APPLICATION FOR THE AUTHORIZATION OF FRUCTOSYLTRANSFERASE FROM *ASPERGILLUS ORYZAE* IN AUSTRALIA AND NEW ZEALAND

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Application for the Authorization of Fructosyltransferase from *Aspergillus oryzae* in Australia and New Zealand

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Application for the Authorization of Fructosyltransferase from *Aspergillus oryzae* in Australia and New Zealand

A. GENERAL REQUIREMENTS

In accordance with Section 3.1.1 (General Requirements) of the Food Standards Australia New Zealand (FSANZ) *Application Handbook* (FSANZ, 2019), the following general information must be provided:

1. Format of the application;
2. Applicant details;
3. Purpose of the application;
4. Justification for the application;
5. Information to support the application;
6. Assessment procedure;
7. Confidential commercial information;
8. Other Confidential information;
9. Exclusive capturable commercial benefit;
10. International and other national standards;
11. Statutory declaration; and
12. Checklist.

Each point is addressed in the following subsections.

A.1 Format of the Application

1. Information Related to Changes to Standard 1.3.3 – Processing Aids

This application for an amendment to Standard 1.3.3 and related Schedules is prepared pursuant to Section 3.3.2 (Processing Aids) of the FSANZ *Application Handbook* (FSANZ, 2019), which requires the following structured format to assess an application for a new processing aid:

A. General information on the application;

B. Technical information on the processing aid;

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

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

E. Information related to the dietary exposure to the processing aid.

The application is presented in this format. At the start of each section (A to D) the information that must be addressed therein is specified in more detail. Additionally, an executive summary for the application is provided as a separate electronic document to this application. The application has been prepared in English and submitted electronically, as required by the FSANZ *Application Handbook* (FSANZ, 2019).

A.2 Applicant Details

Tate & Lyle Solutions USA LLC is a manufacturer of food ingredients. The contact details of

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Address: 5450 Prairie Stone Pkwy,
Hoffmann Estates, IL, 60192
USA

Telephone:
Email:

Contact name:
Address:

Telephone:
Email:

A.3 Purpose of the Application

Tate & Lyle Solutions USA LLC ("Tate & Lyle") is submitting this application to FSANZ to request an amendment to Standard 1.3.3 of the *Food Standards Code* ("the Code") to include fructosyltransferase (EC 2.4.1.9) derived from a non-genetically modified strain of *Aspergillus oryzae* as a processing aid for the production of short-chain fructooligosaccharides (sc-FOS) from sucrose. A related enzyme, β-fructofuranosidase (EC 3.2.1.26), derived from *Aspergillus niger*, *Aspergillus fijiensis*, and *Trichoderma reesei* is currently approved for use in Australia and New Zealand as per Schedule 18. The enzyme utilizes both invertase/β-fructofuranosidase and fructosyltransferase activities to produce sc-FOS. The primary activity of Tate & Lyle's enzyme is fructosyltransferase activity.

A.4 Justification for the Application

A.4.1 Technological Function for the Processing Aid

The fructosyltransferase enzyme catalyses fructosyltransferase reactions in sucrose to produce sc-FOS and glucose. The enzyme also catalyses the hydrolysis of *beta*-fructosyl bonds in sucrose, releasing fructose and glucose. This is a minor activity of the enzyme. Thus, the technical effect of the fructosyltransferase enzyme is to produce sc-FOS from sucrose (granulated sugar) as a raw material.

A.4.2 Costs and Benefits for Industry, Consumers, and Government Associated with Use of the Food Additive

The enzymatic production of sc-FOS is noted to be an effective and economic method, allowing for large-scale production (Flores-Malton *et al.*, 2016). These technological effects cannot be sufficiently met with the use of other enzymes or by other means, and therefore, there exists a technological need for fructosyltransferase in food production. The inclusion of fructosyltransferase derived from *A. oryzae* as a processing aid will provide food manufacturers and food producers with alternative means of sc-FOS production using a non-pathogenic and non-toxigenic source organism.

A.5 Information to Support the Application

Information on the identity of the fructosyltransferase enzyme from *A. oryzae*, the manufacturing process, chemical and physical properties, and product specifications are provided in Section B of the application. Information to support the safety of the processing aid is presented in Section C in accordance with the requirements listed in Section 3.3.2 (Processing Aids) of the FSANZ *Application Handbook* (FSANZ, 2019). The safety of fructosyltransferase was established based on history of use, , anticipated digestion of the enzyme to a significant degree, and lack of toxigenic or pathogenic concerns for the enzyme. The safety is further corroborated through toxicological studies on similar enzymes, lack of enzyme carry-over to the final product, and information related to potential allergenicity of the enzyme.

A.6 Assessment Procedure

Tate & Lyle considers the most appropriate assessment procedure for assessing the application to include *A. oryzae* as a source organism for fructosyltransferase to be the General Procedure. It is anticipated that this application will involve amending Standard 1.3.3 of the Code to include fructosyltransferase derived from *A. oryzae* as a processing aid.

A.7 Confidential Commercial Information (CCI)

Tate & Lyle requests that certain proprietary information be considered confidential commercial information (CCI). General summaries of the proprietary data are provided within this application. Data requested to be treated as CCI have been removed and are summarised in Table A.7-1 below along with verifiable justification and are provided within Appendix A in full. All information presented in Appendix A is requested to remain confidential as it holds significant commercial value to the company, including proprietary details on the manufacture of the food enzyme, the amino acid sequence, and enzyme activity.

Table A.7-1 Information Requested to be Considered as Confidential

Section(s)	Description	Justification
A.2	Applicant and contact person contact details.	The contact details for the person responsible for the dossier are sensitive and should be treated as confidential. Public disclosure of this information is not required for the safety assessment of the processing aid.
Appendix A	Information on molecular mass, amino acid sequence, and optimum temperature and pH conditions. Raw materials and processing aids used in the production process, detailed description of the production process, and process controls. Data on compositional analysis. Taxonomic identity of the production strain. Study demonstrating the absence of viable cells of the production strain and information on genetic stability.	The specific details of the described information and data are considered confidential and proprietary and are of significant commercial value to the applicant. Non-confidential summaries of the information are provided throughout the dossier. The publication of these data would provide a competitive advantage to other manufacturers.
Appendix A-1 Appendix A-2 Appendix A-3 Appendix A-4 Appendix A-5	Enzyme Assay Method Certificates of Analysis Taxonomic Identification Report Certificate of Deposit Absence of Viable Cells of the Strain	These appendices contain sensitive information considered highly confidential and of significant commercial value to the applicant. Public disclosure of this information is not required for the safety assessment of the processing aid and the source organism.

A.8 Other Confidential Information

No other confidential information is contained within this application.

A.9 Exclusive Capturable Commercial Benefit (ECCB)

It is not anticipated that this application would confer Exclusive Capturable Commercial Benefit (ECCB) in accordance with Section 8 of the FSANZ Act as there are other companies that would likely benefit from the approval of fructosyltransferase derived from *A. oryzae*.

A.10 International and Other National Standards

Fructosyltransferase from *A. oryzae* complies with the internationally recognized specifications for enzyme preparations as established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (JECFA, 2006) and the Food Chemicals Codex (FCC, 2024). The fructosyltransferase food enzyme is also referred to as β-fructofuranosidase or invertase as it contains both fructosyltransferase and

fructofuranosidase activities. Β-fructofuranosidase (EC 3.2.1.26) from *Aspergillus niger*, *Aspergillus fijiensis*, and *Trichoderma reesei* is permitted for use in Australia and New Zealand as a processing aid (FSANZ, 2023a). Invertase from *Saccharomyces cerevisiae* intended for general use in food at cGMP was notified as Generally Recognized as Safe (GRAS) to the U.S. FDA and received “no questions” (U.S. FDA, 2002a). Invertase from the same source is also approved for use in the production of confectionary products in France (JORF, 2006). In Canada, invertase from *Saccharomyces* species and *A. fijiensis* are approved from use in confectionary and bakery products, and for the production of sc-FOS, respectively (Health Canada, 2024). In Japan, the β-fructofuranosidase food enzyme is classified as a food additive and is permitted for use under the List of Existing Food Additives, Item No. 33: Invertase (MHLW, 2014a, cited in: FSANZ, [2023]), with no official specification established under the 8th Japanese Specifications and Standards of Food Additives. In the 9th Japanese Specifications and Standards of Food Additives, enzyme was reclassified as “fructosyl transferase,” which catalyses the transfer of fructosyl groups of sugars and is obtained from *Aspergillus genus* and *Penicillium roqueforti* (MHLW, 2014b, cited in: FSANZ, [2023]). In China, fructosyltransferase from *A. oryzae*, β-fructofuranosidase from *A. fijiensis*, and invertase from *S. cerevisiae* are approved for use in food processing (NHFPC/USDA, 2021; 2022).

