A Petition to Amend the Australia New Zealand Food Standards Code with a Invertase Enzyme Preparation produced by a genetically modified strain of *Trichoderma reesei AR-996*

AB Enzymes GmbH

May 30, 2023

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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 an enzyme preparation from ***Trichoderma reesei*** *(T. reesei)* host strain genetically modified to produce a ***T. reesei*** **production strain (AR-996) containing an invertase** (also known as β-Fructofuranosidase) **encoding gene** from *Aspergillus niger*. The enzyme is to be used in the following applications:

- the production of short chain fructooligosaccharides (sc-FOS) and,
- sugar reduction.

Proposed change to Standard 1.3.3 - Processing Aids

The table schedule 18—4, **Permitted processing aids — Permitted Enzymes (section 1.3.3—11)**, is proposed to be amended to include a genetically modified strain of *Trichoderma reesei* as permitted source for **invertase** (EC 3.2.1.26), also known as β-Fructofuranosidase.

This application is submitted under a general assessment procedure.

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 invertase.

Use of the Enzyme and Benefits

The main activity of the *Trichoderma reesei* AR-996 enzyme preparation is invertase (IUBMB 3.2.1.26). The **function** of the invertase enzyme is to catalyse the breakdown of sucrose to fructose and glucose utilizing the hydrolysis of terminal non-reducing β-D-fructofuranoside residues in β-D-fructofuranosides (primary reaction). As a secondary reaction (side-activity of the invertase enzyme) in the production of sc-FOS, the **same enzyme molecule** catalyses fructotransferase reactions that means, that a fructose molecule from one sucrose molecule is transferred to another sucrose molecule to produce sc-FOS and glucose. This is ubiquitous for all invertase enzymes.

For the intended use of invertase in the production of short chain fructooligosaccharides, the substrate is sucrose. Consequently, the substrate for invertase occurs naturally and is therefore a part of the human diet.

The end products or reaction products for invertase are glucose and fructose or short chain fructooligosaccharides, depending on the reaction. All these reaction products are also found in many organisms and occur naturally in food for human consumption.

Enzyme reactions:

Primary[1]**:** $Sucrose + H_2O \rightarrow glucose + fructose$

Secondary[2]: $Sucrose \rightarrow FOS + glucose$

The method to analyze the activity of the enzyme is company specific and is capable of quantifying invertase activity as defined by its IUBMB classification. The enzyme activity is usually reported in GLU/g.

Like any other enzyme, the invertase act as a biocatalyst: with the help of the enzyme, a certain substrate is converted into a certain reaction product. The technical effect on the food or food ingredient is caused by the conversion of the substrate to the reaction product caused by the enzymatic reaction involving invertase. Once the conversion occurs, the enzyme can no longer perform a technological function.

Like most enzymes, the invertase performs its technological function during food processing. The invertase from *Trichoderma reesei* AR-996 object of this dossier is specifically intended to be used in **the production of short chain fructooligosaccharides (sc-FOS)** and **sugar reduction in various foodstuff.**. In the production of sc-FOS, invertase is used as a processing aid in food manufacturing and is not added directly to final foodstuffs.

The production of the described sc-FOS (combination of sucrose and one to four fructose molecules) utilizes sucrose: the **substrate** for invertase is sucrose.

[1] Information on EC 3.2.1.26 - beta-fructofuranosidase - BRENDA Enzyme Database (brenda-enzymes.org)

[2] See footnote 17

The **function** of the invertase enzyme is to catalyse the breakdown of sucrose to fructose and glucose utilizing the hydrolysis of terminal non-reducing β-D-fructofuranoside residues in β-D-fructofuranosides (primary reaction). As a secondary reaction (side-activity of the invertase enzyme), the **same enzyme molecule** catalyses fructotransferase reactions, in which a fructose molecule from one sucrose molecule is transferred to another sucrose molecule to produce sc-FOS and glucose. This is ubiquitous for all invertase enzymes.

Please refer to *Figure 1* below for the reaction diagram.

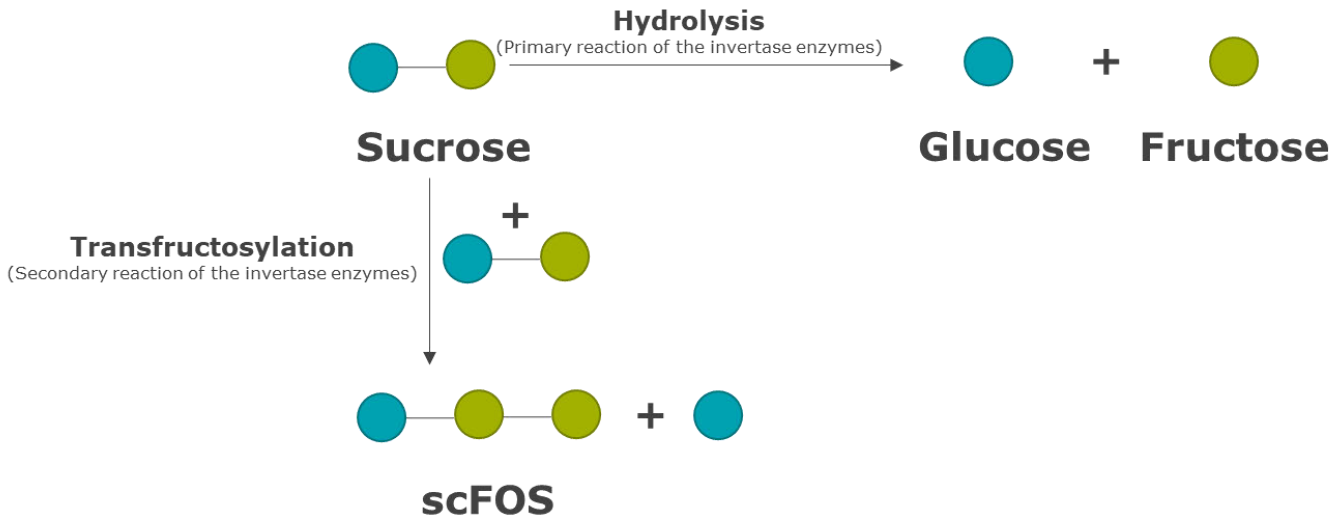

Figure 1: Hydrolysis and Transfructosylation reaction of the Invertase

Invertase from *Trichoderma reesei* AR-996 production strain is intended for use in the following applications:

- Production of sc-FOS from sucrose
- Sugar reduction

sc-FOS Production

The sc-FOS production industry is relatively new and categorizes the substance as a prebiotic ingredient. Prebiotics fall under the functional food category which imply such foods have confirmed or potential health benefits for consumers due to their bio-functional properties (Ojwach et al. 2022; Mutanda et al. 2014). sc-FOS has the ability to stimulate gastrointestinal bacteria such as bifidobacteria (Martins et al. 2019). sc-FOS's history in food began in Japan with food producers adding FOS as a functional ingredient (Martins et al. 2019). Part of the interest in sc-FOS is the biochemical characteristics of the ingredient where compared to other saccharides, sc-FOS has smaller molecular weight, and polymerization (Ojwach et al. 2022). As an oligosaccharide, FOS is found in a number of fruits and vegetables which are part of the human diet, such as banana, barley, garlic, honey, onion, rye, chicory, Jerusalem artichoke, yacon, cereal plants and tomato (Ojwach et al. 2022; Mutanda et al. 2014; Martins et al. 2019).

Use of enzymes in the industrial production of sc-FOS is an alternative to already established acid/chemical methods (Martins et al. 2019). The enzymatic production of sc-FOS in simplified industrial conditions where the enzyme's ability to utilize both invertase and fructosyltransferase activity streamlines the need to avoid additional control steps for sc-FOS production from sucrose.

