FOOD STANDARDS Australia New Zealand
Te Mana Kounga Kai – Ahitereiria me Aotearoa

11 December 2025
372-25

Supporting document

Risk and technical assessment

Application A1304

Endo-1,4-beta-xylanase from *Bacillus licheniformis* (gene donor: *Chryseobacterium cucumeris*) for use as a processing aid

---

Executive summary

Novonesis (previously Novozymes Australia Pty Ltd) has applied to amend the Australia New Zealand Food Standards Code (the Code) to permit the use of the enzyme endo-1,4-beta-xylanase (EC 3.2.1.8), from *Bacillus licheniformis* containing the xylanase gene from *Chryseobacterium cucumeris* as a processing aid in the production of potable alcohol and starch and gluten fractions.

The available evidence provides adequate assurance that the proposed use of endo-1,4-beta-xylanase (EC 3.2.1.8) from *Bacillus licheniformis* is technologically justified. The enzyme does not perform a technological function in the food for sale, therefore it functions as a processing aid for purposes of the Code. There are relevant identity and purity specifications for the enzyme in the Code, and the applicant provided evidence that the enzyme meets these specifications.

No public health or safety concerns were identified concerning the use of the production organism, which is neither pathogenic nor toxigenic. Analysis of the production strain confirmed the presence and stability of the inserted DNA.

The endo-1,4-beta-xylanase that is the subject of this application has a history of safe human use exceeding 2 years. No homology with known toxins or known food allergens was identified, and xylanase was not genotoxic *in vitro* or *in vivo*. The enzyme was well tolerated in a 90-day gavage study in rats. The no observed adverse effect level (NOAEL) was 962 mg total organic solids (TOS)/kg bw/day, the highest dose tested.

The theoretical maximum daily intake (TMDI) of this xylanase was calculated to be 0.47 mg TOS/kg bw/day. A comparison of the NOAEL and the TMDI results in a large margin of exposure (MOE) of approximately 2,000. Based on the reviewed data it is concluded that in the absence of any identifiable hazard an acceptable daily intake (ADI) ‘not specified’ is appropriate.

Overall, FSANZ concludes there are no safety concerns from the use of endo-1,4-beta-xylanase from *Bacillus licheniformis* in the quantity and form proposed to perform its technological function in the production of potable alcohol and starch and gluten fractions.

Table of contents

1 Introduction

Novonesis (then Novozymes Australia Pty Ltd) has applied to amend the Australia New Zealand Food Standards Code (the Code) to permit the use of the enzyme endo-1,4-beta-xylanase (EC 3.2.1.8) from *Bacillus licheniformis* containing the endo-1,4-beta-xylanase gene from *Chryseobacterium cucumeris* as a food processing aid.

The enzyme is intended to be used as a processing aid in the production of distilled alcohol and starch and gluten fractions. The application refers to 'distilled beverages', however the Code refers to 'potable alcohol' in its permissions, and from herein the latter term will be referenced.

The usage level is the minimum level required to achieve the desired effect, in accordance with the principles of Good Manufacturing Practice (GMP)[1].

1.1 Objectives of the assessment

The objectives of this risk and technical assessment were to:

- determine whether the proposed purpose is a solely technological purpose and that the enzyme achieves its technological purpose as a processing aid in the quantity and form proposed to be used
- evaluate potential public health and safety concerns that may arise from the use of this enzyme as a processing aid by considering the:
  - safety and history of use of the production microorganism
  - safety of the enzyme preparation.

2 Food technology assessment

2.1 Identity of the enzyme

The applicant provided relevant information regarding the identity of the enzyme, and this has been verified using the IUBMB[2] enzyme nomenclature reference database (McDonald et al 2009).

Accepted IUBMB name:	Endo-1,4-beta-xylanase
Systematic name:	4-β-D-xylan xylanohydrolase
Other names/common names:	endo-(1→4)-β-xylan 4-xylanohydrolase; endo-1,4-xylanase; xylanase; β-1,4-xylanase; endo-1,4-xylanase; endo-β-1,4-xylanase; endo-1,4-β-D-xylanase; 1,4-β-xylan xylanohydrolase; β-xylanase; β-1,4-xylan xylanohydrolase; endo-1,4-β-xylanase; β-D-xylanase

[1] GMP is defined in the Standard 1.1.2—2 of the Code as follows: *with respect to the addition of substances used as food additives and substances used as processing aids to food, means the practice of:*
(a) limiting the amount of substance that is added to food to the lowest possible level necessary to accomplish its desired effect; and
(b) to the extent reasonably possible, reducing the amount of the substance or its derivatives that:
*(i) remains as a *component of the food as a result of its use in the manufacture, processing or packaging; and*
(ii) is not intended to accomplish any physical or other technical effect in the food itself

[2] International Union of Biochemistry and Molecular Biology.