A.11 Statutory Declaration

Signed Statutory Declarations for Australia and New Zealand are provided in Appendix B.

A.12 Checklist

Completed checklists relating to the information required for submission with this application based on the relevant guidelines in the FSANZ *Application Handbook* are provided in Appendix C.

B. TECHNICAL INFORMATION ON THE PROCESSING AID

In accordance with Section 3.3.1 (Food Additives) of the FSANZ *Application Handbook* (FSANZ, 2019), the following technical information must be provided:

1. Information on the type of processing aid;
2. Information on the identity of the processing aid;
3. Information on the chemical and physical properties of the processing aid;
4. Manufacturing process;
5. Specification for identity and purity; and
6. Analytical method for detection.

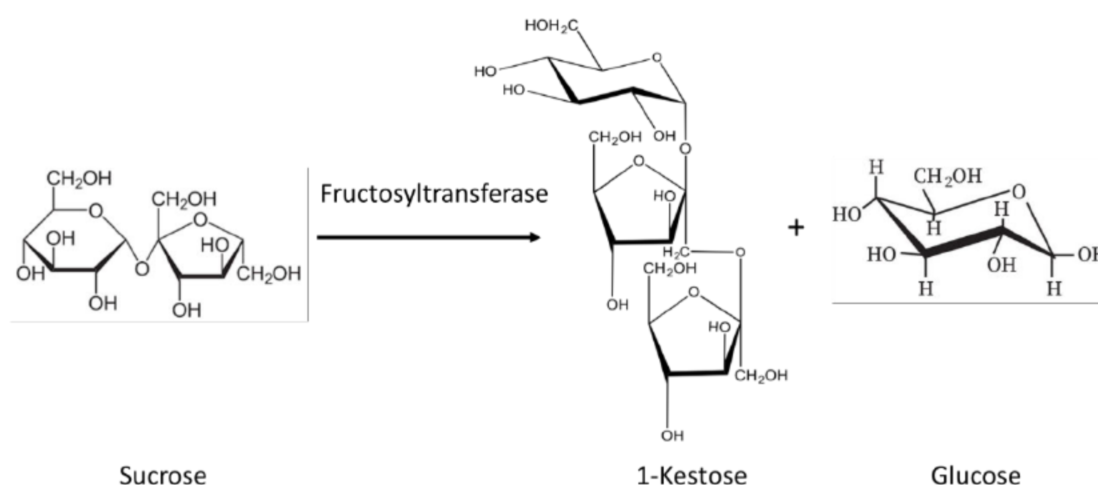
Each point is addressed in the following subsections.

B.1 Information on the Type of Processing Aid

Tate & Lyle's fructosyltransferase produced from *A. oryzae* strain QHT-101 catalyses the transfer of fructose units to sucrose or other fructans, resulting in the production of sc-FOS and glucose (see Figure B.1-1). When the substrate is sucrose, it is first hydrolysed to glucose and fructose. The enzyme reaction does not require any co-factors.

The fructosyltransferase food enzyme derived from *A. oryzae* strain QHT-101 is manufactured as a liquid concentrate that is then immobilized onto food-grade resin. Based on the foregoing, fructosyltransferase from *A. oryzae* would be categorised as an enzyme of microbial origin under Schedule 18. The maximum use level of the enzyme preparation is 412 mg total organic solids (TOS)/kg in sucrose as a raw material (see Section D.1).

Figure B.1-1 Enzymatic Reaction of Fructosyltransferase Catalysing the Fructosyl Transfer from Sucrose to Produce 1-Kestose and Glucose



B.2 Information on the Identity of Fructosyltransferase from *Aspergillus oryzae*

Information on the identity of fructosyltransferase from non-genetically modified *A. oryzae* strain QHT-101, including the source organism, common and systematic name of the food enzyme, synonyms, enzyme classification and chemical abstracts registry numbers, are presented below in Table B.2-1. Tate & Lyle's fructosyltransferase from *A. oryzae* strain QHT-101 has not been protein-engineered and is not modified by a post-translational process.

Table B.2-1 Systemic Name and Registry Numbers of the Food Enzyme

Source (Donor Organism):	*Aspergillus oryzae* strain QHT-101
Common/Accepted Name:	Fructosyltransferase
Other Names:	Sucrose 1-fructosyltransferase; sucrose:2,1-β-D-fructan 1-β-D-fructosyltransferase
Enzyme Classification Number of Enzyme Commission (EC) of the International Union of Biochemistry and Molecular Biology (IUBMB):	2.4.1.9
Chemical/Systematic Name:	Sucrose2→1)-β-D-fructan 1-β-D-fructosyltransferase
Chemical Abstracts Service (CAS) Number:	9030-16-4

B.3 Information on the Chemical and Physical Properties of Fructosyltransferase from *Aspergillus oryzae*

B.3.1 Molecular Mass

The fructosyltransferase food enzyme produced from *A. oryzae* strain QHT-101 is manufactured as a liquid concentrate that is then immobilized onto food-grade resin. The protein pattern of a liquid concentrate of the fructosyltransferase food enzyme (Batch Nos. 5, 6, and 7) prior to immobilisation was evaluated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE). The approximate molecular weight of the fructosyltransferase enzyme is provided in Appendix A.

B.3.2 Amino Acid Sequence

The full amino acid sequence of the fructosyltransferase from *A. oryzae* strain QHT-101 and a determination of the genes encoding the enzyme are provided in Appendix A.

B.3.3 Properties of the Food Enzyme

B.3.3.1 Information on the Principal Enzymatic Activity

The fructosyltransferase food enzyme catalyses the transfer of fructose units to sucrose or other fructans, resulting in the production of sc-FOS such as 1-kestose, nystose, fructosyl-nystose, in addition to glucose (see Figure B.1-1). When the substrate is sucrose, it is first hydrolysed to glucose and fructose. This hydrolytic (fructofuranosidase) activity is a minor activity of the enzyme that is essential for the generation of sc-FOS. The enzyme reaction does not require any co-factors. The fructosyltransferase food enzyme does not have any other subsidiary or side activities.

The fructosyltransferase food enzyme is specifically characterised by its fructosyltransferase activity. Tate & Lyle has established a specification limit for the fructosyltransferase activity, and analytical data supporting the enzyme activity levels of the food enzyme are provided in Section B.5.2.1. Fructosyltransferase activity is measured using the assay method described in a GB/T standard (see Appendix A-1). Fructosyltransferase activity is reported on an enzyme unit per gram basis. One fructosyltransferase unit is defined as the amount of the enzyme that will produce 1 µmole of 1-kestose per minute under the conditions of the assay.

B.3.3.2 Activity of the Food Enzyme Under the Conditions of Intended Use

The optimal pH and temperature conditions for the fructosyltransferase food enzyme produced by *A. oryzae* strain QHT-101 were determined experimentally (see Appendix A).

B.3.3.3 Subsidiary and/or Side Activities

The principal activity of the fructosyltransferase food enzyme is to catalyse the transfer of fructose to sucrose or other fructans, resulting in the production of sc-FOS and glucose. The enzyme reaction does not require any co-factors.

The fructosyltransferase food enzyme does not contain any other subsidiary or side activities (*i.e.*, protease activity, as measured in 3 batches of the immobilised food enzyme using GB 1886.174-2016).

B.3.3.4 Fate of the Enzyme During Food Processing and its Behaviour in the Food Matrix

The fructosyltransferase food enzyme catalyses the transfer of fructose units to sucrose or other fructans, resulting in the production of sc-FOS and glucose. The enzyme, therefore, performs its catalytic function directly on the fructose units of sucrose molecules (*i.e*., granulated white sugar). The enzyme products, sc-FOS, are carbohydrate components that are naturally occurring in fruits and vegetables consumed by humans and have not been associated with any safety concerns. The fructosyltransferase is immobilised, and thus, the presence of residual enzyme during sc-FOS production is not expected. Nonetheless, as demonstrated in Appendix A, any residual fructosyltransferase following its addition during food processing may be heat-denatured at temperatures over 60°C. Thus, residues of the enzyme in finished food products will likely be inactivated under normal food processing conditions that consist of high temperatures such as those that occur during sterilisation. The food application described in Section D.1 include processing steps in which heating over 60°C occurs. In addition, the processing steps for sc-FOS production involve decolourisation and treatment with ion-exchange resins, which would further reduce any residues of the enzyme. As demonstrated by the analytical data summarised in Section D.2, no protein (including enzyme TOS) was detected during the production of sc-FOS following filtration, purification, and in the final sc-FOS product at a limit of quantification of 0.024%. Therefore, on the basis that the enzyme is denatured and/or removed during sc-FOS production, the enzyme would have no technological effect on the final foods as consumed.