Below, the benefits of the use of industrial invertase 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 (such as reduction in complexity, enzymes are managed according to pH and temperature conditions), resulting in better production economy and environmental benefits such as the use of less raw materials (i.e., synthetic chemicals) and the production of less waste. The use of invertase has been recognized as acceptable in the production

of sc-FOS for several years in the USA[3,4], Canada[5], and Australia/New Zealand[6,7] which demonstrates the technological need for such food enzymes in food processes.

In general, the benefits of invertase in sc-FOS production are:

- Higher yields of sc-FOS and lower costs compared to the extraction from plant material (Ibrahim 2021; Wienberg et al. 2022; Choukade and Kango 2021)
- Defined and consistent product composition (Sánchez-Martínez et al. 2020; Wienberg et al. 2022)
- Sucrose widely available and low-cost substrate (Wienberg et al. 2022; Xu et al. 2019)
- No additional substrates as sucrose needed (Martins et al. 2019; Ibrahim 2021)
- FOS production by chemical hydrolysis of inulin uses toxic chemicals and lacks of specificity (Sánchez-Martínez et al. 2020)
- Environmentally friendly, energy saving and production of less by-products (Ojwach et al. 2022; Sánchez-Martínez et al. 2020)

Sugar Reduction

In recent years, numerous studies have shown the negative health effects of high consumption of sugars and the positive health benefits of increasing the soluble dietary fiber in human diets. (Evans 2017; Deliza et al. 2021; Prada et al. 2022; Rippe and Angelopoulos 2016; Respondek et al. 2014; Nobre et al. 2022) In response to these studies and the recommendation to reduce the glycemic load (Augustin et al. 2015), the demand for lower glycemic index foods, which are less sugary and higher in soluble dietary fiber has been increased. To meet this demand, the replacement of traditional sugary carbohydrates like sucrose, glucose or fructose with substitutes like non-nutritive sweeteners, sugar alcohols, glucooligosaccharides and short chain fructooligosaccharides (sc-FOS) has been investigated and applied (Martins et al. 2019; Respondek et al. 2014). Interest has been directed to sc-FOS. These compounds impart mild sweetness,

[3] GRAS Notice GRN 537: short chain fructo-oligosaccharides produced with invertase for use in infant formula

[4] GRAS Notice GRN 1006: short chain fructo-oligosaccharides produced with invertase for general use in food

[5] 5. List of Permitted Food Enzymes (Lists of Permitted Food Additives) - Canada.ca: Invertase is a permitted food additive enzyme for sucrose used in the production of fructooligosaccharides

[6] Application A1055 - Short-chain Fructo-oligosaccharides (foodstandards.gov.au): Invertase from *Aspergillus niger* was approved as a processing aid by FSANZ

[7] A1212 - Beta-fructofuranosidase enzyme from Aspergillus fijiensis (foodstandards.gov.au): Application to update Schedule 18 of FSANZ's Food Code entry for invertase from *Aspergillus niger* to *Aspergillus fijiensis*

but also significantly, they are soluble dietary fibers with health benefits (Flores-Maltos et al. 2016; Respondek et al. 2014; Nobre et al. 2022).

The use of invertase for sugar reduction in fruits and vegetables processing is one example. Some fruit and vegetable raw materials like banana, oranges, apples or carrots contain substantial amounts of sugars. The treatment of fruit and vegetable raw materials with invertase as part of the standard raw material processing leads to sucrose hydrolysis and sc-FOS formation and reduces the endogenous or naturally occurring sugar content. The addition of invertase is possible at several production steps depending on raw material, production process and final product.

In general, the benefits of invertase for sugar reduction are:

- Reduction of total sugars content, the sum of mono - and disaccharides like glucose, fructose and sucrose (Cywińska-Antonik et al. 2023; Gomes et al. 2023)
- Increase of soluble dietary fiber content (Martins et al. 2019; Ibrahim 2021; Nobre et al. 2015; Cywińska-Antonik et al. 2023)
- Decrease of glycemic index (Martins et al. 2019; Respondek et al. 2014)
- Decrease of energy value (calories) (Ibrahim 2021; Gomes et al. 2023)
- Less sweet taste (Ibrahim 2021)
- In situ process for sugar reduction – no removal of valuable other substances like vitamins or organic acids (Cywińska-Antonik et al. 2023; Gomes et al. 2023)

Safety Evaluation

The safety of the invertase produced by the genetically modified *Trichoderma reesei* AR-996 from a toxicological perspective is supported by the historical safety of strain lineage. Toxicological studies were performed on a representative strain (AR-700) which derives from the same recipient strain within the strain lineage of AR-996. Expression constructs of both AR-996 and AR-700 are very similar, only differing by the expression cassette/enzyme gene of interest. As both production strains are free of any harmful sequences or any potential hazards, the expression cassettes are very similar and are stably integrated into the genome of the strains without any additional growth/mutagenesis cycles thereafter, differences

in the genetic modification of AR-996 and AR-700 are not a safety concern. Furthermore, the manufacturing conditions between the two production strains are very similar. The slight changes in pH levels and fermentation medium (food-grade) have been thoroughly assessed. They are considered minor (common industry practice) and do not trigger any additional safety issue.

To add on, enzyme product from AR-996 production strain complies with JECFA specifications for chemical and microbiological purity of food enzymes (Food and Agriculture Organization of the United Nations 2006) which confirms the safety of the production strain AR-996.

The safety of the AR-996 *Trichoderma reesei* production strain is substantiated via three toxicological studies on the *Trichoderma reesei* AR-700 production strain to demonstrate non-toxigenicity of the strain lineage. The toxicological studies conducted include, a reverse mutation assay using bacteria, a Micronucleus Assay in Bone Marrow Cells of the Rat and a 90-day repeated dose oral toxicity study in Wister rats. All three toxicological studies showed negative findings demonstrating the AR-700 production strain to be non-mutagenic, to not induce structural and/or numerical chromosomal damage, and to not cause toxigenic effects on the Wister rats tested in the 90-day oral toxicity study.

The product is free of production strain and DNA.

AB Enzymes is in the process of registering the *Trichoderma reesei* AR-996 invertase production strain in other countries such as Brazil (ANVISA), Canada (Health Canada), Denmark (DVFA), EU (EFSA), and USA (US FDA).

Conclusion

To conclude, the use of the food enzyme invertase from *Trichoderma reesei* AR-996 in the production of food is safe based on the following aspects presented in this dossier:

- Safety data and information of the production strain
- Allergenicity and toxin analysis assessment on the amino acid sequence of food enzyme
- TDMI value based on Budget Method

Trichoderma reesei has been used in the food industry for many years. Strains from the *Trichoderma reesei* microorganism are generally recognized as safe and are recognized to produce a variety of enzymes. *Trichoderma reesei* is listed as a permitted producer of enzymes in multiple global food enzyme positive lists, including in Australia. The safety of the invertase produced by the genetically modified *Trichoderma reesei* AR-996 from a toxicological perspective is supported by the historical safety of strain lineage which is provided in the dossier. We have demonstrated that the enzyme batches containing invertase from *Trichoderma reesei* AR-996 meet the following criteria:

- Absence of Antibiotic and Toxic Compounds & Analysis of Purity and Identity Specifications of the Enzyme Preparation
- Absence of Production strain
- No Detection of DNA

Based on the safety evaluation, AB Enzymes GmbH respectfully request the inclusion of invertase (EC 3.2.1.26) from *Aspergillus niger* expressed a genetically modified strain of *Trichoderma reesei* AR-996 in the table of schedule 18—4, **Permitted processing aids — Permitted Enzymes (section 1.3.3—11)**.

INTRODUCTION

The dossier herein describes a ***Trichoderma reesei (T. reesei)*** host strain, genetically modified to produce a ***Trichoderma reesei*** **production strain which is non-pathogenic and non-toxicogenic, containing an invertase** (also known as β-Fructofuranosidase) **encoding gene** from *Aspergillus niger*.