IUBMB enzyme nomenclature:	EC 3.2.1.8
CAS number:	9025-57-4
Reaction:	Catalyses the endohydrolysis of (1→4)-β-D-xylosidic linkages in xylans present in grains. The hydrolysis reaction scheme for endo-1,4-beta-xylanase is available under its record in the enzyme database BRENDA[3] (Chang et al. 2021).

2.2 Manufacturing process

2.2.1 Production of the enzyme

Enzymes produced from microorganisms are typically produced by controlled fermentation followed by removal of the production microorganism, purification and concentration of the enzyme. Final standardisation with stabilisers, preservatives, carriers, diluents, and other approved food-grade additives and ingredients is carried out after the purification and concentration steps. The formulated enzymes are referred to as enzyme preparations, which, depending upon the application in food, may be a liquid, semi-liquid or dried product. The applicant's product is in the form of a liquid. Enzyme preparations may contain either one major active enzyme that catalyses a specific reaction during food processing or two or more active enzymes that catalyse different reactions (FAO/WHO 2020a).

The enzyme is manufactured in accordance with Good Manufacturing Practice for Food (GMP). Novonesis follow relevant EC regulations for manufacture and packaging of food enzymes and for control and inspection. The quality management system complies with ISO9001:2015 for the development, production and sale of industrial enzymes.

2.2.2 Specifications for identity and purity

There are international general specifications for enzyme preparations used in the production of food. These have been established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in its Compendium of Food Additive Specifications (FAO JECFA Monographs 26 (2021)), explicitly FAO/WHO (2006) and in the Food Chemicals Codex (FCC 2022), referenced in subsection 3—2 of Schedule 3 of the Code. Enzymes used as a processing aid need to meet either of these specifications, or a relevant specification in section S3—3 of Schedule 3. In addition, under JECFA, enzyme preparations must meet the specifications criteria contained in the individual monographs. In the case of endo-1,4-beta-xylanase produced from a strain of *Bacillus licheniformis* containing the xylanase gene from *Chryseobacterium cucumeris*, there is no individual monograph.[4]

Schedule 3 of the Code also includes specifications for arsenic and heavy metals (section S3—4) if they are not already detailed within specifications in sections S3—2 or S3—3.

The applicant provided the results of analysis of three different batches of their enzyme preparation. Table 1 provides a comparison of the results of those analyses with

[3] Information on EC 3.2.1.8 - endo-1,4-beta-xylanase - BRENDA Enzyme Database
[4] For the functional use 'enzyme preparation', the JECFA database can be searched for individual monographs: http://www.fao.org/food/food-safety-quality/scientific-advice/jecfa/jecfa-additives/en/

specifications established by JECFA and Food Chemicals Codex, as well as the Code (as applicable). The applicant's endo-1,4-beta-xylanase met all relevant specifications.

The specification for the enzyme preparation used by the manufacturer (provided in section A.5 of Novonesis's application) includes a test for the absence of the production strain, which was not detected.

Table 1 *Analysis of manufacturer's final enzyme preparation (endo-1,4-beta-xylanase from* Bacillus licheniformis *containing the endo-1,4-beta-xylanase gene from* Chryseobacterium cucumeris*) compared to JECFA, Food Chemicals Codex, and Code specifications for enzymes*

Test parameters	*Novonesis test result**	*Specifications*		
		JECFA	*Food Chemicals Codex*	*The Code - section S3—4*
Lead (mg/kg)	ND (LOD<0.5)**	≤5	≤5	≤2
Arsenic (mg/kg)	ND (LOD<0.3)	-	-	≤1
Cadmium (mg/kg)	ND (LOD<0.05)	-	-	≤1
Mercury (mg/kg)	ND (LOD<0.05)	-	-	≤1
Coliforms (cfu*/g)**	<4	≤30	≤30	-
***Salmonella* (in 25 g)**	Not detected	Absent	Negative	-
***Escherichia coli* (in 25 g)**	Not detected	Absent	-	-
Antimicrobial activity****	Not detected	Absent	-	-
Production Strain****	Not detected			

*All three batch results are the same, only one is listed.
** Limit of detection
***cfu = colony forming units.
****Information provided in confidential appendix.

