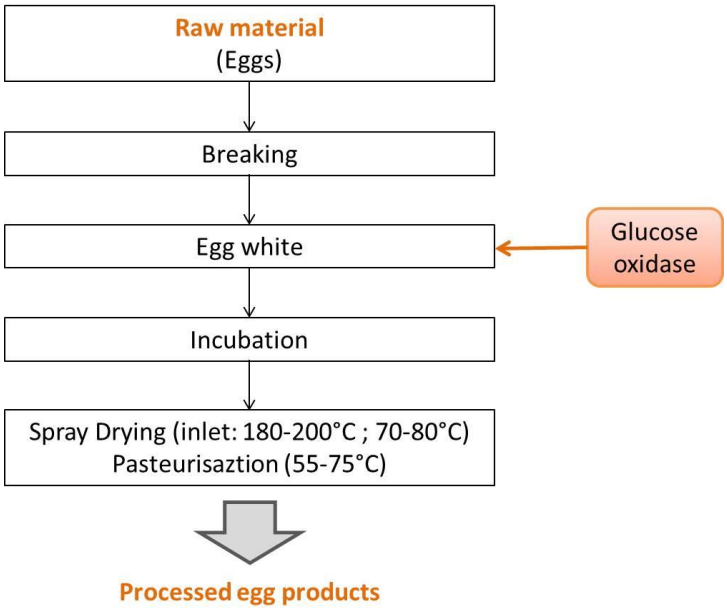
These effects (e.g. browning) are primarily due to the Maillard Reaction in which glucose and protein (or amino acids) react together to give unstable compounds which react further to produce coloured, off-flavoured and insoluble products. Therefore, the glucose present in liquid egg is typically removed before spray drying.

At first, methods of removing glucose by fermentation with live organisms were evolved. These live organism methods are effective but occasionally cause problems in that the inherently high bacterial count may cause undesirable flavours in the final dried egg product. Consequently, in the early 1950's, an enzymatic method of de-sugaring eggs before drying was introduced. Glucose oxidase breaks down the glucose to products which do not cause browning. Glucose oxidase carries out the egg de-sugaring process with the greatest efficiency and the greatest economy of time - faster and easier to control- (Sisak et al. 2006).

Glucose oxidase required the presence of dissolved oxygen to function. As dissolved oxygen in liquid egg is insufficient to complete the reaction, an external source of oxygen is essential. Bubbling with air can be used but addition of hydrogen peroxide as oxygen source (by action of associated catalase[3]) is the more usual method.

The process flow of egg processing is presented below

[3] Catalase decomposes hydrogen peroxide into water and oxygen and therefore provides free oxygen to catalyse the degradation of glucose.



The benefits of the conversion of glucose with the help of glucose oxidase in egg processing can be summarised as follows:

- Enables to increase the temperature during subsequent dry heat pasteurization (hot rooming),
- Enables a faster and easier to control process

In addition, those benefits effects may be associated with beneficial effects on the final ingredient (egg products) that would ensure a better acceptability of the final products:

- Avoids colour changes, which is considered a poor quality of dried egg white and reduces the possibility of sales
- Improves functional properties of dried egg products (whipping characteristics, solubility, odour, and taste).

The fate of the enzyme protein during egg processing*: i*n egg processing, the glucose oxidase is added after egg breaking and will be denatured by heat during drying and pasteurisation steps.

To summarize, the use of glucose oxidase in baking and other cereal-based processes and in egg processing ensures a maximum compatibility with modern industrial processes (also leading to less product variations, hereby ensuring standardised quality products).

A.5. Manufacturing Process

Like all food enzymes, glucose oxidase 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.

T. reesei RF11400 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.5.1.Fermentation

The glucose oxidase enzyme is produced by submerged fermentation of the genetically modified strain of *Trichoderma reesei*. 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.5.2.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, 11th edition, 2018 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.5.3.Materials used in the fermentation process (inoculum, seed and main fermentation)

- Potable water
- A carbon source (e.g. glucose, …)
- A nitrogen source (e.g. wheat derived material, …)
- Salts and minerals (e.g. Ammonium sulphate, Monopotassium phosphate)
- pH adjustment agents
- Foam control agents (e.g. polyalkylene glycols)

[redacted]

A.5.4.Inoculum

A suspension of a pure culture of *T. reesei* RF11400 is aseptically transferred to a shake flask (1 liter) containing fermentation medium.

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

A.5.5.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.5.6.Main fermentation

Biosynthesis of the glucose oxidase enzyme product by the production strain *T. reesei* RF11400 occurs during the main fermentation.

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

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

A.5.7.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.5.8.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.5.9.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.5.10.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.5.11.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.

A.5.12.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.5.13.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.

Glucose oxidase enzyme preparation from *T. reesei* RF11400 is sold mainly as a powdered 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.6. 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 *(Food and Agriculture Organization of the United Nations 2006)* and the Monograph "Enzyme Preparations" Food Chemicals Codex (FCC) 11th edition (2018) for food-grade enzymes. Specifications for the food enzyme preparation have been defined as follows:

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.7. 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.

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 glucose oxidase preparation was confirmed or is under consideration by external expert groups, as follows:

- **France**: The enzyme preparation was safety assessed according to the Guidelines for the evaluation of food enzymes. This resulted in the authorisation of the enzyme product by the French authorities in 2017, Appendix #7.