Invertase from *A. niger* produced in *Trichoderma reesei* is mainly intended to be used in the breakdown of sucrose to fructose and glucose utilizing the hydrolysis of terminal non-reducing β-D-fructofuranoside residues in β-D-fructofuranosides to produce short chain fructooligosaccharides (sc-FOS) and used for sugar reduction.

The following sections describe the genetic modifications implemented in the development of a safe standard host strain, further developed 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 invertase 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 invertase enzyme preparation from *Trichoderma reesei* shows no evidence of pathogenic or toxic effects. Estimates of human consumption and an evaluation of dietary exposure are also included.

III. 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
AB Enzymes GmbH

[redacted]

3.1.3. Purpose of the Application

The table schedule 18—4, **Permitted processing aids — Permitted Enzymes (section 1.3.3—11),** is proposed to be amended to include a genetically modified strain of *Trichoderma reesei* as permitted source for invertase (EC 3.2.1.26), also known as β-Fructofuranosidase.

To explain further, invertase catalyses the breakdown of sucrose to fructose and glucose utilizing the hydrolysis of terminal non-reducing β-D-fructofuranoside residues in β-D-fructofuranosides (primary reaction) for the production of short chain fructooligosaccharides (sc-FOS) and sugar reduction in food processing. As a secondary reaction (side-activity of the invertase enzyme) in the production of sc-FOS, the **same enzyme molecule** catalyses fructotransferase reactions in which a fructose molecule from one sucrose molecule is transferred to another sucrose molecule to produce sc-FOS and glucose. This is ubiquitous for all invertase enzymes.

β-Fructofuranosidase (EC 3.2.1.26) is a permitted enzyme listed in the table schedule 18—4, **Permitted processing aids — Permitted Enzymes (section 1.3.3—11)**.

3.1.4. Justification for the Application

The need for the proposed change:

Trichoderma reesei expressing an invertase gene from *Aspergillus niger* is not present as an approved source in the table to schedule 18—4, **Permitted processing aids — Permitted Enzymes (section 1.3.3—11)**. AB Enzymes GmbH is requesting that this source organism be added. See **Section 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 invertase enzyme is one of AB Enzymes' latest achievements and has showed great potential in food manufacturing as detailed in the 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 food manufacturers. This is a characteristic that is strongly preferred by manufacturers. There is also a cost benefit associated with the use of *Trichoderma reesei* as superior producer of enzymes resulting in a cost benefit that is passed on to the final user of the enzyme. Increased competition in the market is also a desired characteristic in the context of competition laws. It will increase the choice of 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 food applications.

Due to the effectiveness of this enzyme in the above-mentioned food processes, AB Enzymes has submitted our application in Brazil, Canada, Denmark, EU, USA, and plans to submit in China, Thailand, Indonesia and South Korea.

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—4 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 food manufacturers. 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 already have 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 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. Invertase 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 invertase do not have other characteristics than what is expected by the consumer. Consumers could be impacted indirectly by companies able to pass cost savings from utilizing enzymes in food processing on to their customers.

3.1.9. Assessment Procedure

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

3.1.10. Confidential Commercial Information (CCI)

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

3.1.11. Other Confidential Information

n/a

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 publicly 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 food is not restricted by any Codex Alimentarius Commission (Codex) Standards or any other known regulations.

National Standards:

n/a

3.1.14. Statutory Declaration

The Statutory Declaration is included as Appendices #1.3a and #1.3b.

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

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

• SECTION 3.1 – GENERAL REQUIREMENTS

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

Accordingly, the checklist for General Requirements as well as the Processing Aids part of the checklist for Standards related to Substances added to Food was used and is included as Appendix #1.4.

IV. 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

Invertase is a microbial produced enzyme.

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 invertase in production of sc-FOS and sugar reduction. 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	Invertase
Common names	sucrase, β-fructofuranosidase, β-fructosidase, β-(1-2)-fructofuranosidase, β-D-fructofuranosidase, β-D-fructofuranosidase fructohydrolase
Enzyme Commission No. (IUBMB)	EC 3.2.1.26
CAS number	9001-57-4[8]
Host	*Trichoderma reesei* AR-996
Production strain	AR-996
Donor	*Aspergillus niger*

[8] Information on EC 3.2.1.26 - beta-fructofuranosidase - BRENDA Enzyme Database (brenda-enzymes.org)

The classification of the enzyme according to the IUBMB is as follows:

EC 3. is for hydrolases;

EC 3.2. is for glycosylases;

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

EC 3.2.1.26 is for beta-fructofuranosidase.

The enzyme is an invertase enzyme (i.e., acts on sucrose, which is a disaccharide molecule consisting of glucose and fructose, and can be found in many plants, in the roots and fruits as storage of energy). The food enzyme catalyses the breakdown of sucrose into fructose and glucose. Additionally, as all invertases, the enzyme is capable of transferring fructose molecules to sucrose molecule, leading to the formation of short chain fructo-oligosaccharides and glucose.

A.2.2. **Enzyme Preparation**

This dossier includes an invertase enzyme, produced with the help of *Trichoderma reesei* AR-996 containing an invertase enzyme gene from *Aspergillus niger*. The representative current commercial product is ROHALASE® FOS-UP.

A.2.3. **Final Liquid Enzyme preparation composition**

Composition for ROHALASE® FOS-UP	
Constituent	**%**
Invertase enzyme concentrate	**1-2**
Glycerol	50
Sodium Citrate	2.26
Citric Acid	0.45
Water	Reminder

Roles of Formulation Ingredients

In the case of the final enzyme preparations that contain invertase from the AR-996 *Trichoderma* production strain, the following food additives in the final product are present:

- Glycerol (CAS No. 56-81-5): Acts as a carrier in the enzyme preparation
- Potassium chloride (CAS No. 7747-40-7): Acts as a carrier in the enzyme preparation
- Tri-sodium citrate (CAS No. 68-04-2): Acts as a pH adjustment agent in the enzyme preparation

- Citric acid (CAS No. 7732-18-5): Acts as a pH adjustment agent in the enzyme preparation

All substances in the finished enzyme preparation are of food grade quality and conform with the 13th edition of the Food Chemicals Codex (2022) and the *Combined Compendium of Food Additive Specifications* prepared by JECFA.

A.2.4. **Enzyme genetic modification**

The enzyme is from a *Trichoderma reesei* host strain genetically modified with an invertase gene deriving from *Aspergillus niger*. The enzyme is not considered protein engineered.

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 – ROHALASE® FOS-UP

Property	**Requirement**	
Activity	min.	7000 GLU/g
Appearance	Brown liquid	
Density	1.20 g/ml	

The food enzyme catalyses the hydrolysis of sucrose, resulting in the breakdown of sucrose into smaller monosaccharide units (primary reaction), and as a secondary reaction, the food enzyme enables the transfer of a fructose molecule from one sucrose molecule to another sucrose molecule, leading to the formation of short chain fructo-oligosaccharides (sc-FOS) and glucose. Such enzyme activities are widely present in nature and in food ingredients.

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, invertase 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 several 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, lack of water activity, suboptimal pH, etc. The enzyme protein invertase is inactivated by heat in a specific inactivation.

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

Please refer to ROHALASE® FOS-UP product data sheet for shelf-life and storage conditions (Appendix #1). Efficacy data of ROHALASE® FOS-UP is described in Appendix CCI.

The optimal pH and temperature conditions for the activity of the food enzyme are:

- pH: 5.0 – 6.0
- Temperature: 50 - 60ºC

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

A.3.1. Information on the technological need and mechanism of action of the enzyme in food

Like any other enzyme, the invertase act as a biocatalyst: with the help of the enzyme, a certain substrate is converted into a certain reaction product. The technical effect on the food or food ingredient is caused by the conversion of the substrate to the reaction product caused by the enzymatic reaction involving invertase. Once the conversion occurs, the enzyme can no longer perform a technological function.