In addition, the applicant has provided the total organic solids (TOS) value. The TOS encompasses the enzyme component and other organic material originating from the production organism and the manufacturing process, while excluding intentionally added formulation ingredients. Details of the TOS value are included in section 3.4 Dietary exposure assessment.

2.3 Technological purpose

Under the current application, endo-1,4-beta-xylanase is intended for use as a processing aid in the production of potable alcohol and gluten and starch fractions. The applicant requested use of the enzyme at GMP levels. The application states the highest use level can be up to 200 fungal xylanase units per gram (FXU(TB)/g) used in the raw material, being grains and cereals.

The enzyme performs its technological function by catalysing the hydrolysis of endo-1,4-beta-xylanase xylosidic linkages in xylans, including arabinoxylan present in grains, for further processing to produce a range of products including gluten, starch and potable alcohol. Xylans are the main component in hemicellulose, a complex mixture of polysaccharides (Saha 2000).

The technological purpose of the endo-1,4-beta-xylanase enzyme as stated by the applicant includes converting:

- gelatinised starch and dextrins into glucose and other fermentable sugars during potable alcohol production
- xylans, including arabinoxylans, into oligosaccharides during production of starch and gluten fractions.

The technological benefits of using this enzyme in the production of potable alcohol is increased fermentable sugars and for production of gluten and starch fractions, increased yields obtained from targeted degradation of arabinoxylans.

The applicant provided information on the physical and chemical properties of their enzyme preparation. Table 2 summarises this information. The enzyme is denatured at a temperature of 60°C. Therefore, the enzyme is inactivated during the production of potable alcohol and starch and gluten fractions so would have no technological function in the final potable alcohol and starch and gluten fractions.

Table 2 *Endo-1,4-beta-xylanase enzyme preparation physical/chemical properties*

Physical/chemical properties of commercial enzyme preparation	
Enzyme activity	83 *FXU(TB)/g
Appearance	Liquid
Temperature range and optimum	Active up to 60°C, optimum is 42°C at pH 4
Temperature stability	Almost full activity up to 38°C after 30 mins at pH 4. Negligible activity above 60°C
pH range and optimum	Active within pH 2 – 9, optimum is pH 5 at 37°C

**FXU (TB)/g is Fungal Xylanase Units per gram*

The applicant's enzyme preparation is available as a liquid concentrate standardised in fungal xylanase units to an activity of 83 FXU(TB)/g. Endo-1,4-beta-xylanases hydrolyse wheat arabinoxylan to release a reducing carbohydrate. The reaction is stopped by an alkaline reagent that complexes with the reducing sugar producing colour detected at 405nm. The increase in absorbance is proportional to the enzyme activity.

The highest use level is 200 FXU(TB)/kg of grain used in the production of potable alcohol and starch and gluten fractions, with permission requested at levels of GMP.

2.4 Allergen considerations

The applicant stated the micro-organisms used in fermentation utilise most of the raw materials. Remaining amounts of raw materials are removed during downstream processing, including washing and filtration steps. This is supported by analytical results that show no detectable levels of known allergens.

2.5 Food technology conclusion

FSANZ concludes that endo-1,4-beta-xylanase functions as a processing aid in the production of potable alcohol and in the production of starch and gluten fractions, by catalysing the hydrolysis of endo-1,4-beta-xylosidic linkages in xylans. The technological benefits of using the enzyme in these applications include increased fermentable sugars and,

for production of gluten and starch fractions, increased yields obtained from targeted degradation of arabinoxylans.

FSANZ concludes that the evidence presented to support its proposed use provides adequate assurance that endo-1,4-beta-xylanase, in the quantity and form proposed to be used (which must be consistent with GMP), is technologically justified and has been demonstrated to be effective in achieving its stated purpose.