- **Denmark:** The enzyme preparation was safety assessed according to the Guidelines for the evaluation of food enzymes. This resulted in the authorisation of the enzyme product by the authorities in 2017 Appendix #8.
- **USA**: The enzyme preparation was safety assessed according to the Guidelines for the evaluation of food enzymes under GRAS. A GRAS no objection letter determined that the xylanase enzyme reparation is GRAS for its intended use GRAS #707[4]
- **Mexico:** The enzyme preparation was safety assessed according to the Guidelines for the evaluation of food additives. This resulted in the authorisation of the enzyme product by the authorities in 2019 Appendix #9.
- **Canada:** The enzyme preparation was safety assessed according to the Guidelines for the evaluation of food additives. Approved and listed for use in bread, flour, whole wheat flour, liquid egg white, pasta and unstandardized bakery products[5]
- **EFSA/ EU Commission**: A dossier was submitted in 2016 in compliance with Regulation (EC) 1332/2008 and is currently being reviewed by EFSA.

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

As documented below, glucose oxidase from various micro-organisms (including genetically modified ones) are widely accepted for their use in several applications. See accordant table below:

Authority	Food enzyme - GOX	Reference
Australia/NZ	Aspergillus niger Aspergillus oryzae (carrying a glucose oxidase gene from A. niger)	Standard 1.3.3 processing aids
France	Aspergillus niger Aspergillus oryzae (carrying a	Arrêté du 19 octobre 2006

[4] https://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&id=707&sort=GRN_No&order=DESC&startrow=1&type=basic&search=707

[5] https://www.canada.ca/en/health-canada/services/food-nutrition/food-safety/food-additives/lists-permitted/5-enzymes.html

	glucose oxidase gene from A. niger) Aspergillus niger (carrying a glucose oxidase gene from Penicillium chrysogenum)	
USA[1]	Aspergillus oryzae (carrying a glucose oxidase gene from A. niger) Aspergillus niger Penicillium chrysogenum	GRAS Notice Invenory, GRN 106 GRAS Notice Inventory, GRN 089 GRAS Notice Inventory, GRN 509

The enzyme preparation from *Trichoderma reesei* produced glucose oxidase expressing a gene from *Penicillium amagasakiense* 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 glucose oxidase enzyme preparation is safe, see Appendix #10.

[redacted].

C.2.2.Toxicological Studies

This section describes the studies performed to evaluate the safety of the RF11400 glucose oxidase 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 of *T. reesei* are not toxic and since gluocose oxidase is a natural constituent in the environment, it is concluded that the glucose oxidase enzyme from *T. reesei* RF11400 is safe as for use as a food processing aid in various applications.

To further confirm that the glucose oxidase enzyme preparation does not have any toxic properties and to ensure the toxicological safety of the use of the enzyme preparation from *T. reesei*, the following studies were conducted:

- Ames test – Appendix #11

- Micronucleus test, in vitro – Appendix #12
- 90 Day Oral Toxicity Study (Rodents) – Appendix #13

C.2.2.1. Bacterial Reverse Mutation Test

The test, based on OECD Guidelines No. 471 (OECD), was run at Eurofins BioPharma Product Testing Munich GmbH (Planegg/Munich) – Germany. The study was completed on July 13, 2015 and the report is summarized below.

This study was performed to investigate the potential of glucose oxydase produced with *Trichoderma reesei* RF11400 to induce gene mutations according to the plate incorporation test (experiment I) and the pre-incubation test (experiment II) using the *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, TA 100, and TA 102.

Because the principal enzyme activity is a glucose oxidase that in the presence of glucose produces hydrogen peroxide, which is a well-known cytotoxic and mutagenic compound *in vitro* (causes cellular and sub-cellular oxidative damage, including damage to DNA), the glucose oxidase was inactivated by pH shift in order to ensure that secondary genetic mutations arising from potential hydrogen production would not occur.

The assay was performed in two independent experiments both with and without liver 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; 3.16; 1000; 2500; 5000 g/plate.

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

No toxic effect 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 biological relevant increase in revertant colony numbers of any of the five tester strains were observed following treatment with glucose oxidase at any dose level, neither in the presence nor absence of metabolic activation in experiment I and II.

Appropriate reference mutagens were used as positive controls and induced a distinct increase of revertant colonies indicating the validity of the experiments.

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

Therefore, the glucose oxidase from *Trichoderma reesei* RF11400 is considered to be non-mutagenic in this *Salmonella typhimurium* reverse mutation assay.

C.2.2.2. Mammalian Micronucleus Assay in Human Lymphocytes

The test, based on OECD Guidelines No. 487 (OECD, 2014), was run at Eurofins BioPharma Product Testing Munich GmbH (Planegg/Munich) – Germany. The study was completed on May 20th, 2015 and the report is summarized below.

The glucose oxidase from *Trichoderma reesei* RF11400 was assessed for its potential to induce structural micronuclei in human lymphocytes *in vitro* in two independent experiments. The following study design was performed:

	Without S9 mix		With S9 mix
	Experiment I	Experiment II	Experiment III
Exposure period	4 hrs	44 hrs	4 hrs
Cytochalasin B exposure	40 hrs	43 hrs	40 hrs
Preparation interval	44 hrs	44 hrs	44 hrs

Total culture period*	92 hrs	92 hrs	92 hrs

**exposure started 48h after culture initiation*

The selection of the concentrations was based on data from the pre-experiment. In experiment I without metabolic activation a concentration of 15 µg/mL and with metabolic activation a concentration of 275 µg/mL was selected as highest concentration for the microscopic analysis of micronuclei. In experiment II without metabolic activation a concentration of 2.5 µg/mL was selected as highest concentration for the microscopic analysis of micronuclei. The pH value detected with the test item solution was within the physiological range (7.0 ±0.4).