B.4 Manufacturing Process

B.4.1 Manufacturing Process of Fructosyltransferase

B.4.1.1 Identity of Raw Materials and Processing Aids

The list of raw materials, processing aids, and purification aids used in the production of the fructosyltransferase food enzyme is provided in Appendix A. All processing aids used in the manufacture of the food enzyme are of high quality and acceptable for use in the manufacture of the food enzyme.

B.4.1.2 Description of the Key Steps Involved in the Production Process

The fructosyltransferase food enzyme is manufactured in accordance with current Good Manufacturing Practice (cGMP) and Food Safety System Certification (FSSC) 22000. The food enzyme is produced using food-grade materials and using quality-controlled fermentation and purification/recovery processes. The production strain has been deposited in a recognised culture collection. A detailed overview of the manufacturing process for the fructosyltransferase food enzyme is presented in Appendix A.

The production of the immobilised fructosyltransferase follows 3 steps: culture of the production strain, preparation of fructosyltransferase, and immobilisation of the enzyme.

<u>Culture of the Production Strain</u>

In the first step, the production strain is fermented in the cultured medium under the appropriate fermentation conditions. Next, the production strain is streaked onto the same plate slant culture medium and incubated. The production strain is then inoculated into a nutrient broth culture medium consisting of a nutrient broth and incubated.

Preparation of Fructosyltransferase

During culture seeding, the production strain is inoculated in sterilised fermentation media and incubated. In the main fermentation step, the production strain from the seed culture step is inoculated in the same fermentation media. At the end of fermentation, the fermentation broth is centrifuged to separate the cells of the production organism from the broth, washed with water, then the cells are collected and mechanically pulverised, then centrifuged to obtain a supernatant containing the fructosyltransferase enzyme.

Immobilisation of the Enzyme

In order to obtain the immobilised enzyme, the food-grade ion-exchange resin is conditioned with the appropriate substances and added to the supernatant containing the enzyme for adsorption.[1] Following enzyme adsorption, the resin is washed then stored in food-grade packaging drums.

B.4.1.3 Process Controls and Quality Assurance Procedures

A Hazard Analysis and Critical Control Point (HACCP) plan is in place for the manufacture of the fructosyltransferase food enzyme produced with *A. oryzae* strain QHT-101. The critical control points (CCPs) have been identified and measures have been set in place for the prevention of the identified hazards. The HACCP plan consists of series of processes which are identified as a CCP in order to ensure adherence with the established manufacturing process and to produce a high-quality and consistent product. These include measures to ensure that potential residual amounts of the production strain are not transferred to the food enzyme. Furthermore, each manufactured batch of the food enzyme is analysed for conformity to the specifications for the food enzyme set out in Section B.5.1. Batches that do not meet the specifications for the food enzyme are not released for further processing. An outline of the quality control steps is presented in Appendix A.

B.5 Specification for Identity and Purity of Fructosyltransferase from *Aspergillus oryzae*

B.5.1 Product Specifications for Fructosyltransferase from *Aspergillus oryzae* Strain QHT-101

The specifications for the fructosyltransferase from *Aspergillus oryzae* strain QHT-101 are provided in Table B.5.1-1 below. The specifications are compliant with the purity requirements for enzyme preparations established by FCC (2024) and JECFA (2006). All methods of analysis are internationally recognised or validated methods. The enzyme activity for fructosyltransferase is measured using the fructosyltransferase enzyme activity assay described in a GB/T standard, which specifically measures the amount of enzyme required to convert sucrose to 1-kestose (see Section B.3.3). The assay method is provided in Appendix A-1.

Table B.5.1-1 Specifications for the Fructosyltransferase Food Enzyme from *Aspergillus oryzae* Strain QHT-101

Specification Parameter	Specification	Method of Analysis
Escherichia coli	Negative in 25 g	ISO 7251:2005
Total coliforms	NMT 30/g	ISO 4832:2006
Salmonella sp.	Negative in 25 g	ISO 6579-1:2017
Antimicrobial activity	Negative	GB 4789.43-2016

[1] No cross-linking agents are used during the immobilisation process to the ion-exchange resin.

Table B.5.1-1 Specifications for the Fructosyltransferase Food Enzyme from *Aspergillus oryzae* Strain QHT-101

Specification Parameter	Specification	Method of Analysis
Lead	NMT 5 mg/kg	BS EN ISO 17294-2 (2016)
Arsenic	NMT 3 mg/kg	BS EN ISO 17294-2 (2016)

ISO = International Organization for Standardization; NLT = not less than; NMT = not more than.

B.5.2 Product Analysis

B.5.2.1 Batch Analyses

The fructosyltransferase food enzyme is manufactured as a liquid concentrate that is then immobilised onto food-grade resin. Analytical data on 3 non-consecutive production-scale batches (Batch Nos. 1, 2, and 3) of the immobilised fructosyltransferase food enzyme are presented in Table B.5.2.1-1 below and demonstrate that the manufacturing process produces a consistent product that meets the product specifications defined in Section B.5.1. The levels of lead were below the limit of quantitation (LOQ) of 0.05 mg/kg, and all microbiological contaminants were below the limit of determination (LOD) and/or in compliance with the specifications for the food enzyme. The levels of arsenic were detected at very low amounts and complied with the defined product specification of not more than (NMT) 3 mg/kg. In addition, all batches were negative for antimicrobial activity. The certificates of analysis and identities of batch numbers are provided in Appendix A-2.

Table B.5.2.1-1 Batch Analyses for 3 Non-consecutive Production-scale Batches of Fructosyltransferase Food Enzyme from *Aspergillus oryzae* Strain QHT-101

Specification Parameter	Specification Limit	Manufacturing Batch No.			Method of Analysis
		1	2	3	
Microbiological Specifications					
Escherichia coli	Negative in 25 g	Negative	Negative	Negative	ISO 7251:2005
Total coliforms	NMT 30 CFU/g	<10	<10	<10	ISO 4832:2006
Salmonella sp.	Negative in 25 g	Negative	Negative	Negative	ISO 6579-1:2017
Antimicrobial activity	Negative	Negative	Negative	Negative	GB 4789.43-2016
Heavy Metals					
Lead	NMT 5 mg/kg	≤0.05[a]	≤0.05[a]	≤0.05[a]	BS EN ISO 17294-2 (2016)
Arsenic	NMT 3 mg/kg	0.0358	0.0393	0.0471	BS EN ISO 17294-2 (2016)

CFU = colony-forming units; ISO = International Organization for Standardization; NLT = not less than; NMT = not more than.
[a] LOD = 0.05 mg/kg.

Further detailed compositional data on the same 3 non-consecutive production-scale batches of the fructosyltransferase food enzyme are provided in Appendix A. The food enzyme is composed of ash, protein, and water. All methods of analysis are internationally recognised methods. The certificates of analysis are provided in Appendix A-2.

B.5.2.2 Mycotoxins and Secondary Metabolite Analyses

Mycotoxin analyses for 3 non-consecutive production-scale batches (Batch Nos. 1, 2, and 3) of the fructosyltransferase food enzyme demonstrate the absence of aflatoxins (B1, B2, G1, and G2), sterigmatocystin, zearalenone, ochratoxin A, T-2 toxin, HT-2 toxin, deoxynivalenol, and fumonisins B1 and B2 in the final immobilized food enzyme. All mycotoxins were determined to be below the LOD in all 3 batches tested (see Table B.5.2.2-1). These mycotoxin parameters do not form part of the specifications for the food enzyme but were included as part of the batch analyses to confirm absence of potential mycotoxin production. The certificates of analysis are provided in Appendix A-2.

Table B.5.2.2-1 Mycotoxins Analysis of 3 Non-consecutive Production-scale Batches of Fructosyltransferase Food Enzyme from *Aspergillus oryzae* Strain QHT-101

Specification Parameter	LOQ	Manufacturing Batch No.			Method of Analysis
		1	2	3	
Aflatoxins (B1, B2, G1, G2)	8 µg/kg	ND	ND	ND	ISO 16050:2003
Sterigmatocystin	10 µg/kg	ND	ND	ND	Internal Method
Zearalenone	10 µg/kg	ND	ND	ND	Internal Method
Ochratoxin A	0.1 µg/kg	ND	ND	ND	DIN EN 14132-2009
T-2 Toxin	10 µg/kg	ND	ND	ND	Internal Method
HT-2 Toxin	10 µg/kg	ND	ND	ND	Internal Method
Fumonisin B1	20 µg/kg	ND	ND	ND	GB 5009.240-2016
Fumonisin B2	10 µg/kg	ND	ND	ND	GB 5009.240-2016
Deoxynivalenol	20 µg/kg	ND	ND	ND	Internal Method

ISO = International Organization for Standardization; LOQ = limit of quantitation; ND = not detected.