Endo-1,4-beta-xylanase performs its technological purpose during the production of potable alcohol and production of starch and gluten fractions, during which it is inactivated by temperature, and is not performing a technological purpose in the food for sale. It is therefore functioning as a processing aid for the purposes of the Code.

There are relevant identity and purity specifications for the enzyme in the Code, and the applicant provided evidence that the enzyme meets these specifications.

3 Safety assessment

The objective of this safety assessment is to evaluate any potential public health and safety concerns associated with the use of this endo-1,4-beta-xylanase enzyme as a processing aid.

Some information relevant to this section is CCI, so full details cannot be disclosed in this public report.

3.1 Source microorganisms

3.1.1 Host and production organism

B. licheniformis has a long history of safe industrial use, particularly in the production of enzymes for food processing, dating back to 1972 (De Boer et al. 1994; Sewalt et al. 2018; Muras et al. 2021). *B. licheniformis* is a gram-positive, endospore-forming, mesophilic and facultative anaerobic bacterium, present in soil and marine environments. Its optimal growth temperature is around 50°C and it can survive at much higher temperatures. Taxonomically, *B. licheniformis* is classified under the genus *Bacillus*, family *Bacillaceae*, order *Bacillales*, class Bacilli, and phylum *Bacillota* (Whitman 2009).

FSANZ has previously assessed the safety of *B. licheniformis* as the source organism for processing aids. Schedule 18 of Standard 1.3.3 of the Code permits the use of the following enzymes produced by *B. licheniformis*, including: alpha-amylase, chymotrypsin, endo-1,4-beta-xylanase, beta-galactosidase, glycerophospholipid cholesterol acyltransferase, maltotetraohydrolase, pullulanase, and serine proteinase.

B. licheniformis is generally considered non-pathogenic due to the absence of invasive traits (Muras et al. 2021). However, isolates have been identified as the cause of foodborne illness associated with cooked meats, ice cream, cheese, raw milk, infant feed, and prawns (Salkinoja-Salonen et al. 1999). Such incidences of human infection and pathogenicity are rare and typically limited to immunocompromised individuals (Haydushka et al. 2012; Logan 2012).

Both the European Food Safety Authority (EFSA BIOHAZ Panel 2025) and the US Environmental Protection Agency (Federal Register 1999) have classified of *B. licheniformis* as a safe biological agent, provided it does not produce toxins or harbour any acquired antimicrobial resistance genes to clinically relevant antibiotics. The Food and Drug

Administration has affirmed that carbohydrase and protease enzyme products derived from *B. licheniformis* are generally recognized as safe (GRAS) for use in the production of alcoholic beverages, candy, nutritive sweeteners, and protein hydrolysates (Federal Register 1999).

The applicant provided data that adequately demonstrates the production organism's identity as *B. licheniformis.* Using the safe strain concept (Pariza and Johnson 2001), the applicant provided information highlighting the risk of toxin production by the production organism is very low. Microbiological testing was also provided to FSANZ, confirming the absence of the production organism in the final enzyme preparation.

The microbiological assessment undertaken by FSANZ did not identify any public health and safety concerns related to the use of *B. licheniformis* as a production organism for endo-1,4-beta-xylanase.

3.2 Characterisation of the genetic modification

3.2.1 Description of the introduced DNA and method of transformation

The gene encoding the endo-1,4-beta-xylanase enzyme from *C. cucumeris* was synthesised to include several amino acid substitutions relative to the wildtype enzyme. This gene was introduced into the genome of the host *B. licheniformis* as part of an expression cassette containing a hybrid *Bacillus* promoter and a terminator from the *B. licheniformis* α-amylase gene. Data provided by the applicant and assessed by FSANZ confirmed the identity of the endo-1,4-beta-xylanase enzyme.

The expression cassette was integrated at specific integration sites present in the host *B. licheniformis* genome using a vector based on standard *Staphylococcus aureus* vectors. Additional vectors were used to remove marker genes present in the recipient strain.

3.2.2 Characterisation of inserted DNA

Genome sequencing data provided by the applicant confirmed the presence of the inserted DNA at specific loci in the genome of the production strain. The data also confirmed the absence of antibiotic resistance genes, marker genes and other plasmid-derived DNA in the final production strain.

3.2.3 Genetic stability of the inserted gene

The assessment confirmed the inserted gene is stably integrated into the genome of the production strain and does not have the ability to replicate autonomously. The inserted gene is therefore considered to be genetically stable.