Like most enzymes, the invertase performs its technological function during food processing. The invertase from *Trichoderma reesei* AR-996 object of this notice is specifically intended to be used in **the production of short chain fructooligosaccharides (sc-FOS)** and **sugar reduction**. In the production of sc-FOS, invertase is used as a processing aid in food manufacturing and is not added directly to final foodstuffs.

The production of the described sc-FOS (combination of sucrose and one to four fructose molecules) utilizes sucrose: the **substrate** for invertase is sucrose. Sucrose can be found naturally in plants.

Consequently, the substrate for invertase occurs naturally in nature, and in vegetable-based foods and are therefore a natural part of the human diet.

The **function** of the invertase enzyme is to catalyse the breakdown of sucrose to fructose and glucose utilizing the hydrolysis of terminal non-reducing β-D-fructofuranoside residues in β-D-fructofuranosides (primary reaction). As a secondary reaction (side-activity of the invertase enzyme), the **same enzyme molecule** catalyses fructotransferase reactions that means, that a fructose molecule from one sucrose molecule is transferred to another sucrose molecule to produce sc-FOS and glucose. This is ubiquitous for all invertase enzymes.

Please refer to *Figure 1* below for the reaction diagram.

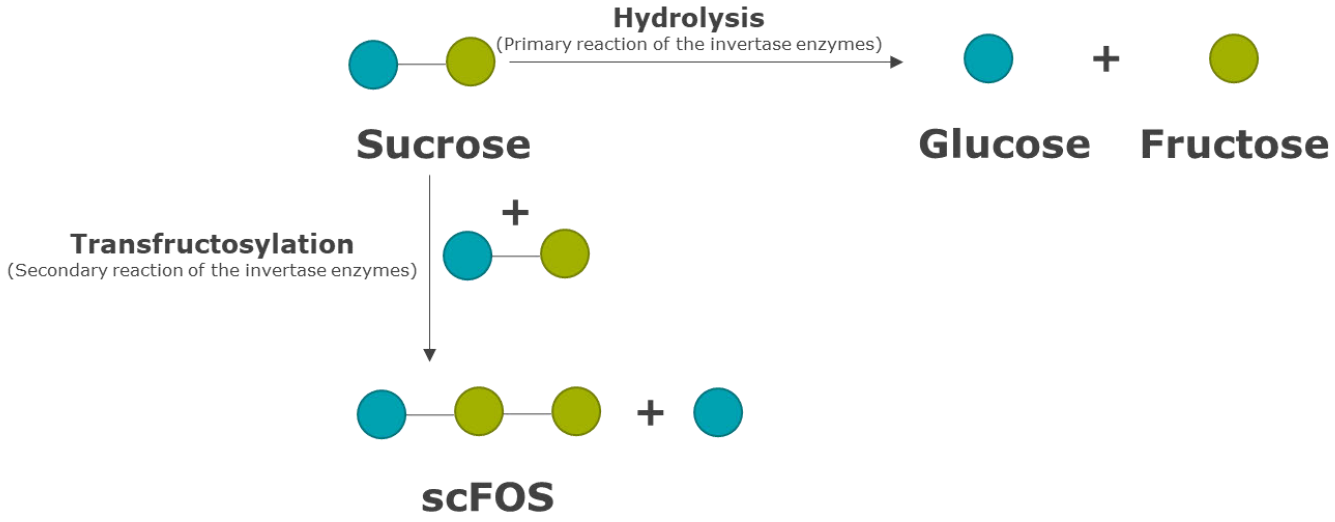

Figure 1: Hydrolysis and Transfructosylation reaction of the Invertase

Invertase from *Trichoderma reesei* AR-996 production strain is intended for use in the following applications:

- Production of sc-FOS from sucrose
- Sugar reduction

sc-FOS Production

The sc-FOS production industry is relatively new and categorizes the substance as a prebiotic ingredient. Prebiotics fall under the functional food category which imply such foods have confirmed or potential health benefits for consumers due to their bio-functional properties (Ojwach et al. 2022; Mutanda et al. 2014). sc-FOS can stimulate gastrointestinal bacteria such as bifidobacteria (Martins et al. 2019). sc-FOS's history in food began in Japan with food producers adding FOS as a functional ingredient (Martins et al. 2019). Part of the interest in sc-FOS is the biochemical characteristics of the ingredient were compared to other saccharides, sc-FOS has smaller molecular weight, and polymerization (Ojwach et al. 2022). As an oligosaccharide, FOS is found in several fruits and vegetables which are part of the human diet, such as banana, barley, garlic, honey, onion, rye, chicory, Jerusalem artichoke, yacon, cereal plants and tomato (Ojwach et al. 2022; Mutanda et al. 2014; Martins et al. 2019).

Use of enzymes in the industrial production of sc-FOS is an alternative to already established acid/chemical methods (Martins et al. 2019). The enzymatic production of sc-FOS in simplified industrial conditions where the enzyme's ability to utilize both invertase and fructosyltransferase activity streamlines the need to avoid additional control steps for sc-FOS production from sucrose.

Below, the benefits of the use of industrial invertase 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 (such as reduction in complexity, enzymes are managed according to pH and temperature conditions), resulting in better production economy and environmental benefits such as the use of less raw materials (i.e., synthetic chemicals) and the production of less waste. The use of invertase has been recognized as acceptable in the production of sc-FOS for several years in the USA[9,10], Canada[11], and Australia/New Zealand[12,13] which demonstrates the technological need for such food enzymes in food processes.

[9] GRAS Notice GRN 537: short chain fructo-oligosaccharides produced with invertase for use in infant formula

[10] GRAS Notice GRN 1006: short chain fructo-oligosaccharides produced with invertase for general use in food

[11] 5. List of Permitted Food Enzymes (Lists of Permitted Food Additives) - Canada.ca: Invertase is a permitted food additive enzyme for sucrose used in the production of fructooligosaccharides

[12] Application A1055 - Short-chain Fructo-oligosaccharides (foodstandards.gov.au): Invertase from *Aspergillus niger* was approved as a processing aid by FSANZ

[13] A1212 - Beta-fructofuranosidase enzyme from Aspergillus fijiensis (foodstandards.gov.au): Application to update Schedule 18 of FSANZ's Food Code entry for invertase from *Aspergillus niger* to *Aspergillus fijensis*

In general, the benefits of invertase in sc-FOS production are:

- Higher yields of sc-FOS and lower costs compared to the extraction from plant material (Ibrahim 2021; Wienberg et al. 2022; Choukade and Kango 2021)
- Defined and consistent product composition (Sánchez-Martínez et al. 2020; Wienberg et al. 2022)
- Sucrose widely available and low-cost substrate (Wienberg et al. 2022; Xu et al. 2019)
- No additional substrates as sucrose needed (Martins et al. 2019; Ibrahim 2021)
- FOS production by chemical hydrolysis of inulin uses toxic chemicals and lacks of specificity (Sánchez-Martínez et al. 2020)
- Environmentally friendly, energy saving and production of less by-products (Ojwach et al. 2022; Sánchez-Martínez et al. 2020)

Sugar Reduction

In recent years, numerous studies have shown the negative health effects of high consumption of sugars and the positive health benefits of increasing the soluble dietary fiber in human diets. (Evans 2017; Deliza et al. 2021; Prada et al. 2022; Rippe and Angelopoulos 2016; Respondek et al. 2014; Nobre et al. 2022) In response to these studies and the recommendation to reduce the glycemic load (Augustin et al. 2015), the demand for lower glycemic index foods, which are less sugary and higher in soluble dietary fiber has been increased. To meet this demand, the replacement of traditional sugary carbohydrates like sucrose, glucose, or fructose with substitutes like non-nutritive sweeteners, sugar alcohols, glucooligosaccharides and short chain fructooligosaccharides (sc-FOS) has been investigated and applied (Martins et al. 2019; Respondek et al. 2014). Interest has been directed to sc-FOS. These compounds impart mild sweetness, but also significantly, they are soluble dietary fibers with health benefits (Flores-Maltos et al. 2016; Respondek et al. 2014; Nobre et al. 2022).