The following concentrations were evaluated for micronuclei frequencies:

Experiment I with short term exposure (4h):

- Without metabolic activation: 5, 10 and 15 µg/mL
- With metabolic activation: 50, 100, 200 and 275 µg/mL

Experiment II with long term exposure (44h):

- Without metabolic activation: 0.10, 0.25, 0.50, 1.0 and 2.5 µg/mL

No precipitate of the test item was noted in all concentrations groups evaluated in the main experiments at the end of the treatment (evaluated by unaided eye).

According to the OECD Guideline 487, the maximum of cytotoxicity should not exceed the limit of 55 ± 5%. Higher levels of cytotoxicity may induce chromosome damage as a secondary effect of cytotoxicity. According to laboratory experience a culture showing reduced cell viability (more than 30% rel. cytostasis) compared to the negative/solvent control displays cytotoxicity. Due to this, the limit of cytotoxicity is ≤70%. This corresponds to ≥30% of re. cytostasis.

In experiment I without metabolic activation no increase of the relative cytostasis above 30% was noted up to concentration of 10 g/mL. At a concentration of 15 µg/mL a relative cytostasis of 32% was noted.

In experiment I with metabolic activation, no increase of the relative cytostasis above 30% was noted up to concentration of 50 µg/mL. At a concentration of 100 µg/mL a relative cytostasis of 33%, at a concentration of 200 µg/mL a relative cytostasis of 48% and at a concentration of 275 µg/mL a relative cytostasis of 60% was observed.

In experiment II without metabolic activation no increase of the relative cytostasis above 30% was noted up to concentration of 0.25 µg/mL. At a concentration of 0.50 µg/mL a relative cytostasis of 31%, at a concentration of 1.0 µg/mL a relative cytostasis of 50% and at a concentration of 2.5 µg/mL a relative cytostasis of 69% was noted.

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

The non-parametric $\chi 2$ Test was performed to verify the results in both experiments. In experiment I without metabolic activation a statistically significant enhancement ($p=0.0191$) of cells with micronuclei was noted at concentration of 10 µg/mL and in experiment I with metabolic activation a statistically significant enhancement ($p=0.0011$) of cells with micronuclei was noted at concentration of 275 µg/mL. However, the frequency of micronucleated cells was within the historical control limits of the negative control and significant cytotoxicity was observed for the concentration of 275 µg/mL with metabolic activation. Therefore, the increases were regarded as not biologically relevant. No statistically significant enhancement ($p<0.05$) of cells with micronuclei was noted in the concentration groups of the test item evaluated in experiment II.

The $\chi 2$ Test for trend was performed to test whether there is a concentration-related increase in the micronucleated cells frequency in the experiment conditions. No statistically significant increase was observed in experiment I and II without metabolic activation. In experiment I with metabolic activation a statistically significant increase of the micronucleated cells frequency was observed. However, since all

values of the micronucleated cell frequency of this experimental condition were within the historical control limits of the negative control, this increase was regarded as not biologically relevant.

Appropriate clastogenic and aneugenic controls were applied. All induced distinct and statistically significant increases of the micronucleus frequency. This demonstrates the validity of the assay.

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

Therefore the glucose oxidase from *Trichoderma reesei* RF11400 is considered to be non-mutagenic with respect to clastogenicity and/or aneugenicity in the *in vitro* Mammalian Cell Micronucleus test. Further *in vivo* tests were not performed, as there was no *in vitro* mutagenicity detected.

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

The test was performed according to the following guidelines: OECD No. 408 at Eurofins BSL Bioservice (Planegg/Munich) – Germany. The study was completed in July 2016 and the report is summarized below.

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

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

The following doses were evaluated:

- Control: 0 mg/kg body weight
- Low Dose: 100 mg/kg body weight
- Medium Dose: 300 mg/kg body weight
- High Dose: 1000 mg/kg body weight

The test item formulations were used not more than 10 days after preparation. The test item was dissolved in aqua ad injectionem and administered daily during a 90-day treatment period to male and female animals. Dose volumes were adjusted individually based on weekly body weight measurements. During the period of administration, the animals were observed precisely each day for signs of toxicity. Animals no 54 and 71 that died was examined macroscopically and, surviving animals were sacrificed at the conclusion of the test and observed macroscopically.

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

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

Summary results:
The acceptance criteria for concentration of test item in dose formulations were met and homogeneity was confirmed.

No test item related mortality and no clinical signs of toxicity were observed in this study. Treatment with Glucose oxidase produced with *Trichoderma reesei* had no influence on neurobehavioural parameters examined at the end of the treatment period. Body weight developed normally in all groups independent of treatment and Glucose oxidase produced with *Trichoderma reesei* did not affect food consumption.

At the end of the treatment period parameters of haematology, blood coagulation and clinical biochemistry were not affected by Glucose oxidase produced with *Trichoderma reesei* in a biologically relevant way. Urinary parameters were not affected by Glucose oxidase produced with *Trichoderma reesei* in this study. At necropsy of the animals macroscopic findings observed in Glucose oxidase produced with *Trichoderma reesei* treated animals were either incidental or also present in control animals. None of them was considered toxicologically relevant.

Differences in organ weight between Glucose oxidase produced with *Trichoderma reesei* treated animals were slight and either not statistically significant or only present in one gender, but in any case not associated with any histopathological findings.

Moreover, Glucose oxidase produced with *Trichoderma reesei*, produced no histomorphologic evidence of toxicological properties in any organs and tissues examined.

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

There was no indicator for toxicity in any of the dose levels tested. Therefore, the NOEL may be established at 1000 mg/kg/day. Thus, the dose level of 1000 mg/kg/day also marks the NOAEL in this study.