To provide further evidence of the stability of the inserted endo-1,4-beta-xylanase gene, the applicant provided phenotypic data from large-scale fermentation of the production strain, which confirmed the gene is expressed stably over multiple generations.

3.3 Safety of the triacylglycerol lipase enzyme

3.3.1 History of safe use of the enzyme

Xylanases have been used as processing aids in the production of human food since the 1980s. The specific enzyme that is the subject of this application is used in countries that have no restrictions on the use of enzyme processing aids. In addition, it has been approved

for use in Denmark for over a year and has been approved for use in France for more than two years. Confidential data confirming a history of commercial use have been provided.

3.3.2 Bioinformatics concerning homology with known toxins

A report of a recent bioinformatics search for homology with known proteins was provided. The amino acid sequence of the enzyme was compared by FASTA sequence comparison to all proteins identified as toxins in the NCBI Identical Protein Groups resource[5]. No homology with known toxins was found. Nineteen hits that had E-values < 0.00001 were identified, all of which were xylanases of various organisms.

3.3.3 Toxicology data

Toxicology data provided in support of the safety assessment include a bacterial reverse mutation assay (Ames test), an *in vivo* micronucleus test in mice, and a 13-week oral gavage study in rats. All the studies were conducted under Good Laboratory Practice (GLP) and in compliance with appropriate OECD test guidelines.

No evidence of genotoxicity was found in either the bacterial reverse mutation assay or the *in vivo* micronucleus test. The maximum dose administered in the micronucleus test was 2000 mg TOS/kg bw/day. No adverse effects of the test article were observed in the 13-week study, and a no observed adverse effect level (NOAEL) of 962 mg TOS/kg bw/day, the highest dose tested, was identified. The studies are described in detail in Appendix 1.

3.3.4 Potential for allergenicity

The amino acid sequence of the xylanase was compared to those of known allergens in the Comprehensive Protein Allergen Resource[6] (COMPARE). Search strategies included 35% identity over 80 amino acids, with and without scaling, full length alignment, and 100% identity over eight amino acids. Only one match was found using a sliding window search for 80 consecutive amino acids. The allergen is Phl p 4, an enzyme from timothy grass (*Phleum pratense*). This enzyme is a respiratory allergen in occupational settings. Respiratory allergens do not appear to elicit allergic reactions when ingested (Bindslev-Jensen *et al.* 2006) and therefore this homology is not considered to be of concern.

3.3.5 Assessments by other regulatory agencies

No safety assessment reports by other regulatory agencies are available.

3.4 Dietary exposure assessment

The objective of the dietary exposure assessment was to review the budget method calculation presented by the applicant as a 'worst-case scenario' approach to estimating likely levels of dietary exposure, assuming that all of the TOS from the xylanase enzyme preparation remained in the food.

The budget method is a valid screening tool for estimating the theoretical maximum daily intake (TMDI) of a food additive (Douglass et al 1997). The calculation is based on physiological food and liquid requirements, the food additive concentration in foods and beverages, and the proportion of foods and beverages that may contain the food additive. The TMDI can then be compared to an acceptable daily intake (ADI) or a NOAEL to estimate a margin of exposure (MOE) for risk characterisation purposes. Whilst the budget method

[5] https://www.ncbi.nlm.nih.gov/ipg/
[6] https://comparedatabase.org

was originally developed for use in assessing food additives, it is also appropriate to use for estimating the TMDI for processing aids (FAO/WHO 2020). The method is used by overseas regulatory bodies and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (FAO/WHO 2021) for dietary exposure assessments for processing aids.

In their budget method calculation, the applicant made the following assumptions:

- the maximum physiological requirement for solid food (including milk) is 25 g/kg body weight/day
- all solid foods contain the maximum use level of 50.6 mg TOS per kg cereal (or cereal-derived) dry matter
- 50% of solid food is processed
- all processed food contains 25% cereal (or cereal-derived) dry matter
- the maximum physiological requirement for liquid is 100 mL/kg body weight/day (the standard level used in a budget method calculation for non-milk beverages)
- all liquid foods contain the maximum use level of 50.6 mg TOS per kg starch (or starch-derived) dry matter
- 25% of non-milk beverages are processed
- all processed beverages contain 12% starch (or starch-derived) dry matter
- the densities of non-milk beverages are ~1
- all of the TOS from the enzyme preparation remains in the final food.