B.6 Analytical Method for Detection

According to Section 3.3.2 (Processing Aids) of the FSANZ *Application Handbook* (FSANZ, 2019), an analytical method for detection is not required in the case of an enzymatic processing aid, and this section is therefore not relevant to fructosyltransferase derived from *A. oryzae* strain QHT-101.

C. INFORMATION RELATED TO THE SAFETY OF AN ENZYME PROCESSING AID

In accordance with Parts C and D of Section 3.3.2 (Processing Aids) of the FSANZ *Application Handbook* (FSANZ, 2019), the following safety information must be provided for enzyme processing aids:

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

2. Information on the potential toxicity of the enzyme processing aid;

3. Information on the potential allergenicity of the enzyme processing aid;

4. Safety assessment reports prepared by international agencies or other national government agencies, if applicable;

5. Information on the source microorganism;

6. Information on the potential pathogenicity and toxicity of the source microorganism; and

7. Information on the genetic stability of the source organism.

Each point is addressed in the following subsections.

C.1 General Information on the Use of the Enzyme as a Food Processing Aid in Other Countries

Fructosyltransferase, also known as β-fructofuranosidase or invertase, is authorised for usage in various jurisdictions. Details regarding the authorised food enzymes, the enzyme source, and permitted uses are summarised in Table C.1-1 below.

Table C.1-1 Similar Authorised Food Enzymes

Jurisdiction	Evaluating/ Authoritative Body	Enzyme Name	Enzyme Source	Permitted Uses	Reference
Australia and New Zealand	FSANZ	β-Fructofuranosidase (EC 3.2.1.26)	*Aspergillus niger*	Processing aid	FSANZ (2024)
			Aspergillus fijiensis		
			Trichoderma reesei		
Canada	Health Canada	Invertase	*Aspergillus fijiensis*	Sucrose used in the production of fructooligosaccharides	Health Canada (2024)
			Saccharomyces sp.	Unstandardised soft-centred and liquid centred confectionary and bakery products	
China	Ministry of Health Commission	β-Fructofuranosidase	*Aspergillus fijiensis*	In food processing (uses not specified)	NHFPC/USDA (2022)
		Sucrose 1-fructosyltransferase	*Aspergillus oryzae*	In food processing (uses not specified)	NHFPC/USDA (2021)
		Invertase	*Saccharomyces cerevisiae*	In food processing (uses not specified)	
France	L'Agence francaise de sécurité sanitaire des aliments	Invertase	*Saccharomyces cerevisiae*	Processing aid in confectionary products	JORF (2006)
Japan	MHLW	Fructosyltransferase	*Aspergillus genus; Penicillium roqueforti*	Food additive (uses not specified)	MHLW (2014b)
		Invertase	*Aspergillus aculeatus, Aspergillus awamori, Aspergillus niger,* and *Aspergillus japonicus; Kluyveromyces lactis* and *Saccharomyces cerevisiae;* and *bacteria* (limited to species of the genera *Arthrobacter* and *Bacillus).*		
U.S.	FDA	Invertase	*Saccharomyces cerevisiae*	Use in foods in general at levels in accordance with current Good Manufacturing Practice	U.S FDA (2002a)

Table C.1-1 Similar Authorised Food Enzymes

Jurisdiction	Evaluating/ Authoritative Body	Enzyme Name	Enzyme Source	Permitted Uses	Reference

EC = Enzyme Commission; FDA = United States Food and Drug Administration; FSANZ = Food Standards Australia New Zealand; JORF = Journal officiel de la République française; MHLW = Ministry of Health, Labour and Welfare; NHFPC = National Health and Family Planning Commission of the People's Republic of China; U.S. = United States.

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

C.2.1 Toxicity of Fructosyltransferase from *Aspergillus Oryzae*

The potential toxicity of the fructosyltransferase enzyme was addressed according to the requirements laid out in Section 3.3.2 (C.2) of the *Application Handbook*, including information on the history of consumption of the food enzyme, information on the similarity of the amino acid sequence to known protein toxins, and information on the stability of the enzyme in digestion models. The history of consumption of fructosyltransferase can be established based on use of the enzyme in other countries (See Section C.1). The fructosyltransferase food enzyme is also referred to as β-fructofuranosidase or invertase as it contains both fructosyltransferase and fructofuranosidase activities. As such, approvals for all aforementioned enzymes are relevant to use of fructosyltransferase with fructosyltransferase activity. The additional requirements to address the potential toxicity of fructosyltransferase are discussed in detail herein.

The digestion of the fructosyltransferase enzyme from *A. oryzae* strain QHT-101 was predicted *in silico* using Expasy's PeptideCutter.[2] The potential cleavage sites in the amino acid sequence of the food enzyme were analysed using pepsin at pH 1.3 and >2 to simulate gastric conditions and using trypsin to simulate intestinal conditions. The number of cleavages estimated in the 525-residue sequence using pepsin at pH 1.3, pH >2, and for trypsin were 96, 147, and 27, respectively. Thus, it can be concluded that any residual fructosyltransferase enzyme consumed would be extensively digested in the stomach, and further degraded in the small intestine.

Additionally, a toxin sequence alignment query of the amino acid sequence of the fructosyltransferase enzyme from *A. oryzae* strain QHT-101 was conducted using the BLAST program maintained by the NCBI. The sequence was searched against downloaded protein sequences obtained from a curated database of 8,572 venom proteins and toxins[3] maintained by UniProt. Sequence alignments were considered significant using the criteria reported by the European Food Safety Authority (EFSA) (EFSA, 2021) for identifying genes of concern (*i.e.,* ≥80% identity and ≥70% coverage). No significant similarity to any toxins was identified from the sequence homology searches, indicating that the fructosyltransferase enzyme from *A. oryzae* strain QHT-101 is not expected to pose any toxigenic or pathogenic concerns.

Toxicological studies were not conducted using the fructosyltransferase enzyme from *A. oryzae* strain QHT-101 on the basis that the food enzyme is immobilised, and therefore, dietary exposure to the food enzyme is not expected. As discussed in Section D.2 below, experimental data was generated to demonstrate that transfer of enzyme TOS does not occur throughout the production process of sc-FOS. The levels of protein in the starting material, in-process/intermediate products, and final sc-FOS product were not significantly changed (*i.e.,* no protein was detected at a limit of quantification of 0.024%), indicating that no significant amounts of enzyme TOS is transferred into the final sc-FOS product.

[2] https://web.expasy.org/peptide_cutter/.

[3] UniProtKB/Swiss-Prot; available at: https://www.uniprot.org/ using search terms: ((cc_tissue_specificity:venom) OR (keyword:KW-0800)) AND (reviewed:true).

An acute oral toxicity study and a battery of genotoxicity tests were conducted with a powdered form of the fructosyltransferase concentrate from *A. oryzae* strain QHT-101 according to GB 15193-2014. Although not considered pivotal to the safety assessment of the immoblised fructosyltransferase, brief summaries of the unpublished data have been included in Appendix A for completeness of the data package. In summary, the median lethal dose in mice was concluded to be >20 g/kg body weight/day, and the enzyme was not mutagenic or clastogenic in an *in vivo* micronucleus test, in vitro mammalian chromosome aberration test, and a bacterial reverse mutation test. Overall, the safety of the immobilised fructosyltransferase enzyme from *A. oryzae* strain QHT-101 was established based on the history of consumption of the enzyme, the significant digestion of the enzyme predicted using *in silico* gastric models, and lack of toxigenic or pathogenic concerns for the enzyme.

C.2.2 Published Toxicological Studies on Fructosyltransferase Enzymes

Toxicological evaluations of fructosyltransferase/*beta*-fructofuranosidase enzymes from *Aspergillus* species have been published in the literature or reviewed as part of an application to FSANZ. The safety of Shin Nihon Chemical Co., Ltd.'s β-fructofuranosidase from *Aspergillus brunneoviolaceus* was evaluated in a series of published toxicology studies, including an evaluation of the mutagenicity and genotoxicity potential using the *in vitro* bacterial reverse mutation and mammalian chromosomal aberration assays, as well as systemic toxicity in a 90-day oral subchronic toxicity study (Vo *et al*., 2021). The activity of the *beta*-fructofuranosidase concentrate from *A. brunneoviolaceus* was 158,800 U/mL or 120 mg total organic solids (TOS)/mL. FSANZ reviewed a bacterial reverse mutation test, an *in vitro* micronucleus assay, and a 90-day oral toxicity study employing a β-fructofuranosidase food enzyme from *A. fijiensis* strain ATCC 20611 (FSANZ, [2023]). The test substance was identified as a spray-dried powder concentrate with 92% TOS content. No additional information on the enzyme properties or composition was available for either source. It was noted by FSANZ that *A. fijiensis* is phylogenetically related to *A. brunneoviolaceus,* leading to the classification of the two species as equivalent.