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 1,000 mg TOS/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 non-redundant protein sequences database using the BLAST-P (protein – protein BLAST) program, v. 2.6.1+ (http://blast.ncbi.nlm.nih.gov/). The amino acid sequence of the glucose oxidase ([redacted]) 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 it can be concluded that the glucose oxidase protein does not shown 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 gluocose oxidase gene from *Penicillium amagasakiense* expressed in *T.reesei*.

Name of the enzyme protein: **Glucose oxidase**

Production strain: *Trichoderma reesei* RF11400

C.4.2.Donor

The glucose oxidase gene is from *Penicillium amagasakiense*.

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

As some enzymes manufactured for use in food have been reported to cause inhalation allergy in workers exposed to enzyme dust in manufacturing facilities, glucose oxidase may also cause such occupational allergy in sensitive individuals. However, the possibility of an allergic reaction to the glucose oxidase residues in food seems remote. In order to address allergenicity by ingestion, it may be taken into account that:

- 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 comprised enzymes produced by wild-type and genetically modified strains as well as wild-type enzymes and protein engineered variants and comprised 400 patients with a diagnosed allergy to inhalation allergens, food allergens, bee or wasp. It was concluded from this study that ingestion of food enzymes in general is not likely to be a concern with regard to food allergy.
- Previously, the AMFEP Working Group on Consumer Allergy Risk from Enzyme Residues in Food performed an in-depth analysis of the allergenicity of enzyme products (Daurvin et al. 1998). The overall conclusion is that exposure to enzyme proteins by ingestion, as opposed to exposure by inhalation, are not potent allergens and that sensitization to ingested enzymes is rare.

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

Additional considerations supporting the assumptions that the ingestion of an enzyme protein is not a concern for food allergy should also be taken into account:

- 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.
- The food enzyme is used in small amounts during food processing, resulting in very small amounts of the enzyme protein in the final food. A high concentration generally equals a higher

risk of sensitization, whereas a low level in the final food equals a lower risk (Goodman et al. 2008).

- In the case where proteins are denatured - which is the case for this glucose oxidase- due to the food process conditions (i.e starch process), the tertiary conformation of the enzyme molecule is destroyed. In general, these alterations in conformation are associated with decrease in the antigenic reactivity in humans: in the vast majority of investigated cases, denatured proteins are much less immunogenic than the corresponding native proteins (Valenta and Kraft 2002; Valenta 2002; Takai et al. 1997; Takai et al. 2000; Nakazawa et al. 2005; Kikuchi et al. 2006)
- In addition, residual enzyme still present in the final food will be subjected to digestion in the gastro-intestinal system, which reduces further the risk of enzyme allergenicity. While stability to digestion is considered as a potential risk factor of allergenicity, it is believed that small protein fragments resulting from digestion are less likely to be allergenic
- Finally, 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.

In order to specifically evaluate the risk that glucose oxidase enzyme would cross react with known allergens and induce a reaction in an already sensitized individual, sequence homology testing to known allergens was performed.

A sequence homology comparison test was then performed using a database of allergens from the Food Allergy Research and Resource Program (FARRP), University of Nebraska, Allergen Database (Version 14, January 20, 2014), which contains the amino acid sequences of known and putative allergenic proteins ([redacted]

The resulting alignments of the full-length glucose oxidase protein to any allergenic proteins in the allergen database showed an identity of 30.7 % with Mala s 12 allergen precursors produced by the fungal species *Malassezia sympodialis*. Aalberse suggested "*cross-reactivity is rare below 50% amino acid*

identity and in most situations requires more than 70% identity" (Aalberse 2000) making it unlikely that the glucose oxidase in question can be presumed to be allergenic based on full-length sequence relatedness to known allergens.

Using the 80-mer sliding window analysis the glucose oxidase protein sequence, one identity match of 41.2% with Mala s 12 allergen precursor produced by the fungal species *Malassezia sympodialis* was identified. As recommended by the FAO/WHO, a possible cross-reactivity has to be considered, when there is more than 35% identity in the amino acid sequence of the expressed protein using an 80 amino acids window and a suitable gap penalty (Food and Agriculture Organization of the United Nations January/2001). This recommendation was challenged however recently. According to Ladics et al. (2007) by 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 glucose oxidase in question would be below threshold even using the 80-mer sliding window approach.

In addition, the glucose oxidase protein sequence showed no perfect match to any known allergen when searching for a straight stretch of eight amino acids that could serve as potential IgE binding sites.

In summary therefore the bioinformatics approach to estimate potential allergenicity based on relatedness to known allergens and taking into account the most recent scientific recommendations on the interpretation of such data leads us to conclude that the glucose oxidase produced by *Trichoderma reesei* RF11400 is of no concern.

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 the glucose oxidase produced by *Trichoderma reesei* RF11400 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 glucose oxidase is the fungus *Trichoderma reesei*.

Scientific name:

Genus:Trichoderma

Species: *Trichoderma reesei*

Taxonomy: *Trichoderma reesei* is a hypercellulolytic fungus which was found on deteriorating military fabrics such as tents and clothing. This isolate, designated as QM6a, was initially named *Trichoderma viride*. Approximately 20 years later, QM6a was re-classified as *Trichoderma reesei*. In the 1980s, it was suggested that *Trichoderma reesei* should be placed into synonymy with *Trichoderma longibrachiatum* (Bissett 1991). Later however, evidence appeared that the two species were not identical (Meyer et al. 1992) and it was decided to go back to the *Trichoderma reesei* name. It is of relevance to note that enzymes have been approved that are produced by *T. reesei* under the name of *T. longibrachiatum*.