Based on these assumptions, the applicant calculated the TMDI of the TOS from the enzyme preparation to be 0.310 mg TOS/kg body weight/day.

As assumptions made by the applicant differ from those that FSANZ would have made in applying the budget method, FSANZ independently calculated the TMDI using the following assumptions that are conservative and reflective of a first tier in estimating dietary exposure.

- The maximum physiological requirement for solid food (including milk) is 50 g/kg body weight/day (the standard level used in a budget method calculation where there is potential for the enzyme preparation to be in baby foods or general purpose foods that would be consumed by infants).
- FSANZ would generally assume 12.5% of solid foods contain the enzyme based on commonly used default proportions noted in the FAO/WHO Environmental Health Criteria (EHC) 240 Chapter 6 on dietary exposure assessment (FAO/WHO 2009). However, the applicant has assumed a higher proportion of 50% based on the nature and extent of use of the enzyme and therefore FSANZ has also used this proportion for solid foods as a worst-case scenario.

All other inputs and assumptions used by FSANZ remained as per those used by the applicant. The TMDI of the TOS from the enzyme preparation based on FSANZ's calculations for solid food and non-milk beverages is 0.47 mg TOS/kg bw/day.

Both FSANZ and the applicant's estimates of the TMDI will be overestimates of the dietary exposure given the conservatisms in the budget method. This includes that it was assumed that all of the TOS from the enzyme preparation remains in the final foods and beverages whereas the applicant has stated that it is likely to either be reduced or removed during processing, or would be present in insignificant quantities. The enzyme is denatured by heat during grain processing for the production of potable alcohol and starch and gluten fractions, so would perform no function in the final food to which the ingredient is added.

4 Discussion and Conclusion

FSANZ concludes that the use of this endo-1,4-beta-xylanase as a processing aid for use in the production of potable alcohol and production of starch and gluten fractions is consistent with its typical function of catalysing the hydrolysis of endo-1,4-beta-xylosidic linkages in xylan found in grains. The use of the enzyme in the production of potable alcohol includes increased fermentable sugars and for production of gluten and starch fractions, increased yields. The enzyme functions as a processing aid for the purposes of the Code and does not perform a technological purpose in the food for sale. The evidence presented to support its proposed use provides adequate assurance that the enzyme, in the quantity and form proposed to be used (which must be consistent with GMP), is technologically justified and effective in achieving its stated purpose.

No public health or safety concerns were identified concerning the use of the production organism, which is neither pathogenic nor toxigenic. Analysis of the production strain confirmed the presence and stability of the inserted DNA.

Xylanases have a long history of safe human use for food production. This specific xylanase has been used for more than two years. Xylanase is not genotoxic *in vitro* or *in vivo*, and no homology was found with any known toxins or food allergens.

A NOAEL of 962 mg TOS/kg bw/day was identified in a 90-day oral toxicity study in rats. The TMDI was calculated by FSANZ to be 0.47 mg TOS/kg bw/day. A comparison of the NOAEL and the TMDI results in a large margin of exposure (MOE) of approximately 2,000.

Based on the reviewed data it is concluded that in the absence of any identifiable hazard an ADI ‘not specified’ is appropriate.

5 References

Bindslev-Jensen C, Skov PS, Roggen EL, Hvass P, Brinch DS (2006). Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry. Food and Chemical Toxicology 44: 1909–1915

Chang A., Jeske L., Ulbrich S., Hofmann J., Koblitz J., Schomburg I., Neumann-Schaal M., Jahn D., Schomburg D. (2021) BRENDA, the ELIXIR core data resource in 2021: new developments and updates. *Nucleic Acids Res.*, 49:D498-D508.

De Boer AS, Priest F and Diderichsen B (1994) 'On the industrial use of Bacillus licheniformis: A review', *Applied Microbiology and* Biotechnology, 40(4):595–598, doi:10.1007/BF00173313.

Douglass JS, Barraj LM, Tennant DR, Long WR and Chaisson CF (1997) 'Evaluation of the Budget Method for screening food additive intakes, Food Additives and Contaminants' 14:791-802.