The use of invertase for sugar reduction in fruits and vegetables processing is one example. Some fruit and vegetable raw materials like banana, oranges, apples or carrots contain substantial amounts of sugar. The treatment of fruit and vegetable raw materials with invertase as part of the standard raw material processing leads to sucrose hydrolysis and sc-FOS formation and reduces the endogenous or naturally

occurring sugar content. The addition of invertase is possible at several production steps depending on raw material, production process and final product.

In general, the benefits of invertase for sugar reduction are:

- Reduction of total sugars content, the sum of mono - and disaccharides like glucose, fructose, and sucrose (Cywińska-Antonik et al. 2023; Gomes et al. 2023)
- Increase of soluble dietary fiber content (Martins et al. 2019; Ibrahim 2021; Nobre et al. 2015; Cywińska-Antonik et al. 2023)
- Decrease of glycemic index (Martins et al. 2019; Respondek et al. 2014)
- Decrease of energy value (calories) (Ibrahim 2021; Gomes et al. 2023)
- Less sweet taste (Ibrahim 2021)
- In situ process for sugar reduction – no removal of valuable other substances like vitamins or organic acids (Cywińska-Antonik et al. 2023; Gomes et al. 2023)

Fate of the Enzyme in Food

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

Invertase performs its technological function during food processing as a processing aid. The enzyme acts as both an invertase on sucrose molecules and a fructosyltransferase between sucrose molecules and fructofuranosyl-sucrose molecules (i.e., comprising fructose chains with a terminal glucose). Once this reaction is complete, the ingredient undergoes several inactivation and filtration steps to ensure the enzyme is inactivated and no residual enzyme protein remains in the final food product. Since the enzyme is not present in the final FOS ingredient, the food enzyme will also not be present in the final food product (confectionary, jams, beverages, etc.).

To be able to perform a technological function in the final food, several conditions must be fulfilled at the same time:

- the enzyme protein must be in its 'native' (non-denatured) form, AND

- the substrate must still be present, AND
- the enzyme must be free to move (able to reach the substrate), AND

conditions like pH, temperature and water content must be favorable

A.4. Manufacturing Process

The food enzyme is produced by ROAL Oy through submerged fermentation of *Trichoderma reesei* AR-996 in accordance with current Good Manufacturing Practices for Food (GMP) and the principles of Hazard Analysis of Critical Control Points (HACCP). As it is run in the EU, it is also subject to the Food Hygiene Regulation (852/2004). Quality certificates are provided in Appendix #2.

The enzyme preparation described herein is produced by controlled batch submerged fermentation. The production process involves the fermentation process, recovery (downstream processing) and formulation and packaging. Finally, measures are taken to comply with cGMPs and HACCP. The manufacturing flow-chart is presented in Appendix #3.

Note that the fermentation process of microbial food enzymes is substantially equivalent across the world. This is also true for the recovery process: in most cases, the enzyme protein is only partially separated from the other organic material present in the food enzyme.

A.4.1. **Fermentation**

The invertase 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 food enzymes production for decades. The main fermentation steps are:

- Inoculum
- Seed fermentation
- Main fermentation

A.4.2. **Raw materials**

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

The raw materials used for the formulation are of food grade quality. Please refer to Appendix CCI on the composition of the fermentation media specific to produce invertase from *T. reesei* AR-996.

A.4.3. **Inoculum**

A suspension of a pure culture of *T. reesei* AR-996 is aseptically transferred to shake flasks containing fermentation medium. When enough biomass is obtained the shake flasks cultures are combined to be used to inoculate the seed fermentor.

A.4.4. **Seed fermentation**

After sufficient growth, the biomass is aseptically transferred to a seed fermentor, where further growth takes place under agitation and aeration at a constant temperature and a fixed pH.

A.4.5. **Main fermentation**

Biosynthesis of the invertase enzyme product by the production strain *T. reesei* AR-996 occurs during the main fermentation.

The contents of the seed fermentor are aseptically transferred into the main fermentor, where enzyme production will take place. The main fermentation is run under specified pH, temperature and aeration conditions, until sufficient enzyme production has taken place.

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

A.4.6. **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 producing microorganisms 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 several types of unit operations described below may vary, depending on the specific enzyme production plant.

A.4.7. **Materials**

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

- Flocculants
- Filter aids
- pH adjustment agents

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

A.4.8. **Pre-Treatment**

Flocculants and/or filter aids are added to the fermentation broth in order to get clear filtrates and to facilitate the primary solid/ liquid separation.

A.4.9. **Primary solid/liquid separation**

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

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

A.4.10. **Concentration**

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

A.4.11. **Polish and Germ Filtration**

After concentration, for removal of residual cells of the production strain and as a general precaution against microbial contamination, filtration on dedicated germ filters is applied at various stages during the recovery process. Pre-filtration (polish filtration) is included if needed to remove insoluble substances and facilitate the germ filtration. The final polish and germ filtration at the end of the recovery process results in a concentrated enzyme solution free of the production strain and insoluble substances.

A.4.12. **General Production Controls and Specifications**

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

Identity and purity of the producing microorganism:

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

determined by checking identity, viability, microbial purity and productivity of the WCB. The accepted WCB is used as seed material for the inoculum.

Microbiological hygiene:

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

- Hygienic design of equipment:
- Cleaning and sterilization:
    - Validated standard cleaning and sterilization procedures of the production area and equipment
    - Sterilization of all fermentation media
    - Use of sterile air for aeration of the fermentor
- Hygienic processing:
    - Aseptical transfer of the content of the WCB ampoule, inoculum flask or seed fermentor
    - Maintaining a positive pressure in the fermentor
- Germ filtration

Chemical contaminants:

It is ensured that all raw materials used in production of food enzymes are of food grade quality or have been assessed to be fit for their intended use and comply with agreed specifications.

In addition to these control measures, in-process testing and monitoring are performed to guarantee an optimal and efficient enzyme production process and a high-quality product (cGMPs). The whole process is controlled with a computer control system which reduces the probability of human errors in critical process steps.

These in-process controls comprise:

Microbial controls:

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

Monitoring of fermentation parameters may include:

- pH
- Temperature
- Aeration conditions

The measured values of these parameters are constantly monitored during the fermentation process. The values indicate whether sufficient biomass or enzyme protein has been developed and the fermentation process evolves according to plan.

Deviations from the pre-defined values lead to adjustment, ensuring an optimal and consistent process.

Enzyme activity and other relevant analyses (like dry matter, refraction index or viscosity):

This is monitored at regular intervals and at critical steps during the whole food enzyme production process.

A.4.13. **Formulation and Packaging**

The enzyme solution before formulation is defined as "food enzyme". Subsequently, the food enzyme is formulated. The resulting product is defined as a 'food enzyme preparation'. For all kinds of food enzyme preparations, the food enzyme is adjusted to the desired activity and is standardized and preserved with food-grade ingredients or additives.

The food enzyme preparation is tested by Quality Control for all related aspects, like expected enzyme activity and the general JECFA Specifications 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.

A.4.14. Stability of the Enzyme during Storage and Prior to Use

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

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

A.5. Specification for the Purity and Identity

The food enzyme invertase complies with the internationally accepted JECFA specifications for chemical and microbiological purity of food enzymes (FAO/WHO 2006):

Lead:	No more than 5 mg/kg
Salmonella sp.:	Absent in 25 g of sample
Total coliforms:	Not more than 30 per gram
Escherichia coli:	Absent in 25 g of sample
Antimicrobial activity:	Not detected
Mycotoxins:	No significant levels[14]

Analytical data is provided in Appendix CCI.