Taxonomic studies have shown that the species *Trichoderma reesei* consists only of this single isolate QM6a and its derivatives (e.g. Rut Series, Montenecourt and Eveleigh, 1977, 1979; QM9123 and QM9414, Mandels et al, 1971 – as reviewed by Nevalainen et al. (1994)). The American Type Culture Collection (ATCC) designation for this original strain of *Trichoderma reesei* QM6a is ATCC 13631.

Synonyms[6]**:** T*richoderma reesei* is the species name given to the anamorphic form (the form which reproduces asexually) of the fungus whose telemorphic form (the form which reproduces sexually) is now understood to be *Hypocrea jecorina* (Kuhls et al. 1996; Seidl et al. 2008). *Trichoderma reesei* was formerly known as *Trichoderma longibrachiatum*.

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

Species belonging to the genus *Trichoderma* are common in soil as well as on vegetable debris and they are widespread all over the world. *Trichoderma reesei* strains have been isolated from soil (compost material) only at low altitudes and within a narrow belt around the equator (± 20 degrees altitude; (Kubicek et al. 2008). The original isolate, QM6a (MANDELS and REESE 1957) was isolated from the Solomon Islands in 1944. As *T. reesei is a goo*d producer of cellulases, it has been widely studied in several laboratories and developed as industrial enzyme producer using random mutagenesis and genetic engineering. The original isolate, QM6a is the initial parent of practically all currently industrially relevant food enzyme production strains, including our strain RF11400.

Trichoderma reesei has a long history (more than 30 years) of safe use in industrial-scale enzyme production (Nevalainen et al. 1994; Blumenthal 2004). E.g. cellulases, hemicellulases, β-glucanases, pectinases and xylanases produced by this fungus are used in food, animal feed, pharmaceutical, textile, detergent, bioethanol and pulp and paper industries.

Food enzymes derived *Trichoderma reesei* strains (including recombinant *T. reesei* strains) have been evaluated by JECFA and many countries which regulate the use of food enzymes, such as the USA,

[6] Reference: Mycobank taxonomic database - Search Term "Trichoderma reesei" (see: http://www.mycobank.org/Biolomics.aspx?Table=Mycobank&Page=200&ViewMode=Basic).

France, Denmark, Australia and Canada, resulting in the approval of the use of food enzymes from *Trichoderma reesei* in the production of various foods, such as baking, brewing, juice production, wine production and the production dairy products.

Pathogenicity:

Trichoderma reesei strains are non-pathogenic for healthy humans and animals (Nevalainen et al. 1994).

Trichoderma reesei is not listed in Annex III of Directive 2000/54/EC – which lists microorganisms for which safety concerns for workers exist-as it is globally regarded as a safe microorganism:

- In the USA, *Trichoderma reesei* is not listed as a Class 2 or higher Containment Agent under the National Institute of Health Guidelines for Recombinant DNA Molecules. Data submitted in Generally Recognized as Safe (GRAS) petitions to the Food and Drug Administration (FDA) for numerous enzyme preparations from T. *reesei* for human and animal consumption demonstrate that the enzymes are nontoxic. The Environmental Protection Institute (EPA) completed a risk assessment on *T. reesei* in 2011 resulting in a Proposed Rule in 2012, concluding that it is appropriate to consider *T. reesei* as a recipient microorganism eligible for exemptions from full reporting requirements[7], if this fungus was to be used in submerged standard industrial fermentation for enzyme production.
- In Europe, *Trichoderma reesei* is classified as a low-risk-class microorganism, as exemplified by being listed as Risk Group 1 in the microorganism classification lists of the German Federal Institute for Occupational Safety and Health (BAuA[8]) and the Federal Office of Consumer Protection and Food Safety (BVL), and not appearing on the list of pathogens from Belgium (Belgian Biosafety Server, 2010[9]).

As a result, *Trichoderma reesei* can be used under the lowest containment level at large scale, GILSP, as defined by OECD (ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT 1992).

[7] reporting procedures in place under the Toxic Substances Control Act (TSCA) for new micro-organisms that are being manufactured for introduction into the commerce. https://www.gpo.gov/fdsys/pkg/FR-2012-09-05/html/2012-21843.htm

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

[9] https://www.biosafety.be/content/tools-belgian-classification-micro-organisms-based-their-biological-risks

Secondary metabolites in *Trichoderma reesei* (*Hypocrea jecorina*) strains:

The safety of *Trichoderma reesei* has been discussed in several review papers (Nevalainen et al. 1994; Blumenthal 2004; Kubicek et al. 2011; Peterson and Nevalainen 2012). *T. reesei* has been described not to produce mycotoxins or antibiotics under conditions used for enzyme production.

It is recognized that *Trichoderma reesei* is capable of producing peptaibols (e.g. paracelcin) and that the *Trichoderma reesei* genome contain genes for two peptaibol synthases (Kubicek et al. 2011). However, the bulk of the literature investigating the capability of *Trichoderma reesei* to produce peptaibols is based on fermentation conditions designed either to mimic natural (and stressful) growth conditions or attempt to optimize the conditions for secondary metabolite production. These methods are not representative of the conditions used in controlled industrial fermentation practices:

- Under controlled industrial fermentation conditions, the organisms are not subjected to significant stress: the literature indicates that the biosynthesis of peptaibols is a defence response against other fungi when subjected to environmental stress such as the lack of nutrients (Tisch and Schmoll 2010; Komon-Zelazowska et al. 2007).
- Standard industrial fermentation process times are short for peptaibols induction: peptaibols have mostly been isolated from very old cultures of *Trichoderma*, at least 15 days of cultivation (Kubicek et al. 2007). Industrial fermentation processes for *Trichoderma reesei* can be up to 10 days, but is typically shorter (3-8 days).