The studies for each enzyme are summarized in detail in the sections below as corroborating evidence of safety for Tate & Lyle's fructosyltransferase enzyme. The results of the genotoxicity studies indicate that the enzymes do not pose mutagenic or clastogenic concerns. The NOAELs from the 90-day oral toxicity studies using *beta*-fructofuranosidase from *A. brunneoviolaceus* and β-fructofuranosidase from *A. fijiensis* strain ATCC 20611 were concluded to be 1,200 mg TOS/kg body weight/day and 920 mg TOS/kg body weight/day, respectively, the highest doses tested in each study.

C.2.2.1 beta-Fructofuranosidase from Aspergillus brunneoviolaceus

Bacterial Reverse Mutation Test

The mutagenic potential of Shin Nihon's ultra-filtered *beta*-fructofuranosidase concentrate was assessed through a bacterial reverse mutation assay, both with and without metabolic activation, using strains *Salmonella typhimurium* TA100, TA98, TA1535, TA1537, and *Escherichia coli* WP2uvrA (Vo *et al.*, 2021). This evaluation adhered to the Organisation for Economic Co-operation and Development (OECD) Test Guideline 471 (*Bacterial reverse mutation Test*; OECD, 1997) and was carried out under the principles of Good Laboratory Practice (GLP) (OECD, 1998a). Initially, a concentration range-finding study identified the doses for the main examination. Subsequently, the *beta*-fructofuranosidase concentrate was examined in triplicate at doses of 375, 750, 1,500, 3,000, 6,000, and 12,000 µg TOS/plate, both with and without metabolic activation, across all bacterial strains. Distilled water served as the negative control under all testing conditions, and appropriate positive controls were used. The analysis revealed a significant increase in revertant colony numbers in the positive controls, exceeding twice the number observed in the negative controls. Conversely, the groups exposed to the *beta*-fructofuranosidase concentrate exhibited revertant colony increases of less than 2-fold relative to the negative controls, irrespective of metabolic activation presence. Based on the findings, it was concluded that the *beta*-fructofuranosidase concentrate lacks mutagenic activity.

In Vitro Chromosome Aberration Test

An examination of the clastogenic effects of *beta*-fructofuranosidase concentrate was evaluated by Vo *et al.* (2021) utilizing an *in vitro* chromosomal aberration assay, conducted according to OECD Test Guideline 473 (*In vitro mammalian chromosomal aberration test*; OECD, 2014) and GLP standards (OECD, 1998a). This evaluation utilized human lymphocytes obtained from healthy, non-smoker donors. The enzyme's concentration effects were tested in a short-term assay in duplicate across a range from 750 to 12,000 µg TOS/mL with metabolic activation and from 1,500 to 12,000 µg TOS/mL without metabolic activation. Additionally, in a continuous exposure assay, the concentrate's effects were assessed in duplicate at doses ranging from 93.8 to 3,000 µg TOS/mL over a 24-hour period without metabolic activation. Distilled water was utilized as both the solvent for the test substance and the negative control. Mitomycin C was applied in both the absence of metabolic activation for short-term assays and the continuous assay, whereas Cyclophosphamide was employed in the presence of metabolic activation for the short-term assay. To assess chromosomal aberrations, microscopic analyses were performed on groups treated with 3,000, 6,000, and 12,000 µg TOS/mL in the short-term assays, and with 375, 750, and 1,500 µg TOS/mL in the continuous assay, based on the calculated mitotic index for each treatment group. Comparative analysis of chromosomal aberrations and the presence of polyploid cells across all tested concentrations and assays revealed no significant differences from the negative controls. It was therefore concluded that the *beta*-fructofuranosidase enzyme exhibits no clastogenic effects.

90-day Subchronic Toxicity Study

A comprehensive 90-day oral toxicity assessment of *beta*-fructofuranosidase was performed in accordance with OECD Test Guideline 408 (*Repeated dose 90-day oral toxicity study in rodents*; OECD, 1998b) and GLP principles (OECD, 1998a) as detailed by Vo *et al.* (2021). Following the outcomes of a preliminary 2-week repeated dose oral toxicity evaluation, Crl:CD (SD) [SPF] rats (n=10/sex/group) received varying doses of the β-fructofuranosidase solution *via* oral gavage: 0 (control with distilled water), 133 (low dose), 400 (medium dose), and 1,200 (high dose) mg TOS/kg body weight/day for a duration of 90 days. The rats had free access to food pellets and water throughout the study. Observations for clinical signs were recorded daily pre- and post-administration. Body weight and food intake were measured weekly for all subjects. During the final week, assessments including urinalysis, eye exams, and a functional observation battery (FOB) were performed. The study concluded with the collection of blood for haematology and biochemistry analyses and a comprehensive pathological evaluation, which included weighing organs, along with macroscopic and microscopic tissue examinations. The oestrous cycle was also monitored from Day 85 to necropsy by analysing vaginal smears under a microscope. A male from the medium dose group died on Day 33, with necropsy attributing death to pulmonary congestive edema likely caused by gavage mishap; The finding was deemed incidental and not related to the test substance. Haematological analysis indicated significantly increased haematocrit, haemoglobin levels, and red blood cell counts in mid-dose female rats compared to controls. Conversely, alanine aminotransferase and sodium levels were notably lower in high-dose males, while chloride levels were elevated in low-dose males. In females receiving the medium dose, significant rises in the albumin/γ-globulin ratio and γ-globulin levels were observed. These haematology and urinalysis outcomes were not deemed associated with the treatment as they lacked dose-dependency and cross-sex consistency. The significant changes in male organ weights, both absolute and relative, was interpreted as unrelated to treatment due to the absence of dose-correlation and histopathological evidence. Females showed no notable variations in organ weights across any group. Macroscopic findings were determined to be spontaneous and incidental, reinforced by a lack of histopathological correlation and the lack of dose-response correlation. The absence of dose-dependent effects and related histopathological alterations established the NOAEL for *beta*-fructofuranosidase concentrate at 1,200 mg TOS/kg body weight/day, the maximum dose administered.

C.2.2.2 beta-Fructofuranosidase from Aspergillus fijiensis Strain ATCC 20611

The genotoxicity studies and 90-day oral toxicity study on β-fructofuranosidase from *A. fijiensis* strain ATCC 20611 were reviewed by FSANZ as part of application A1212, seeking to revise Schedule 18 of the Food Standards Code to include *A. fijiensis* as a source organism (FSANZ, 2021, 2022, 2024). The agency concluded that based on the NOAEL of 920 mg TOS/kg body weight/day and maximum calculated intake of 0.52 mg TOS/kg bw/day for the enzyme in adults, the resulting margin of exposure (MOE) of more than 1,700 does not pose any safety concerns.

Bacterial Reverse Mutation Test

A mutagenic assessment of β-fructofuranosidase from *A. fijiensis* strain ATCC 20611 was undertaken using the bacterial reverse mutation assay (BoZo Research Center Inc., 2014a, cited in: FSANZ, [2023]). The study was conducted in compliance with the principles of GLP and the guidelines set forth by the Japanese Ministry of Health and Welfare Ordinance No. 21 and 114 (MHLW, 1997, 2008, cited in: FSANZ, [2023]) and OECD Test Guideline 471 (OECD, 1997). Concentrations of 156 to 5,000 µg/plate of β-fructofuranosidase were tested on *S. typhimurium* strains TA98, TA100, TA1535, and TA1537, as well as *E. coli* strain WP2uvrA, both with and without metabolic activation. Water served as both the negative and vehicle control for all bacterial strains, and the appropriate positive controls were used. The findings from this study led to the conclusion that the β-fructofuranosidase enzyme did not exhibit mutagenicity.

In Vitro Mammalian Cell Micronucleus Test

The assessment of potential clastogenic and aneugenic effects of β-fructofuranosidase extracted from the *A. fijiensis* strain ATCC 20611 was conducted on cultured mouse lymphoma cells (L5178Y tk+/- – clone 3.7.2c) (BoZo Research Center Inc., 2014b, cited in: FSANZ, [2023]). The study was conducted in compliance with the principles of GLP (OECD, 1998a) and the Japanese Ministry of Health and Welfare Ordinance No. 21 and 114 (MHLW, 1997, 2008, cited in: FSANZ, [2023]), and performed in accordance with OECD Test Guideline 487 (*In Vitro mammalian cell micronucleus test*; OECD, 2010) and the Japanese Ministry of Health, Labour, and Welfare Guidance for the Evaluation of Genetic Toxicity Studies of Pharmaceuticals (MHLW, 2012). The mouse lymphoma cells underwent exposure to β-fructofuranosidase across concentrations ranging from 2,500 to 4,500 µg/mL and 2,000 to 5,000 µg/mL, without and with metabolic activation, respectively, for a duration of 3 hours. Additionally, in a 24-hour exposure scenario, the cells were treated with dosages from 500 to 2,500 µg/mL in the absence of metabolic activation. The detection of micronuclei was carried out through microscopic examination of a minimum of 1,000 cells per slide, conducted in duplicate for each dosage level and in a blinded fashion. The findings led to the conclusion that β-fructofuranosidase does not exhibit clastogenic or aneugenic effects within the context of this study.