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

A.6. Analytical Method for Detection

Please refer to Appendix CCI.

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

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

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

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

Dossiers have been submitted to the Brazil (ANVISA), Canada (Health Canada), Denmark (DVFA), EU (EFSA), and USA (US FDA) and there are plans to be submit in China, Indonesia, South Korea and Thailand.

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

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

The safety of *Trichoderma reesei* as an enzyme producer has been reviewed by Frisvad et al. (2018), Nevalainen et al. (1994), Nevalainen et al.; Olempska-Beer et al. (1994; 2006) and Blumenthal (2004). *T. reesei* is regarded as a safe organism for production of industrial enzymes.

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

Non-exhaustive list of authorized food enzymes (other than invertases) produced by *Trichoderma reesei*		
Authority	**Food Enzyme**	**Reference**
JECFA	Cellulase Beta-glucanase Glucoamylase	FAS 30-JECFA 39/15 and FAS 22-JECFA 31/31 FAS 22-JECFA 31/25, JECFA monograph gluco amylase
Australia/New Zealand	Cellulase Glucan 1-3 beta-glucosidase Beta-glucanase Hemicellulase complex Gluco-amylase Endo 1,4-beta- xylanase	Australia New Zealand Food Standards Code – Schedule 18 – Processing aids (legislation.gov.au)

	Pectinases	
Canada	Cellulase Glucanase Pentosanase Xylanase Protease Pectinase	5. List of Permitted Food Enzymes (Lists of Permitted Food Additives)
France	Alpha-amylase (GM) Amyloglucosidase (GM) Beta-glucanase (GM) Xylanase Cellulase Lysophospholipase (GM)	Arrêté du 19 octobre 2006
USA[15]	Pectin lyase Transglucosidase (GM) Glucoamylase Phospholipase A Polygalacturonase Pectin esterase Mannanase Endo-1,4-beta-xylanase Lipase Lysophospholipase Glucose oxidase Serine endopeptidase	GRAS Notice Inventory, GRN 32 GRAS Notice Inventory, GRN 315 GRAS Notice Inventory, GRN 372 GRAS Notice Inventory, GRN 524 GRAS Notice Inventory, GRN 557 GRAS Notice Inventory, GRN 558 GRAS Notice Inventory, GRN 566 GRAS Notice Inventory, GRN 628 GRAS Notice Inventory, GRN 631 GRAS Notice Inventory, GRN 653 GRAS Notice Inventory, GRN 707 GRAS Notice Inventory, GRN 817

[15] GRAS affirmations and GRAS notifications

Non-exhaustive list of authorized invertase from production organisms other than *Trichoderma reesei*		
Authority	**Production organisms**	**Reference**
Australia/NZ[16]	*Aspergillus niger* *Aspergillus fijiensis ATCC 20611* *Saccharomyces cerevisiae*	Australia New Zealand Food Standards Code – Schedule 18 – Processing aids (legislation.gov.au)
France	*Aspergillus niger*	Arrêté du 19 octobre 2006
USA	*Saccharomyces cervisiae*	GRAS Notice Inventory, GRN 88
Brazil	*Aspergillus niger* *Bacillus subtilis* *Kluyveromyces fragilis* *Saccharpmyces carlsbergensis* *Saccharomyces cerevisiae*	RDC Nº 728 July 1, 2022
Canada	*Aspergillus fijiensis* *Saccharomyces sp.*	5. List of Permitted Food Enzymes (Lists of Permitted Food Additives)
South Korea	*Aspergillus aculeatus & variants* *Aspergillus awamori & variants* *Aspergillus niger & variants* *Bacillus genus* *Kluyveromyces lactis & variants* *Saccharomyces cerevisiae & variants*	South Korean Food Code Invertase

[16] Invertase is listed as ß-Fructofuranosidase

JECFA	*Saccharomyces cerevisiae*	JECFA Evaluations-INVERTASE FROM SACCHAROMYCES CEREVISIAE- (inchem.org)

C.2.2. **Toxicological Studies**

This section describes the studies performed to evaluate the safety of the invertase enzyme preparation.

Safe Strain Lineage

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

Enzyme preparations meet the JECFA definition of Safe Food Enzyme Production Strain[17] or a Presumed Progeny Strain[18] when appropriate toxicological testing (i.e., repeated-dose toxicity and genotoxicity testing) are conducted on enzymes from closely related strains derived from the same parental organism.

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

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

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

The safety of the invertase produced by the genetically modified *Trichoderma reesei* AR-996 from a toxicological perspective is supported by the historical safety of strain lineage. Toxicological studies were performed on a strain (AR-700) which derives from the same recipient strain within the strain lineage of AR-996.

The following studies were performed on a *Trichoderma reesei* AR-700 from the AR-996 strain lineage:

- Reverse Mutation Assay using Bacteria (*Salmonella typhimurium and Escherichia Coli*) with *Trichoderma reesei* produced phytase
- Micronucleus Assay in Bone Marrow Cells of the Rat with *Trichoderma reesei* produced phytase
- *Trichoderma reesei* produced phytase: 90-Day Oral Toxicity (Gavage) Study in the Wistar Rat

All tests were performed according to the principles of Good Laboratory Practices (GLP) and the current OECD and EU guidelines.

Please refer to the toxicological studies reports in Appendix CCI.

As mentioned above both the AR-996 and AR-700 have been developed from the same recipient strain. Expression constructs are similar, only differing by the expression cassette/enzyme gene of interest. As both production strains are free of any harmful sequences or any potential hazards, the expression cassettes are remarkably similar and are stably integrated into the genome of the strains without any additional growth/mutagenesis cycles thereafter, differences in the genetic modification of AR-996 and AR-700 are not a safety concern.

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

To add on, enzyme product from AR-996 production strain complies with JECFA specifications for chemical and microbiological purity of food enzymes (Food and Agriculture Organization of the United Nations 2006) which confirms the safety of the production strain AR-996.

Safety of the Production strain (SSL):

Please refer to Appendix #4 for more details on the safety of the *Trichoderma reesei* AR-996 production strain including:

- **Pariza and Johnson Decision Tree**
- **JECFA Safe Progeny Strain statement**
- **Differences between toxicological tested strain and AR-996 production strain**
- **Diagram on Strain Lineage**

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

AB Enzymes conducted a prediction of toxicity of invertase using bioinformatics tools. A homology search was performed from the NCBI Identical Protein Groups (IPG) database using the BLAST-P. The amino acid sequence of the invertase (as shown in Appendix CCI) was used as the query sequence in the searches.

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

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

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

C.4.1. The source of the Enzyme Processing Aid

The dossier concerns an invertase gene from *Aspergillus niger* expressed in *Trichoderma reesei*.

Name of the enzyme protein: Invertase

Production strain: *Trichoderma reesei* AR-996

C.4.2. **Donor**

Name of the Donor: *Aspergillus niger*

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

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

- The allergenic potential of enzymes was studied by (Bindslev-Jensen et al. 2006) and reported in the publication: "*Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry*". The investigation conducted involved enzymes produced by wild-type and genetically modified strains as well as wild-type enzymes and Protein Engineered variants. To add on, the investigation comprised 400 patients with a diagnosed allergy to inhalation allergens, food allergens, bee or wasp. The conclusion from the study was that ingestion of food enzymes in general is not likely to be a concern regarding food allergy.
- In the past, the AMFEP Working Group on Consumer Allergy Risk from Enzyme Residues in Food performed an in-depth analysis of the allergenicity of enzyme food products (Daurvin et al. 1998). The overall conclusion is that exposure to enzyme proteins by ingestion, as opposed to exposure by inhalation, are not potent allergens and that sensitization to ingested enzymes is rare.
- Enzymes when used as digestive (Abad et al. 2010) aids are ingested daily, over many years, at much higher amounts when compared to enzymes present in food (up to 1 million times more).