From what is described above, it can be concluded that the production of peptaibols by *Trichoderma reesei* strains under controlled and optimized industrial fermentation conditions is of insignificant concern.

It is relevant to note that during recent years, genetic engineering techniques have extensively been used to improve the industrial production strains of *T. reesei*, and in addition, considerable experience of safe use of recombinant *T. reesei* strains in industrial scale has accumulated. Furthermore, food enzymes from *Trichoderma reesei* have been subjected to several testings as part of their safety assessment for the use in food products manufacturing processes including 90-day toxicological tests.

T. reesei RF11400 enzyme fermentation extracts have been subjected to several tests as part of their safety assessment for the production of food products. In toxicological tests that have been performed, including a 90-day repeated dose study, no toxicity of glucose oxidase fermentation product as produced by the present production strain *Trichoderma* reesei RF11400 was detected (see **Section C**). These results show that there is no need for any toxicological concern with fermentation products as produced by use of *Trichoderma reesei*.

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 [redacted]

E.2. Host/recipient organism

The *T. reesei* recipient is a classical mutant strain originating from *T. reesei* QM6a. The identification of the strain as *T. reesei* has been confirmed by the Centraalbureau voor Schimmelcultures (CBS) in the Netherlands.

E.3. Donor

The *Trichoderma reesei* host strain is genetically modified with a glucose oxidase gene deriving from *Penicillium amagasakiense*.

E.4. Genetic modification

Trichoderma reesei strain RF11400 was constructed for production of *Penicillium amagasakiense* glucose oxidase, by transformation of a *T. reesei* host mutant strain with a purified DNA fragment isolated from a plasmid consisting of the fungal expression cassette and a pUC19 vector backbone.

The plasmid was digested with *Not*I and the expression cassette containing the glucose oxidase gene was isolated. The purified expression cassette devoid of pUC19 elements was used for transformation of *T. reesei* RF10310.

The transformation of the recipient strain with the glucose oxidase expression cassette was performed as described in Penttilä et al. (1987) with the modifications described in *Karhunen et al. (1993)*. The transformants were selected according to their ability to grow on acetamide plates. The expression cassette is integrated of at least one copy into the *T. reesei* genome.

The cassettes contain the following genetic materials:

The expression cassette consists of a *T. reesei* promoter and terminator, the glucose oxidase gene from *Penicillium amagasakiense* and *Aspergillus nidulans amdS* gene sequence (as a selection marker).

According to Southern blot analysis multiple copies of the glucose oxidase expression cassette are present in the genome of RF11400.

The DNA fragments that have been transformed into the *T. reesei* mutant host strain are well characterized, the sequences of the genes are known, and the fragments are free of any harmful sequences.

E.5. Stability of the transformed genetic sequence

T. reesei strains are widely used in biotechnological processes because of their known stability. The inserted DNA does not include any mobile genetic elements. Additionally, it should be highlighted that

T. reesei genome lacks a significant repetitive DNA component and no extant functional transposable elements have been found in the genome (Kubicek et al. 2011; Martinez et al. 2008). This results to low risk of transfer of genetic material.

The stability and potential for transfer of genetic material was assessed as a component of the safety evaluation of the production microorganism. Southern blot analyses were performed to the genome of the *T. reesei* production strain RF1400. Results indicated that at least 1 copy of the expression cassettes were integrated in the genome of strain RF11400 and that the production strain is stable in terms of genetic traits.

[redacted]

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

Glucose oxidase can be used in the manufacturing of cereal based products such as, but not limited to, bread, biscuits, steamed bread, cakes, pancakes, tortillas, wafers and waffles, as well as egg processing.

Like any other enzyme, glucose oxidase 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 glucose oxidase from *T. reesei* RF11400 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[10] (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 glucose oxidase is to be used.

Foods Uses for Glucose Oxidase	
Food Grouping	**Proposed Food Uses**
Cereal-based products and dishes	Used in the manufacturing of bakery products such as, but not limited to, bread, biscuits, steamed bread, cakes, pancakes, tortillas, wafers and waffles. Pastas, noodles
Egg products and dishes	Liquid egg-white (liquid albumen), liquid whole egg or liquid yolk, destined for drying

Application and Raw Material	**Raw Material**	**Maximal recommended use levels (mg TOS/kg RM)**

[10] 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.

Baking and other cereal based processes	Flour	10
Egg products and dishes	Egg	10

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 glucose oxidease 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)	Suggested recommended use level (mg TOS/kg RM)	Final food (FF)	Ratio RM/FF*	Suggested level in final food (mg TOS/kg food)
SOLID FOODS	Baking	Flour	10	Bread; Flour; Whole wheat flour/ Unstandardized bakery products	0.71	7.1
	Egg processing	Egg/egg white	10	Liquid egg-white (liquid albumen), liquid whole egg or liquid yolk, destined for drying	0.48	4.8

* Assumptions behind ratios of raw material to final food:

Baking:

Bakery products fall in the category of solid foods.

Flour is the raw material for bakery product and the yield will vary depending on the type of final food produced.

From 1 kg of flour you would have 4 kg of cakes, 1.4 kg of bread or 1.1 kg of cracker. Cracker may represent the most conservative input from the bakery processes. However, consumption of bread is higher than that of cracker, this is why bread is used as the assumption for the calculation of dietary exposure from bakery processes.

The yield of 1.4 kg of bread per 1 kg of flour correspond to a RM/FF ratio of 0.71 kg of flour per kg bakery product is used.