90-day Subchronic Toxicity Test

A comprehensive 90-day oral toxicity evaluation of β-fructofuranosidase was conducted according to OECD Test Guideline 408 (OECD, 1998b) and in compliance with the principles of GLP established by the OECD (1998a) and the Japanese Ministry of Health and Welfare Ordinance No. 21 and 114 (MHLW, 1997, 2008). Sprague-Dawley SPF rats (10/sex/group) were administered β-fructofuranosidase at levels of 0, 100 (low-dose), 300 (mid-dose), or 1,000 (high-dose) mg/kg body weight/day *via* gavage for 91 days. Distilled water was used as the vehicle control. Clinical observations were conducted daily, and detailed clinical observations were made weekly. FOB tests were conducted in Week 12. Body weight was measured before the start of the study and on Day 1, 4 and 7 and twice weekly thereafter. Food consumption was assessed prior to dosing and on Day 1 and 7 and once weekly thereafter. Ophthalmology and urinalysis were performed prior to the exposure period and in Week 13. Water intake was assessed at the time of urinalysis. Blood samples were collected on the final day of the study period for analysis of haematology, biochemistry, and clinical chemistry parameters. Gross pathology, measurement of organ weights and histopathological examinations were conducted on all animals at study termination. No mortality was reported throughout the study. No treatment related effects were observed in body weight, food consumption, FOB, urinalysis, haematology, blood chemistry or ophthalmology examinations in any of the test animals. No macroscopic or histopathological changes related to treatment were observed at necropsy. The NOAEL for β-fructofuranosidase in both male and female rats was determined to be 1,000 mg/kg body weight/day, the highest dose tested (equivalent to 920 mg TOS/kg body weight/day).

C.3 Information on the Potential Allergenicity of the Enzyme Processing Aid

Although Pariza and Foster (1983) have indicated that there have been no confirmed reports of allergies as a result of enzymes used in food processing, the potential allergenicity of the fructosyltransferase from *Aspergillus oryzae* strain QHT-101 was assessed by investigating sequence homology between the enzyme and known food allergens using the methodology described by FAO/WHO (2001), Codex Alimentarius (2003, 2009), and EFSA GMO Panel (2017).

To investigate the possibility of allergenic cross-reactivity of fructosyltransferase from *A. oryzae* strain QHT-101, an *in silico* assessment of sequence homology of the amino acid sequence of the food enzyme against known allergens was conducted using the methodology described by FAO/WHO (2001),[4] Codex Alimentarius (2003, 2009),[5] and EFSA GMO Panel (2017).[6] In accordance with these guidelines, the following sequence homology searches were conducted against the AllergenOnline database (Version 22)[7]:

1. A "sliding window" of 80-amino acid sequences (*e.g.,* segments 1-80, 2-81, 3-82, *etc*.) derived from each full-length amino acid sequence of the food enzyme (search parameters: minimum identity cut-off = 35%);

2. An 8-amino acid exact match; and

3. The full-length amino acid sequence (search parameters: FASTA36; E-value cut-off = 1 and maximum alignments of 20).

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[4] https://www.who.int/publications/m/item/evaluation-of-allergenicity-of-genetically-modified-foodsreport-of-a-joint-fao-who-expert-consultation-on-allergenicity-of-foods-derived-from-biotechnology.
[5] https://www.fao.org/fao-who-codexalimentarius/sh-proxy/es/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FStandards%252FCXG%2B46-2003%252FCXG_046e.pdf; https://www.fao.org/3/a1554e/a1554e00.htm.
[6] https://www.efsa.europa.eu/en/efsajournal/pub/4862.
[7] http://www.allergenonline.org.

The 80-amino acid alignment searches were conducted using the FASTA search algorithm hosted by the AllergenOnline Allergenic Protein Sequence Search program[8] with default search parameters, and results were reviewed for any protein sharing ≥35% identity over a "sliding window" of 80 amino acids. Matches greater than 35% over 80 amino acids are suggestive of potential cross-reactivity with putative allergens; similarly, sequences sharing ≥35% identity over a window of 80 amino acids are common for many highly conserved proteins (Abdelmoteleb *et al.,* 2021). Although Abdelmoteleb *et al.* (2021) found that all major and minor allergens were identified using an E-value threshold of 1×10^{-7}, the degree of false positives obtained suggests that this threshold may not be sufficiently selective for use in risk assessment. Although EFSA CEP Panel (2023) suggests that 1×10^{-7} may be a suitable E-value threshold, this has not yet garnered scientific consensus, and thus was not used as a threshold in the present assessment of allergenicity. Nonetheless, E-values were considered in the weight of evidence when assessing the relevance of sequences sharing ≥35% identity over at least 1 sliding window of 80 amino acids such that E-values $>1 \times 10^{-7}$ over the full-length sequence are considered unlikely to pose a risk of allergenicity. It is further noted that the FAO/WHO (2001), Codex Alimentarius (2003, 2009), and EFSA GMO Panel (2017) guidelines recommend searches with the 80-amino acid sliding window and 8-amino acid exact matches, and do not include a recommendation to conduct searches with the full-length sequence. However, given that structural similarity between folded proteins may be evaluated using the full-length amino acid sequences, as noted by Aalberse (2000), Goodman *et al.* (2008), and Abdelmoteleb *et al.* (2021), any matches identified from the 80-amino acid sliding window or the 8-amino acid exact match searches were further evaluated for the degree of significance and identity over the full sequence and only considered further in the present allergenicity assessment if the match also had a percent identity >50% over the full sequence.

From the sliding window searches of 80 amino acids with fructosyltransferase, 2 matches were identified that shared ≥35% identity over 80 amino acids with a known allergenic protein from AllergenOnline. The results of the search are summarised in Table C.3-1 below. The "best" identity match was 47.50% over 53 to 56 possible 80-amino acid sliding windows from the fructosyltransferase amino acid sequence. Similarly, the full-length sequence search identified matches that shared between 26% and 28% identity to the same 2 allergenic proteins; the E-values for these identified matches ranged between 1.1 and 7.0×10^{-21}. No 8-amino acid exact matches were identified. Considering that the highest identity match in the 80-amino acid sliding window search was 47.50% and the full-length search was well below the 50% threshold, these results are not suggestive of allergenic cross-reactivity of the fructosyltransferase to known allergens. These findings are consistent with those identified by EFSA for β-fructofuranosidase from *Saccharomyces cerevisiae* strain INV, wherein EFSA concluded that the risk for allergenicity of this enzyme is low (EFSA CEP Panel, 2023[9]).

Table C.3-1 AllergenOnline Search Results for Fructosyltransferase from *Aspergillus oryzae* Strain QHT-101

Protein	Species	80-amino Acid Sliding Window Search		Full-length Sequence Search			Accession Number
		Best % Identity	# Hits >35% Identity	E-value	% Identity	Length	
Minor allergen β-fructofuranosidase	*Solanum lycopersicum* (*Lycopersicon esculentum*)	47.50%	56/446	7.0×10^{-21}	26.40%	552	18542115
Minor allergen β-fructofuranosidase	*Solanum lycopersicum* (*Lycopersicon esculentum*)	47.50%	53/446	1.1×10^{-21}	27.80%	425	18542113

[8] http://www.allergenonline.org/databasefasta.shtml.
[9] https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2023.7833.

The allergenicity of fructosyltransferase was further considered through a search of available scientific literature. Search terms included synonyms for allergenicity (allerg* or hypersensitiv* OR hypersensitiz* OR hypersensitis* or "*crossreacti*" or "*cross-reacti*") and common names characterising the food enzyme (*e.g.*, Fructosyltransferase or Fructosyl transferase or "9030-16-4" or "E.C. 2.4.1.9"). The search was conducted on 27 February 2024 using the following databases: AdisInsight: Trials, AGRICOLA, AGRIS, Allied & Complementary Medicine™, BIOSIS® Toxicology, BIOSIS Previews®, CAB ABSTRACTS, Embase®, Foodline®: SCIENCE, FSTA®, MEDLINE®, NTIS: National Technical Information Service, ToxFile®, and Toxicology Abstracts.