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

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

- The majority of proteins are not food allergens and based on previous experience, the enzyme industry is not aware of any enzyme proteins used in food that are homologous to known food allergens[19].
- Only a small amount of the food enzyme is used during food processing, which leads to very small amount of enzyme protein present in the final food. A high concentration generally equals a higher risk of sensitization, whereas a low level in final food equals a lower risk (Goodman et al. 2008).
- For cases where the proteins are denatured which is the case for this enzyme due to the food process conditions, the tertiary conformation of the enzyme molecule is destroyed. These types of alterations to the conformation in general, are associated with a decrease in the antigenic reactivity in humans. In the clear majority of investigated human cases, denatured proteins are much less immunogenic than the corresponding native proteins (Valenta and Kraft 2002; Valenta 2002; Takai et al. 2000; Nakazawa et al. 2005; Kikuchi et al. 2006).
- To add on, residual enzyme still present in the final food will be subjected to digestion in the gastro-intestinal system, that reduces further the risk of enzyme allergenicity. While stability to digestion is considered as a potential risk factor of allergenicity, it is believed that small protein fragments resulting from digestion are less likely to be allergenic (FAO/WHO 2001; Goodman et al. 2008).

Lastly, enzymes have a long history of safe use in food processing, with no indication of adverse effects or reactions. Moreover, a wide variety of enzyme classes (and structures) are naturally present in food. This is in contrast with most known food allergens, which are naturally present in a narrow range of foods.

Allergenicity Search

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

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

The alignment methods used in the searches are:

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

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

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

Results of Allergenicity searches:

Type of Search	Outcome
Alignment of the Invertase mature amino acid sequence to sequences in allergen online databases	No matches having greater than 35 % identity were found from the AllergenOnline database using the full-length search
Alignment of sliding 80-amino acid window of the query protein to known protein allergens	No matches having greater that 35 % identity were found from the AllergenOnline database using the 80-mer sliding window search
Search of 8 amino acid exact matches	The search modus used a number of 679 8mers as query. None of them delivered a hit in the database.

According to the results obtained from the alignments and the most recent scientific recommendations on the interpretation of such data, the invertase enzyme is of no concern.

To summarize, the bioinformatics approach to estimate potential allergenicity and cross-reactivity based on relatedness to known allergens and considering the most recent scientific recommendations on the interpretation of such data leads us to conclude that the **invertase** produced by *Trichoderma reesei* AR-996 is of no concern.

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 to produce invertase is the fungus *Trichoderma reesei*.

Scientific name:

Genus: *Trichoderma reesei*

Species: *Trichoderma reesei*

Taxonomy:

Trichoderma reesei is a hyper cellulolytic fungus which was found on deteriorating military fabrics such as tents and clothing. This original 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 in synonym with *Trichoderma longibrachiatum* (Bissett 1991). Later however, evidence appeared that the two species were not identical, (Goodfellow et al. 2005; Meyer et al. 1992) and it was decided to go back to the *Trichoderma reesei* name. For a summary of *T. reesei´s* taxonomy, see Druzhinina et al. (2005).

Taxonomic studies have shown that the industrially relevant *Trichoderma reesei* species 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)).

Synonyms[20]**:** *Trichoderma longibrachiatum*

D.2. Information on the Pathogenicity and Toxicity of the Source Microorganism

Trichoderma reesei strains are non-pathogenic for healthy humans and animals (Nevalainen et al. 1994; Frisvad et al. 2018). *Trichoderma reesei* is not present on the list of pathogens in the EU (Directive Council Directive 2000/54/EC) and is present in major culture collections worldwide.

***Trichoderma reesei* is globally regarded as a safe microorganism:**

- In the USA, *Trichoderma reesei* is not listed as a Class 2 or higher Containment Agent under the National Institute of Health (NIH, 1998) Guidelines for Recombinant DNA Molecules. Data submitted in Generally Recognized as Safe (GRAS) petitions to the Food and Drug Administration (FDA) for numerous enzyme preparations from *T. reesei* for human and animal consumption demonstrate that the enzymes are nontoxic. The Environmental Protection Institute (EPA) completed a risk assessment on *T. reesei* in 2011 resulting in a Proposed Rule in 2012, concluding that it is appropriate to consider *T. reesei* as a recipient microorganism eligible for exemptions from full reporting requirements[21], if this fungus was to be used in submerged standard industrial fermentation for enzyme production. To add on in March 2020, the EPA issued a final rule on **Microorganisms; General Exemptions From Reporting Requirements; Revisions to Recipient Organisms Eligible for Tier I and Tier II Exemptions**[22] as part of the 40 Code of Federal Regulations Part 725 where *Trichoderma reesei* is classified as a Tier I organism.

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

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

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

- The Public Health Agency of Canada (PHAC) assigned the species *T. reesei* to 'Risk Group 1' (low individual risk, low community risk) for both humans and terrestrial animals[23]. *T. reesei* is not considered to be an aquatic animal pathogen, nor a regulated plant pest in Canada by the Canadian Food Inspection Agency (CFIA).
- Health Canada's List of Permitted Food Enzymes sets out permitted source organisms (including *T. reesei* A83 (previously named *T. longibrachiatum* A83) and *T. reesei* QM9414 (previously named *T. longibrachiatum* QM9414) for enzymes that may be used as food additives. As per section B.01.045, Part B, of the Food and Drug Regulations, food additives are required to meet specifications set out in these regulations and where no specifications are set out in Part B, the additive must meet specifications set out in the most recent edition of the Food Chemicals Codex (FCC). For food enzymes, the FCC specifications for enzyme preparations would apply.
- FSANZ has approved applications from AB Enzymes in which our *Trichoderma strain* platform has been utilized as the production strain – application A1162 (Lipase from *Trichoderma reesei)*, A1153 (Xylanase from *Trichoderma reeesei*), A1183 (Glucose oxidase from *Trichoderma reesei*).

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

The genus *Trichoderma* contains filamentous fungi which are frequently found on decaying wood and in soil. Industrial *T. reesei* strains have a long history of safe use and several of the *Trichoderma* based products have been approved for food and feed applications[24]. *T. reesei* is listed as a "Risk Group 1" organism according to German TRBA classification (Federal Institute for Occupational Safety and Health, www.baua.de) and as "Biosafety Level 1" organism by the American Type Culture Collection (www.atcc.org). *Trichoderma reesei* strains are non-pathogenic for healthy humans and animals. The DNA based identification methods have shown that *T. reesei* is taxonomically different from the other *Trichoderma* species of the section *Longibrachiatum* (Druzhinina et al. 2005).

[23] https://www.canada.ca/en/environment-climate-change/services/evaluating-existing-substances/screening-assessment-trichoderma-reesei.html#toc6

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

Some species belonging to *Trichoderma* genus can secrete several types of antibiotics in laboratory cultures. However, strains of *T. reesei* used in industrial applications are proven to be devoid of antibiotic activities (Coenen et al. 1995; Hjortkjaer et al. 1986). The absence of antibiotic activities, according to the specifications recommended by JECFA (FAO/WHO 2006), was also confirmed for AR-996. The analyzed data are presented in Appendix CCI.

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

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

- Results of the toxicological studies provided in the narrative (Appendix #4);
- Safety and history of use of the production organism *Trichoderma reesei;*
- Mycotoxin testing results presented in the composition report (Appendix CCI).

With the use of safe strain lineage, we have substantiated the safety of the AR-996 *Trichoderma reesei* production strain via three toxicological studies on the *Trichoderma reesei* AR-700 production strain to demonstrate non-toxigenicity of the strain lineage. The toxicological studies conducted include, a reverse mutation assay using bacteria, a Micronucleus Assay in Bone Marrow Cells of the Rat and a 90-day repeated dose oral toxicity study in Wister rats. All three toxicological studies showed negative findings demonstrating the AR-700 production strain to be non-mutagenic, to not induce structural and/or numerical chromosomal damage, and to not cause toxigenic effects on the Wister rats tested in the 90-day oral toxicity study. For more details on the results of the toxicological studies conducted on the production strain, please refer to Appendix CCI.