Egg processing:

Egg products are used in a huge variety of food products, such as dairy products, confectionary, bakery, pastas, dressings and mayonnaises…

Mayonnaise may represent the most conservative input for the egg yolk processing, with 1 kg egg-yolk used to produce 20 kg mayonnaise[11]. This corresponds to a RM/FF of 0.05.

Angel food cake, which is a type of sponge cake made with stiffly beaten egg whites with no butter added) is the most conservative example for the white egg processing, as containing 48% of liquid egg-white[12]. This corresponds to a RM/FF of 0.48, which will be used as the RM/FF for egg processing.

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)
7 x 0.0125 = 0.088	0 x 0.025 = 0	**0.088**

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

- It is assumed that ALL producers of the above-mentioned foodstuffs use the specific enzyme glucose oxidase from *Trichoderma reesei*;
- 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;

[11] http://www.mvo.nl/media/gezondheid/code_of_practice_en_mayonaise.pdf

[12] Baking Science & Technology (4th Edition) van E Pyler, Sosland Publishing, ISBN 978-0-9820239-1-4

- 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 is 0.088 mg TOS/kg body weight/day. Consequently, the MoS is:

- MoS = 1,000 / 0.088 = 11,429

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 glucose oxidase *from Trichoderma reesei* RF11400 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 glucose oxidase from *Trichoderma reesei* RF11400 can be used *Quantum Satis* in bakery products and dried eggs destined for baking.

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 glucose oxidase 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

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Section 3.2

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[redacted]

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10. AMES
11. Chromosomal aberration
12. 90 Day Oral Toxicity Study

[redacted]

II. Publication bibliography

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

Bankar, Sandip B.; Bule, Mahesh V.; Singhal, Rekha S.; Ananthanarayan, Laxmi (2009): Glucose oxidase--an overview. In *Biotechnology advances* 27 (4), pp. 489–501. DOI: 10.1016/j.biotechadv.2009.04.003.

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

Birgbauer, E.; Chun, J. (2006): New developments in the biological functions of lysophospholipids. In *Cellular and molecular life sciences : CMLS* 63 (23), pp. 2695–2701. DOI: 10.1007/s00018-006-6155-y.

Bissett, John (1991): A revision of the genus Trichoderma. II. Infrageneric classification. Canadian Journal of Botany. In *Can. J. Bot.* 69 (11), pp. 2357–2372. DOI: 10.1139/b91-297.

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

Bonet, A.; Rosell, C. M.; Caballero, P. A.; Gómez, M.; Pérez-Munuera, I.; Lluch, M. A. (2006): Glucose oxidase effect on dough rheology and bread quality. A study from macroscopic to molecular level. In *Food Chemistry* 99 (2), pp. 408–415. DOI: 10.1016/j.foodchem.2005.07.043.

Bruneel, Charlotte; Pareyt, Bram; Brijs, Kristof; Delcour, Jan A. (2010): The impact of the protein network on the pasting and cooking properties of dry pasta products. In *Food Chemistry* 120 (2), pp. 371–378. DOI: 10.1016/j.foodchem.2009.09.069.

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

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

Eugenia Steffolani, María; Ribotta, Pablo D.; Pérez, Gabriela T.; León, Alberto E. (2012): Combinations of glucose oxidase, α-amylase and xylanase affect dough properties and bread quality. In *International Journal of Food Science & Technology* 47 (3), pp. 525–534. DOI: 10.1111/j.1365-2621.2011.02873.x.

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

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

Fuad, Tina; Prabhasankar, P. (2010): Role of ingredients in pasta product quality: a review on recent developments. In *Critical reviews in food science and nutrition* 50 (8), pp. 787–798. DOI: 10.1080/10408390903001693.

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

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

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

Karhunen, T.; Mäntylä, A.; Nevalainen, K. M.; Suominen, P. L. (1993): High frequency one-step gene replacement in Trichoderma reesei. I. Endoglucanase I overproduction. In *Molecular & general genetics : MGG* 241 (5-6), pp. 515–522.

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

Komon-Zelazowska, Monika; Neuhof, Torsten; Dieckmann, Ralf; Döhren, Hans von; Herrera-Estrella, Alfredo; Kubicek, Christian P.; Druzhinina, Irina S. (2007): Formation of atroviridin by Hypocrea atroviridis is conidiation associated and positively regulated by blue light and the G protein GNA3. In *Eukaryotic cell* 6 (12), pp. 2332–2342. DOI: 10.1128/EC.00143-07.

Kubicek, Christian P.; Herrera-Estrella, Alfredo; Seidl-Seiboth, Verena; Martinez, Diego A.; Druzhinina, Irina S.; Thon, Michael et al. (2011): Comparative genome sequence analysis underscores mycoparasitism as the ancestral life style of Trichoderma. In *Genome biology* 12 (4), pp. R40. DOI: 10.1186/gb-2011-12-4-r40.

Kubicek, Christian P.; Komon-Zelazowska, Monika; Druzhinina, Irina S. (2008): Fungal genus Hypocrea/Trichoderma: from barcodes to biodiversity. In *Journal of Zhejiang University. Science. B* 9 (10), pp. 753–763. DOI: 10.1631/jzus.B0860015.

Kubicek, Christian P.; Komoń-Zelazowska, Monika; Sándor, Erzsébet; Druzhinina, Irina S. (2007): Facts and challenges in the understanding of the biosynthesis of peptaibols by Trichoderma. In *Chemistry & biodiversity* 4 (6), pp. 1068–1082. DOI: 10.1002/cbdv.200790097.