EFSA BIOHAZ Panel (2025) 'Updated list of QPS-recommended microorganisms for safety risk assessments carried out by EFSA', *Zenodo*, accessed 12 September 2025. <https://doi.org/10.5281/zenodo.15827398>.

FAO/WHO (2009) 'Environmental Health Criteria 240. Principles and Methods for the Risk Assessment of Chemicals in Food' Chapter 6 – Dietary exposure assessment of chemicals in food, WHO, Geneva.

FAO/WHO (2020) Environmental Health Criteria 240. Principles and Methods for the Risk Assessment of Chemicals in Food. Chapter 6: Dietary exposure assessment of chemicals in food. Second Edition 2020. WHO, Geneva. https://www.who.int/docs/default-source/food-safety/publications/chapter6-dietary-exposure.pdf?sfvrsn=26d37b15_6

FAO/WHO (2021) Evaluation of certain food additives: eighty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives. WHO Technical Report Series, No. 1027.

Federal Register (1999) 'Carbohydrase and protease enzyme preparations derived from Bacillus subtilis or Bacillus amyloliquefaciens; Affirmation of GRAS status as direct food ingredients', *Federal Register*, 64(78), accessed 12 September 2025. <https://www.govinfo.gov/content/pkg/FR-1999-04-23/pdf/99-10220.pdf>.

Haydushka IA, Markova N, Kirina V and Atanassova M (2012) 'Recurrent sepsis due to Bacillus licheniformis', *Journal of Global Infectious Diseases*, 4(1):82–83, DOI:10.4103/0974-777X.93768.

Logan NA (2012), 'Bacillus and relatives in foodborne illness', *Journal of Applied Microbiology*, 112(3):417–429, doi:10.1111/j.1365-2672.2011.05166.x.

Muras A, Romero M, Mayer C and Otero A (2021) 'Biotechnological application of Bacillus licheniformis', Critical Reviews in Biotechnology, 40(4):609–627, doi:10.1080/07388551.2021.1873239

Pariza MW and Johnson EA (2001) Evaluating the safety of microbial enzyme preparations used in food processing: update for a new century, *Regulatory Toxicology and Pharmacology*, 33(2):173–186, doi:10.1006/rtph.2001.1466.

Saha BC (2000). Research Review paper: α-L-Arabinofuranosidases: biochemistry, molecular biology and application in biotechnology. Biotechnology Advances 18(5), 402-423

Salkinoja-Salonen MS, Vuorio R, Andersson MA, Kämpfer P, Andersson MC, Honkanen-Buzalski T & Scoging AC (1999) 'Toxigenic strains of Bacillus licheniformis related to food poisoning', *Applied and Environmental Microbiology*, 65(10):4637–4645, doi:10.1128/AEM.65.10.4637-4645.1999.

Sewalt VJ, Reyes TF & Bui Q (2018) 'Safety evaluation of two α-amylase enzyme preparations derived from Bacillus licheniformis expressing an α-amylase gene from Cytophaga species', *Regulatory Toxicology and Pharmacology*, 98:140–150, doi:10.1016/j.yrtph.2018.07.015.

Whitman, WB (ed.) 2009, Bergey's manual of systematic bacteriology: Volume 3: The Firmicutes, 2nd edn, Springer, New York.

6 Appendix 1. Toxicity studies.

Both genotoxicity studies were conducted under GLP.

***Bacterial reverse mutation assay of xylanase from* Bacillus licheniformis[7]**

Test	*Test system*	*Concentration*	*Purity (% total organic solids)*	*Results*
Bacterial reverse mutation assay (OECD TG 471, [1997])	*Salmonella enteridis* var. Typhimurium test strains TA98, TA100, TA1535 and TA1537; and *Escherichia coli* strain WP2 *uvr*A	Experiment I[1]: 6.9, 20.7, 69, 207, 690, 2070, 6900 µg/mL Experiment II[2]: 69, 207, 690, 2070, 6900 µg/mL	6.9%	Negative ± S9

[1] Test conducted in triplicate.
[2] Test conducted in triplicate.

Mammalian Erythrocyte Micronucleus Test of Xylanase, Batch PPQ69045 in Swiss albino mice. Bioneeds India Private Limited 2022. Regulatory status: GLP, *in compliance with OECD Test Guideline 474.*

The test article was provided as a liquid with a TOS content of 9.3% w/w. The vehicle/negative control article was distilled water. Cyclophosphamide monohydrate was used as a positive control article.