To add on, as mentioned in in this section of the dossier, the *Trichoderma reesei* as a production organism has a long history of use for the production of industrial food enzymes. Food enzymes, including those derived from recombinant *Trichoderma reesei* strains, have been evaluated by JECFA and many countries which regulate the use of food enzymes such as France, Denmark, Australia, and Canada, apart from the USA. Also, AB Enzymes has used *Trichoderma reesei* strains for food enzyme production for many years without any safety problems. Lastly, we have demonstrated the low presence of the mycotoxins produced

by the *Trichoderma reesei* microorganism. The composition report (Appendix CCI) provided demonstrates mycotoxin values below the levels of quantification (LoQs) for the enzyme concentrate batches tested.

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

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. Detailed information is provided in the Appendix CCI.

E.2. Host/recipient organism

The recipient strain used in the genetic modification for the construction of the production strain is *Trichoderma reesei* AR-407. The recipient strain was created from parental strain AR-256. This is further described in Appendix CCI.

Therefore, the recipient can be described as followed:

Kingdom: Fungi

Division: *Ascomycota*

Class:	*Sordariomycetes*
Order:	*Hypocreales*
Family:	*Hypocreaceae*
Genus:	*Trichoderma*
Species:	*Trichoderma reesei*
Strain:	*Trichoderma reesei* AR-407

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

E.3. Donor

The *Trichoderma reesei* host strain is genetically modified with invertase gene deriving from *Aspergillus niger*.

E.4. Genetic modification

T. reesei AR-996 was constructed for invertase production. The production strain differs from the recipient strain AR-407 in its high invertase production capacity due to expression of the invertase gene from the expression cassette integrated into the genome of recipient strain. Besides the heterologous invertase production, no other changes in phenotype are made. The genetic modification is described in Appendix CCI.

E.5. Stability of the Transformed Genetic Sequence

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 production microorganism's safety evaluation. Southern blot analyses were performed to the genome of the *T. reesei* production strain AR-996. Further information is described in Appendix CCI.

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

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

Commercial food enzyme preparations are generally used following the *Quantum Satis* (QS) principle, i.e., at a level not higher than the necessary dosage to achieve the desired enzymatic reaction, according to Good Manufacturing Practice. The amount of enzyme activity added to the raw material by the individual food manufacturer must be determined case by case, based on the desired effect and process conditions. Therefore, the enzyme manufacturer can only issue a recommended enzyme dosage range. Such a dosage range is the starting point for the individual food producer to fine-tune this process and determine the amount of enzyme that will provide the desired effect and nothing more. Consequently, from a technological point of view, there are no 'normal or maximal use levels' and **invertase** is used according to the QS principle. A food producer who would add much higher doses than the needed ones would experience untenable costs as well as negative technological consequences.

The dosage of a food enzyme depends on the activity of the enzyme protein present in the final food enzyme preparation (i.e., the formulated food enzyme). However, the activity units do not indicate the amount of food enzyme added.

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

The table below shows the range of recommended use levels for each application where the **invertase** from *Trichoderma reesei* AR-996 may be used:

Food Application	Raw material (RM)	Suggested recommended use levels (mg TOS/kg RM)
Short chain-fructooligosaccharides (sc-FOS) production	Sucrose	7
Sugar reduction	Various foodstuff	14

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

The Invertase is strictly used as a processing aid and is removed during the production of FOS by inactivation and dedicated removal steps, as such it is expected that there will be negligible residues in the FOS ingredient, However, to ensure that all requirements are met under the FSANZ guidelines, we have calculated a theoretical maximum potential of the residual enzyme level in foods (choosing the most likely highest type of food consumed) using the Budget Method.

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*

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

Applications		**Raw Material**	**Maximum recommended use level (mg TOS/kg RM)**	**Final food (FF)**	**Ratio RM/FF ***	**Maximal level in final food (mg TOS/kg food)**
Liquid foods	Sugar reduction	Beverages	14	For example, fruit and vegetable juices including citrus juices	1.3	**18.2**
Solid foods	Sugar reduction	Solid foodstuff	14	For example, purees, jams confectionary	1	**14**
Applications		**Raw Material**	**Maximum recommended use level (mg TOS/kg RM)**	**Final food (FF)**	**Ratio RM/FF ***	**Maximal level in final food (mg TOS/kg food)**
Liquid foods	FOS	Non-milk beverages	7	beverages (FOS syrup)	1	**7**
Solid foods	FOS	Solid foodstuff	7	For example, purees, jams confectionary	1.2	**8.4**

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

- *We assume the highest consumption would be fruit and vegetable beverages: For fruit juices, we assume that a RM/FF ratio of 1.3 kg fruit per L of fruit juice will be used (typically 0.75-0.9 l juice is produced per kg of fruit thus the range for RM/FF will be 1.1-1.3 kg fruit per L of fruit juice).*

- *We assume the highest consumption would be fruit products: For fruit purees and jams, we assume a RR/FF of 1 (1 kg of fruits / kg of puree).*
- *For FOS production, we assume that FOS would be utilized as either a FOS syrup or powder in various solid foods and beverages.*

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

The Total TMDI will consequently be calculated as follows:

TMDI in food (mg TOS/kg body weight/day)	**TDMI in beverage (mg TOS/kg body weight/day)**	**Total TMDI (mg TOS/kg body weight/day)**
14 x 0.0125 = 0.175	18.2 x 0.025 = 0.455	**0.630**

The Total TMDI is based on conservative assumptions and represents a highly exaggerated value as per the following

- It is assumed that ALL producers of the above-mentioned foodstuffs (and beverages) use specific invertase from *Trichoderma reesei* AR-996;
- It is assumed that ALL producers apply the HIGHEST use level per application;
- For the calculation of the TMDI's in food, only the above foodstuffs were selected containing the highest theoretical amount of TOS. Therefore, foodstuffs containing lower theoretical amounts were not included;
- It is assumed that the final food containing the calculated theoretical amount of TOS is consumed DAILY over the course of a lifetime;
- Assumptions regarding food and beverage intake of the general population are overestimates of the actual average levels (Douglass et al. 1997).

Dietary exposure is calculated on the basis of the total organic solids (TOS) content in the final (commercial) enzyme preparation and is usually expressed in milligrams or micrograms of TOS per kilogram of body weight per day. TOS encompasses the enzyme component and other organic material

originating from the production organism and the manufacturing process, while excluding intentionally added formulation ingredients.

Margin of Exposure (MoE)

The Margin of Exposure (MoE)[25] for human consumption can be calculated through the division of the NOAEL (no-observed adverse effect) value by the TMDI (Total Theoretical Maximal Daily Intake). Total TMDI of the food invertase is calculated to be 0.560 mg TOS/kg body weight/day.

As a result, the MoE is:

MoE =1000/0.630 =**1587**.

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

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

Conclusion:

To conclude, the use of the food enzyme invertase from *Aspergillus oryzae* AR-996 in the production of food is safe. Considering the high safety value determined by the MoE, even when calculating using means of overestimation of intake via the Budget method, there is no need to restrict the use of the enzyme in food. The suggested dosage for food manufacturers is not a restrictive value and could be higher or lower depending on usage within cGMPs.

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 invertase 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.

V. List of Appendices

Section 3.1

1.1 Customer Support Letter
1.2 Formal Request for Confidential Information (CCI)
1.3a Statutory Declaration Australia
1.3b Statutory Declaration New Zealand
1.4 Checklist 3.1 and 3.3

Section 3.2

1. Product Data Sheet – ROHALASE® FOS-UP
2. Quality Certificates
3. Manufacturing Flow-chart
4. Safe Strain Lineage

Appendix CCI – Treated as confidential information

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