Kuhls, K.; Lieckfeldt, E.; Samuels, G. J.; Kovacs, W.; Meyer, W.; Petrini, O. et al. (1996): Molecular evidence that the asexual industrial fungus Trichoderma reesei is a clonal derivative of the ascomycete Hypocrea jecorina. In *Proceedings of the National Academy of Sciences of the United States of America* 93 (15), pp. 7755–7760.

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

MANDELS, M.; REESE, E. T. (1957): Induction of cellulase in Trichoderma viride as influenced by carbon sources and metals. In *Journal of bacteriology* 73 (2), pp. 269–278.

Martinez, Diego; Berka, Randy M.; Henrissat, Bernard; Saloheimo, Markku; Arvas, Mikko; Baker, Scott E. et al. (2008): Genome sequencing and analysis of the biomass-degrading fungus Trichoderma reesei (syn. Hypocrea jecorina). In *Nature biotechnology* 26 (5), pp. 553–560. DOI: 10.1038/nbt1403.

Meyer, Wieland; Morawetz, Renate; Börner, Thomas; Kubicek, Christian P. (1992): The use of DNA-fingerprint analysis in the classification of some species of the Trichoderma aggregate. In *Current Genetics* 21 (1), pp. 27–30. DOI: 10.1007/BF00318650.

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

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

ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT (1992): Safety Considerations for Biotechnology. OECD, pp. 1–45.

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

Peterson, Robyn; Nevalainen, Helena (2012): Trichoderma reesei RUT-C30--thirty years of strain improvement. In *Microbiology (Reading, England)* 158 (Pt 1), pp. 58–68. DOI: 10.1099/mic.0.054031-0.

Poutanen, K. (1997): Enzymes. An important tool in the improvement of the quality of cereal foods. In *Trends in Food Science & Technology* 8 (9), pp. 300–306. DOI: 10.1016/S0924-2244(97)01063-7.

Primo-Martín, Cristina; Wang, Mingwei; Lichtendonk, Wim J.; Plijter, Johan J.; Hamer, Robert J. (2005): An explanation for the combined effect of xylanase-glucose oxidase in dough systems. In *J. Sci. Food Agric.* 85 (7), pp. 1186–1196. DOI: 10.1002/jsfa.2107.

Ramachandran, Sumitra; Fontanille, P.; Pandey, Ashok; Larroche, Christian (2006): Gluconic Acid: Properties, Applications and Microbial Production. In *A Review, Food Technol. Biotechnol.* 44 (2), pp. 185–195.

Rasiah, I. A.; Sutton, K. H.; Low, F. L.; Lin, H.-M.; Gerrard, J. A. (2005): Crosslinking of wheat dough proteins by glucose oxidase and the resulting effects on bread and croissants. In *Food Chemistry* 89 (3), pp. 325–332. DOI: 10.1016/j.foodchem.2004.02.052.

Resmini P; Pagani M.A (1983): Ultrastructure Studies of Pasta. A Review. In *Food Microstructure* 2, pp. 1–12.

Rosell, Cristina M.; Haros, Monica; Escrivá, Consuelo; Benedito de Barber, Carmen (2001): Experimental Approach To Optimize the Use of α-Amylases in Breadmaking. In *J. Agric. Food Chem.* 49 (6), pp. 2973–2977. DOI: 10.1021/jf010012j.

Seidl, Verena; Gamauf, Christian; Druzhinina, Irina S.; Seiboth, Bernhard; Hartl, Lukas; Kubicek, Christian P. (2008): The Hypocrea jecorina (Trichoderma reesei) hypercellulolytic mutant RUT C30 lacks a 85 kb (29 gene-encoding) region of the wild-type genome. In *BMC genomics* 9, p. 327. DOI: 10.1186/1471-2164-9-327.

Sisak, C.; Csanádi, Z.; Rónay, E.; Szajáni, B. (2006): Elimination of glucose in egg white using immobilized glucose oxidase. In *Enzyme and Microbial Technology* 39 (5), pp. 1002–1007. DOI: 10.1016/j.enzmictec.2006.02.010.

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

Takai, T.; Yokota, T.; Yasue, M.; Nishiyama, C.; Yuuki, T.; Mori, A. et al. (1997): Engineering of the major house dust mite allergen Der f 2 for allergen-specific immunotherapy. In *Nature biotechnology* 15 (8), pp. 754–758. DOI: 10.1038/nbt0897-754.

Täufel, A. (1988): Enzymes and Their Role in Cereal Technology. Herausgegeben von J. E. Kruger, D. Lineback und C. E. Stauffer. 403 Seiten, zahlr. Abb. und Tab. American Association of Cereal Chemists, Inc., St. Paul, Minnesota, USA, 1987. Preis. 90,— $. In *Nahrung* 32 (1), p. 83. DOI: 10.1002/food.19880320133.

Tisch, Doris; Schmoll, Monika (2010): Light regulation of metabolic pathways in fungi. In *Applied Microbiology and Biotechnology* 85 (5), pp. 1259–1277. DOI: 10.1007/s00253-009-2320-1.

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

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

Vemulapalli, V.; Hoseney, R. C. (1998): Glucose Oxidase Effects on Gluten and Water Solubles 1. In *Cereal Chemistry* 75 (6), pp. 859–862. DOI: 10.1094/CCHEM.1998.75.6.859.

Wong, Chun Ming; Wong, Kwun Hei; Chen, Xiao Dong (2008): Glucose oxidase: natural occurrence, function, properties and industrial applications. In *Applied Microbiology and Biotechnology* 78 (6), pp. 927–938. DOI: 10.1007/s00253-008-1407-4.