AB Enzymes GmbH – Feldbergstrasse 78 , D-64293 Darmstadt

A Petition to Amend the Australia New Zealand Food Standards Code with an Invertase Enzyme Preparation produced by *Trichoderma reesei*

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

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

[2] See footnote 17

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.

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.

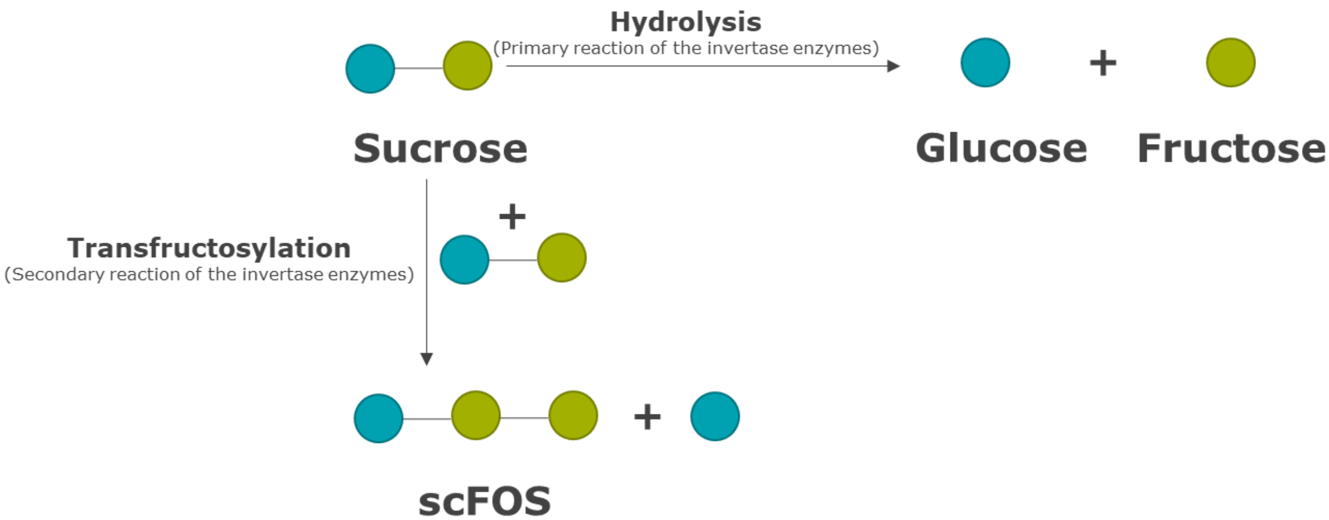

Figure 1: Hydrolysis and Transfructosylation reaction of the Invertase

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

[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

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;

[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 fijensis*

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 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)**.

Publication bibliography

Augustin, L. S. A.; Kendall, C. W. C.; Jenkins, D. J. A.; Willett, W. C.; Astrup, A.; Barclay, A. W. et al. (2015): Glycemic index, glycemic load and glycemic response: An International Scientific Consensus Summit from the International Carbohydrate Quality Consortium (ICQC). In *Nutrition, metabolism, and cardiovascular diseases : NMCD* 25 (9), pp. 795–815. DOI: 10.1016/j.numecd.2015.05.005.

Choukade, R.; Kango, N. (2021): Production, properties, and applications of fructosyltransferase: a current appraisal. In *Critical reviews in biotechnology* 41 (1), pp. 1178–1193. Available online at https://doi.org/10.1080/07388551.2021.1922352.

Cywińska-Antonik, M.; Chen, Z.; Groele, B.; Marszałek, K. (2023): Application of Emerging Techniques in Reduction of the Sugar Content of Fruit Juice: Current Challenges and Future Perspectives. In *Foods* 12 (6), Article 1181. Available online at https://doi.org/10.3390/foods12061181.

Deliza, Rosires; Lima, Mayara F.; Ares, Gastón (2021): Rethinking sugar reduction in processed foods. In *Current Opinion in Food Science* 40, pp. 58–66. DOI: 10.1016/j.cofs.2021.01.010.

Evans, Charlotte Elizabeth Louise (2017): Sugars and health: a review of current evidence and future policy. In *The Proceedings of the Nutrition Society* 76 (3), pp. 400–407. DOI: 10.1017/S0029665116002846.

Flores-Maltos, Dulce A.; Mussatto, Solange I.; Contreras-Esquivel, Juan C.; Rodríguez-Herrera, Raúl; Teixeira, José A.; Aguilar, Cristóbal N. (2016): Biotechnological production and application of fructooligosaccharides. In *Critical reviews in biotechnology* 36 (2), pp. 259–267. DOI: 10.3109/07388551.2014.953443.