Upon review of the literature search results, no information was identified on the oral or respiratory sensitisation or elicitation reactions of fructosyltransferase from *A. oryzae* strain QHT-101. As noted above, β-fructofuranosidase from tomato (*S. lycopersicum*) has been reported to be a minor food allergen that is listed on the AllergenOnline database. However, only the natural variant (*i.e.,* not the recombinant variant expressed in *Escherichia coli*) elicited IgE-reactivity in the sera of patients sensitised to tomato (Westphal *et al.,* 2003). As such, the study authors reported that the IgE-reactivity likely depends on the glycan structure of the glycosylated protein. Although the sequence homology search of fructosyltransferase from *A. oryzae* strain QHT-101 revealed two matches with tomato β-fructofuranosidases sharing >35% identity over 80 amino acids, the degree of sequence homology over the full sequence was low (26.4% and 27.8%) and is therefore unlikely indicative of a true risk of cross-reactivity. β-fructofuranosidase was also reported to demonstrate IgE reactivity in individuals allergic to moulds (Horner *et al.,* 2008). However, several studies have shown that adults sensitised to mould *via* inhalation may be able to ingest the corresponding allergen without acquiring clinical symptoms of food allergy (Brisman, 2002; Poulsen, 2004; Armentia *et al.*, 2009). As such, the possibility of fructosyltransferase from *A. oryzae* strain QHT-101 to pose a risk of allergenicity to final consumers is low, considering that it is intended for use as a food enzyme in the production of sc-FOS, which, as described in Section D, undergoes several purification steps which effectively reduces the potential dietary exposure to the enzyme. In addition, De Canio *et al.* (2009) noted that fructosyltransferase from ryegrass (*Lolium perenne*) may be involved in polysensitisation in individuals suffering from seasonal allergies. However, the study authors noted that profilin, not fructosyltransferase, was the major cross-reactive allergen from pollen. Furthermore, allergic reactions to ryegrass pollen are limited to seasonal allergies elicited *via* inhalation of the allergenic component (De Canio *et al.,* 2009). No other relevant information was identified. There is no evidence from the available literature to indicate that ingestion of the fructosyltransferase produced by *A. oryzae* strain QHT-101 would be allergenic.

Based on the information, no evidence was identified that would suggest that the fructosyltransferase from *A. oryzae* strain QHT-101 would cross-react with known allergens. Although the risk of allergic reactions by dietary exposure to this food enzyme cannot be excluded, based on the intended conditions of its use in food processing, this risk is considered low. Furthermore, it should be noted that the enzyme would only be available as an immobilised enzyme, and as described in Section D, there is negligible carryover of enzyme total organic solids into the final product, thus, further limiting potential exposure to the enzyme. There is no evidence from the available scientific literature indicating allergenicity to fructosyltransferase in consumers. Furthermore, it is expected that the enzyme would be denatured if carried over into the final sc-FOS product under the conditions of food processing, and therefore would not have the potential to result in allergenicity in consumers of the final foods. Based on this information, no evidence exists that might indicate that the fructosyltransferase from *A. oryzae* strain QHT-101 would produce an allergenic response following its use in food processing.

C.4 Safety Assessment Reports Prepared by International Agencies or Other National Government Agencies

The fructosyltransferase food enzyme, also referred to as β-fructofuranosidase or invertase, derived from various sources is permitted for use in several jurisdictions. β-fructofuranosidase (EC 3.2.1.26)

from *A. niger*, *A. fijiensis*, and *T. reesei* is permitted for use in Australia and New Zealand as a processing aid (Schedule 18) (FSANZ, 2024). In the United States (U.S.), the GRAS status of an invertase enzyme from *Saccharomyces cerevisiae* intended for general use in food at cGMP was notified to the U.S. FDA and received "no questions" (U.S. FDA, 2002a). Invertase from the same source is also approved for use in the production of confectionary products in France (JORF, 2006). In Canada, invertase from *Saccharomyces* species and *A. fijiensis* are approved from use in confectionary and bakery products, and for the production of sc-FOS, respectively (Health Canada, 2024). In Japan, the β-fructofuranosidase food enzyme is currently classified as a food additive and is permitted for use under the List of Existing Food Additives, Item No. 33: Invertase (MHLW, 2014a, cited in: FSANZ, [2023]), with no official specification established under the 8th Japanese Specifications and Standards of Food Additives. The 9th Japanese Specifications and Standards of Food Additives, however, reclassified the enzyme as "fructosyl transferase," establishing it as an enzyme that transfers the fructosyl group of sugars and is obtained from the mould cultures, *Aspergillus genus* and *Penicillium roqueforti* (MHLW, 2014b, cited in: FSANZ, [2023]). In China, fructosyltransferase from *A. oryzae*, β-fructofuranosidase from *A. fijiensis*, and invertase from *S. cerevisiae* are approved for use in food processing (NHFPC/USDA, 2021; 2022).

In the European Union, one application for the authorisation of the β-fructofuranosidase food enzyme from *Saccharomyces cerevisiae* strain INV has been submitted to the European Commission by DSM Food Specialties B.V. (EFSA Q 2021-00695) and received a positive opinion by EFSA CEP Panel (2023).

C.5 Information on the Source Microorganism

The production strain from which the fructosyltransferase food enzyme is produced is a non-genetically modified strain of the filamentous fungus *A. oryzae*, designated as strain QHT-101. Strain QHT-101 was isolated from a food source and was selected as the production strain based on its capacity to produce high levels of fructosyltransferase activity, its cell viability, and its suitability for the industrial production of food enzymes, owing to its lack of pathogenicity and toxigenicity (*i.e.,* mycotoxin production).

C.5.1 Taxonomic Identity of the Production Strain

The taxonomic identification of the production strain was performed using genomic analyses of the internal transcribed spacer (ITS) rDNA and the *BenA* gene (*beta*-tubulin). In addition, identity of the production strain was evaluated *via* morphological analysis. The details of the methodologies and findings are provided in Appendix A, and the study report is provided in Appendix A-3. The results demonstrate that the production strain is taxonomically classified as *A. oryzae*. The certificate of deposit is provided in Appendix A-4. The taxonomic classification of this organism is presented in Figure C.5.1-1.

Figure C.5.1-1 Taxonomy of Source Organism

Kingdom: Fungi
- Phylum: Ascomycota
  - Class: Eurotiomycetes
    - Order: Eurotiales
      - Family: Aspergillaceae
        - Genus: *Aspergillus*
          - Species: *oryzae*
            - Strain: QHT-101

C.5.2 Details of Documented History of Use with Absence of Human Health Adverse Effects

A. oryzae is a filamentous fungus with a long history of safe use in food production and the production of enzymes used in food processing. Globally, the species represents one of the most important sources of enzymes for the food industry. Food enzymes produced by *A. oryzae* include amylases,

aminopeptidases, asparaginases, glucanases, glucose oxidases, laccases, lactases, lipases, phospholipase, pectinesterases, phytase, proteases, and xylanases (U.S. FDA, 2002a,b,c; Amfep, 2015; FSANZ, 2024; Health Canada, 2024). In the EU specifically, *A. oryzae* is currently listed as an authorised source of lactase and various other enzyme preparations in France (Article Annexe I C of *Arrêté du 19 octobre 2006 relatif à l'emploi d'auxiliaires technologiques dans la fabrication de certaines denrées alimentaires* [JORF, 2006]). *A. oryzae* is an approved source of lactase in Japan as indicated in Japan's *Specifications and Standards for Food Additives* (MHLW, 2021). According to JECFA, *A. oryzae* is considered a production organism that is safe for human consumption (FAO/WHO, 2007), and in 2020, JECFA concluded that *A. oryzae* is a safe source of food enzymes based on their review of a number of food enzyme preparations from this microorganism without any documented adverse or toxic effect (FAO/WHO, 2020). As such, *A. oryzae* has a history of safe use in food enzyme production on a global basis. Furthermore, the U.S. Environmental Protection Agency (EPA) recognises that "*the experience of safe commercial use of A. oryzae is extraordinarily well established*" (U.S. EPA, 1997). In addition to its uses in the production of food enzymes, *A. oryzae* has been used for centuries in Asia as a fermentation organism for the production of miso, soya sauce, and sake liquors (Bourdichon *et al.*, 2012).

C.6 Information on the Pathogenicity and Toxicity of the Source Microorganism

According to the guidelines developed by Pariza and Foster (1983), Pariza and Johnson (2001), and the International Food Biotechnology Council (IFBC, 1990), the primary consideration in evaluating the safety of an enzyme preparation derived from a microbial source is the safety of the production strain. The safety of a production strain is addressed primarily by evaluating its toxigenic potential; for filamentous fungi, such as *A. oryzae*, the oral toxins of concern are mycotoxins (small molecular weight organic molecules, usually less than 1,000 Da in size) (Pariza and Johnson, 2001). Additional considerations for evaluating the safety of a production strain include pathogenicity and antibiotic production. According to the established guidelines, the safety of the enzyme preparation itself also should be assessed in part *via* analytical testing to ensure absence of toxic constituents (*e.g.*, mycotoxins) and antibiotic activity. The potential toxigenicity and pathogenicity of *A. oryzae* and the impact on the manufactured fructosyltransferase food enzyme are discussed below.