The dose range-finding study was conducted at dose levels of 0 or 2000 mg TOS/kg bw/day. Each group comprised 2 mice/sex. Xylanase was administered twice daily by gavage, with an interval of 4 h ± 10 min between doses. Dosing was conducted for two consecutive days. Mice were observed for clinical signs and mortality. At scheduled termination on Day 3, 18 to 24 hours after the second dosing on Day 2, mice were killed and subjected to gross necropsy and collection of bone marrow from femurs. Three smears of bone marrow cells were examined from each mouse, and 500 erythrocytes were examined for calculation of the ratio of Polychromatic Erythrocytes (PCE) versus Normochromatic Erythrocytes (NCE).

No abnormal clinical signs or unscheduled mortality was observed. A biologically significant toxicity to the bone marrow is identified if there is a reduction in PCE: total erythrocytes ratio >50%. In the dose range-finding study, there was a group mean reduction in PCE: total erythrocytes ratio of 6.00% in treated males compared to controls, while in females the corresponding value was 5.88%. It was concluded that there was no treatment-related toxicity to the bone marrow.

Based on the results from the dose range-finding study, doses of 0, 500, 1000 or 2000 mg TOS/kg bw/day were selected for the definitive study, and as there were no observed sex-related differences, only male mice, 5/group, were used for the definitive study. The same twice-daily dosing schedule for two consecutive days was used. A positive control group was treated with cyclophosphamide monohydrate at 100 mg/kg bw/day according to the same

[7] Xylanase, Batch PPQ69045: Bacterial Reverse Mutation Test (Treat-and-wash method) Covance study number 8445324, January 2021

schedule. Mice were killed by cervical dislocation approximately 19 hours after the final dosing. Bone marrow cells were collected and examined for determination of PCE:total erythrocytes ratio, and number of MNPCEs per 4000 PCEs scored.

No clinical signs or unscheduled deaths were observed in any group. There was no statistically significant increase in the group mean value for number of MNPCEs/4000 PCEs in any of the test item dosed groups in comparison with the vehicle control. In the positive control group, there was a significant increase in MNPCEs/4000 PCEs when compared to the vehicle control group, confirming the validity of the assay. It was concluded that the xylanase was non-mutagenic under the conditions of this study.

Xylanase, Batch PPQ69045: Toxicity Study by Oral Gavage Administration to Han Wistar Rats for 13 Weeks. Covance Laboratories Ltd 2021. Regulatory status: GLP, in accordance with OECD Test Guideline 408.

The test article was provided frozen and thawed for administration either as supplied or following dilution. The highest dose represented the undiluted liquid. Stability of the dose formulations for 24 hours was confirmed by the sponsor. The vehicle/negative control article was reverse osmosis water. Han Wistar rats were assigned to four groups of 10/sex/group. Rats were dosed by oral gavage with 0, 96.2, 317 or 962 mg TOS/kg bw/day. Parameters recorded during the in-life phase included weekly detailed physical examination and arena observations, body weights and bodyweight changes, and food consumption. Sensory reactivity observations, motor activity and grip strength were recorded for all rats in Week 12. Rats were subject to ophthalmic examinations prestudy and during Week 12. Blood was collected from Week 13 for haematology, clinical chemistry and clotting parameters, and prior to scheduled kill for thyroid hormone analysis. The stage of oestrus was determined by vaginal smears from female rats over the last four days prior to scheduled termination. Rats were killed and fresh organ weights, gross necropsy findings and histopathological findings recorded. Sperm were collected from the left vas deferens of males for assessment of motility and morphology.

Treatment with xylanase had no effects on survival, clinical observations, sensory reactivity observations, motor activity, grip strength, ophthalmic findings, haematology, clinical chemistry, clotting parameters, thyroid hormones, oestrous cycle, sperm parameters, organ weights, gross necropsy findings or histopathology. The group mean value for total bodyweight gain of females dosed with 962 mg TOS/kg bw/day was significantly lower than that of female controls, but interpreted as within the range of normal variation, and there was no corresponding effect in males. The no observed adverse effect level (NOAEL) was 962 mg TOS/kg bw/day, the highest dose tested.