Gomes, A.; Bourbon, A. I.; Peixoto, A. R.; Silva, A. S.; Tasso, A.; Almeida, C. et al. (2023): Chapter 9 - Strategies for the reduction of sugar in food products. Book Food Structure Engineering and Design for Improved Nutrition, Health and Well-Being: Academic Press (Food Structure Engineering and Design for Improved Nutrition, Health and Well-Being). Available online at https://www.sciencedirect.com/science/article/abs/pii/B9780323855136000086?via%3Dihub.

Ibrahim, O. (2021): Technological Aspects of Fructo-Oligosaccharides (FOS), Production Processes, Physiological Properties, Applications and Health Benefits. In *J Food Chem Nanotechnol* 7 (2), pp. 41–46. Available online at https://doi.org/10.17756/jfcn.2021-111.

Martins, Gonçalo N.; Ureta, Maria Micaela; Tymczyszyn, E. Elizabeth; Castilho, Paula C.; Gomez-Zavaglia, Andrea (2019): Technological Aspects of the Production of Fructo and Galacto-Oligosaccharides. Enzymatic Synthesis and Hydrolysis. In *Frontiers in nutrition* 6, p. 78. DOI: 10.3389/fnut.2019.00078.

Mutanda, T.; Mokoena, M. P.; Olaniran, A. O.; Wilhelmi, B. S.; Whiteley, C. G. (2014): Microbial enzymatic production and applications of short-chain fructooligosaccharides and inulooligosaccharides: recent advances and current perspectives. In *Journal of industrial microbiology & biotechnology* 41 (6), pp. 893–906. DOI: 10.1007/s10295-014-1452-1.

Nobre, C.; Cerqueira, M. A.; Rodrigues, L. R.; Vicente, A. A.; Teixeira, J. A. (2015): Chapter 19 - Production and Extraction of Polysaccharides and Oligosaccharides and Their Use as New Food Additives: Elsevier (Industrial Biorefineries & White Biotechnology). Available online at dx.doi.org/10.1016/B978-0-444-63453-5.00021-5.

Nobre, Clarisse; Simões, Lívia S.; Gonçalves, Daniela A.; Berni, Paulo; Teixeira, José A. (2022): Fructooligosaccharides production and the health benefits of prebiotics. In : Current Developments in Biotechnology and Bioengineering: Elsevier, pp. 109–138.

Ojwach, Jeff; Adetunji, Adegoke Isiaka; Mutanda, Taurai; Mukaratirwa, Samson (2022): Oligosaccharides production from coprophilous fungi: An emerging functional food with potential health-promoting properties. In *Biotechnology Reports* 33, e00702. DOI: 10.1016/j.btre.2022.e00702.

Prada, Marília; Saraiva, Magda; Garrido, Margarida V.; Sério, Ana; Teixeira, Ana; Lopes, Diniz et al. (2022): Perceived Associations between Excessive Sugar Intake and Health Conditions. In *Nutrients* 14 (3). DOI: 10.3390/nu14030640.

Respondek, F.; Hilpipre, C.; Chauveau, P.; Cazaubiel, M.; Gendre, D.; Maudet, C.; Wagner, A. (2014): Digestive tolerance and postprandial glycaemic and insulinaemic responses after consumption of dairy desserts containing maltitol and fructo-oligosaccharides in adults. In *European journal of clinical nutrition* 68 (5), pp. 575–580. DOI: 10.1038/ejcn.2014.30.

Rippe, James M.; Angelopoulos, Theodore J. (2016): Relationship between Added Sugars Consumption and Chronic Disease Risk Factors: Current Understanding. In *Nutrients* 8 (11). DOI: 10.3390/nu8110697.

Sánchez-Martínez, M. J.; Soto-Jover, S.; Antolinos, V.; Martínez-Hernández, G. B.; López-Gómez, A. (2020): Manufacturing of Short-Chain Fructooligosaccharides: from Laboratory to Industrial Scale. In *Food Engineering Reviews* 12, pp. 149–172. Available online at https://link.springer.com/article/10.1007/s12393-020-09209-0.

Wienberg, F.; Hövels, M.; Deppenmeier, U. (2022): High-yield production and purification of prebiotic inulin-type fructooligosaccharides. In *ABM Express* 12, Article 144. Available online at https://amb-express.springeropen.com/articles/10.1186/s13568-022-01485-9.

Xu, W.; Ni, D.; Zhang, W.; Guang, C.; Zhang, T.; Mu, W. (2019): Recent advances in Levansucrase and Inulosucrase: evolution, characteristics, and application. In *Crit Rev Food Sci Nutr.* 59 (22), pp. 3630–3647. Available online at https://doi.org/10.1080/10408398.2018.1506421.