C.6.1 Production Strain Pathogenicity and Toxigenicity

The production strain is *A. oryzae* strain QHT-101, a filamentous fungus. Filamentous fungi are known as potential producers of toxic secondary metabolites known as mycotoxins. A search of the scientific literature was performed to identify publications pertaining to the pathogenicity and toxigenicity potential of the production organism. As highlighted by Pariza and Johnson (2001), the pathogenicity potential of a microorganism may be considered to be low if the final food enzyme does not contain viable cells of the production organism. As discussed in Section C.6.2 below, the absence of viable cells of the production organism was demonstrated in 3 production batches of the fructosyltransferase food enzyme. Furthermore, to date, there have not been any known reports of pathogenicity and/or virulence of *A. oryzae* in the scientific literature, despite its extensive history of use in food production. Therefore, the pathogenic potential of the production strain, *A. oryzae* strain QHT-101, is considered to be very low.

As discussed in Section B.5.2.2, 3 production batches of the food enzyme produced from *A. oryzae* strain QHT-101 were analysed for the standard list of mycotoxins required by JECFA to be tested for all enzyme preparations (*i.e*., ochratoxin A, aflatoxins [B1, B2, G1, and G2], zearalenone, sterigmatocystin, T-2 toxin) (JECFA, 1991), as well as additional secondary metabolites reported by EFSA (2017), including deoxynivalenol, fumonisin B1 and B2, and HT-2 toxin. The results of the analysis demonstrate the levels of these compounds to be below their respective limit of detection (LOD)/limit of quantitation (LOQ) values. Batch analyses also confirm absence of detectable levels of antimicrobial activity in the food enzyme. Additionally, the specifications for the fructosyltransferase food enzyme ensure that no antibacterial activity is present in the manufactured food enzyme. Therefore, the available analytical information indicates the toxigenicity potential of the production strain to be low, which is consistent with the lack of reports in the scientific literature on the toxigenic nature of *A. oryzae* as well as the conclusions of regulatory and scientific bodies such as JECFA and the U.S. EPA.

C.6.2 Absence of the Production Strain

The absence of viable cells of the production strain (*A. oryzae* strain QHT-101) in the fructosyltransferase enzyme was confirmed analytically. The experiment was conducted according to the recommendations set out in EFSA's *Scientific Guidance for the submission of dossiers of Food Enzymes* (EFSA CEP Panel, 2021). The full details of the methodology are provided in Appendix A and the study report is provided in Appendix A-5.

Three non-consecutive batches (Batch Nos. 1, 2, and 3) of the immobilised fructosyltransferase food enzyme produced using the industrial-scale manufacturing process were collected for analysis. Test samples were inoculated on culture plates, while positive control plates of the fructosyltransferase food enzyme were spiked with low counts of viable cells of *A. oryzae* strain QHT-101 to demonstrate normal growth of the production strain. All plates were examined for colonies visually. No viable cells were detected in the cultures of the 3 batches of the fructosyltransferase food enzyme. Cell growth was observed in the positive control plates, confirming that the medium and cultivation conditions were suitable for cell growth.

C.7 Information on the Genetic Stability of the Source Organism

The source organism is not genetically modified. The production strain, *A. oryzae* strain QHT-101, was selected as the production strain based on its capacity to produce high levels of fructosyltransferase activity, its viability, and its suitability for industrial production, including minimal production of secondary metabolites. The organism is stored at a recognised culture collection until needed and maintained in a microbial collection at -80°C at the production facility. Tate & Lyle maintains a well-defined cell bank system using master cell bank (MCB) and working cell bank (WCB) to store the production strain. Details on the monitoring of the production strain are provided in Appendix A.

The production process of the fructosyltransferase food enzyme complies with HACCP, and includes quality control steps throughout the production process, including the fermentation steps, to ensure that certain criteria are met. Batches that do not meet the specifications for the food enzyme are not released for further processing.

D. INFORMATION RELATED TO THE DIETARY EXPOSURE TO THE PROCESSING AID

In accordance with Section 3.3.2 (Processing Aids) of the FSANZ *Application Handbook* (FSANZ, 2019), the following dietary exposure information must be provided:

- A list of foods or food groups likely to contain the processing aid or its metabolites;
- The levels of residues of the processing aid or its metabolites for each food or food group;
- 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;
- 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;
- Information relating to the levels of residues in foods in other countries; and
- For foods where consumption has changed in recent years, information on likely current food consumption.

Each point is addressed in the following subsections.

D.1 A list of Foods or Food Groups Likely to Contain the Processing Aid

The fructosyltransferase food enzyme is intended for use in the production of sc-FOS at a maximum use level up to 412 mg TOS/kg sucrose. A dietary exposure assessment for fructosyltransferase from *A. oryzae* strain QHT-101 was not conducted due to the absence of enzyme TOS demonstrated in the final FOS product (see Section D.2).

D.2 The Levels of Residues of the Processing Aid or its Metabolites for Each Food or Food Group

During the production of sc-FOS, the immobilised fructosyltransferase enzyme is added to soluble sugar at the start of the food manufacturing process. Following the enzymatic reaction, the syrup is filtered, then desalinated and decolourised which effectively removes proteins and other substances in the sc-FOS syrup. The syrup is then subjected to a filter purification step. These purification processes effectively remove enzyme TOS from the final sc-FOS product. The removal of enzyme TOS was demonstrated analytically by measuring the total protein content during various stages of the food manufacturing process. The protein content of the starting material (granulated white sugar), in-process samples of sc-FOS syrup (following filtration and concentrated syrup after purification), and the final product (sc-FOS syrup containing >95% sc-FOS) was measured using a GB/T method. The LOQ for this method is 0.024%. The tested samples are described in Table D.2-1 below.

Table D.2-1 Description of Samples (Starting Material, In-process, and Final Product) Tested for Protein Content

Sample	Description	Batch No. (Test 1)	Batch No. (Test 2)	Batch No. (Test 3)
Sugar	Starting material	Batch 7a	Batch 8a	Batch 9a
Samples after filtration	In-process sample	Filtration 1	Filtration 2	Filtration 3
Concentrated samples after purification	In process sample	Concentrated 1	Concentrated 2	Concentrated 3
QHT-FOS-P95S	Final product	Batch 7b	Batch 8b	Batch 9b

The results of the analysis are summarised in Table D.2-2 below. The protein content was below the LOQ in the starting material, in-process samples, and final product, indicating that there was no significant amount of enzyme TOS transferred into the final sc-FOS product. As a result, a dietary exposure assessment of the fructosyltransferase was not performed.

Table D.2-2 Results of Analysis for Protein Content in Test Samples (Starting Material, In-process, and Final Product)

Sample	Batch No.	Protein Content
Test 1		
Sugar	Batch 7a	<LOQ
Samples after filtration	Filtration 1	Not detected
Concentrated samples after purification	Concentrated 1	<LOQ
QHT-FOS-P95S	Batch 7b	<LOQ
Test 2		
Sugar	Batch 8a	<LOQ
Samples after filtration	Filtration 2	Not detected
Concentrated samples after purification	Concentrated 2	<LOQ
QHT-FOS-P95S	Batch 8b	<LOQ

Table D.2-2 Results of Analysis for Protein Content in Test Samples (Starting Material, In-process, and Final Product)

Sample	Batch No.	Protein Content
Test 3		
Sugar	Batch 9a	<LOQ
Samples after filtration	Filtration 3	Not detected
Concentrated samples after purification	Concentrated 3	<LOQ
QHT-FOS-P95S	Batch 9b	<LOQ

LOQ = limit of quantification.

D.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.

D.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

β-Fructofuranosidase from *A. niger*, *A. fijiensis*, and *T. reesei* are currently permitted for use as a processing aid in the production for FOS in Australia and New Zealand. As the enzyme contains both fructofuranosidase and transferase activities that facilitate the production of FOS, it is anticipated that Tate & Lyle's fructosyltransferase from *A. oryzae* strain QHT-101 will provide food manufacturers and food producers with an alternative processing aid for the production of FOS. Therefore, approval for fructosyltransferase from *A. oryzae* strain QHT-101 is not expected to increase the existing usage of β-Fructofuranosidase/fructosyltransferase in food production.

D.5 Information Relating to the Levels of Residues in Foods in Other Countries

As discussed in Section D.2, no enzyme residues are expected during the production of sc-FOS due to the purification steps employed. Marginal amounts of enzyme residue carried over would not pose any safety concerns due to the high margins of safety determined from the safety data presented in Section C.2.2.

D.6 For Foods Where Consumption Has Changed in Recent Years, Information on Likely Current Food Consumption

The fructosyltransferase food enzyme is intended for use in the production of sc-FOS, which can in turn be added to a variety of food products such as baked goods, juices, milk-based beverages, snack foods, *etc.* It is not anticipated that the intakes of these food products have drastically changed in recent years.

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