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

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Supporting document 1

Risk and technical assessment report – Application A1250

Pullulanase from GM *Bacillus subtilis* (gene donor: *Bacillus deramificans*) *as a processing aid*

Executive summary

Novozymes Australia Pty Ltd (Novozymes) has applied to amend the Australia New Zealand Food Standards Code (the Code) to permit the use of the enzyme pullulanase (EC 3.2.1.41) as a processing aid in starch processing for the production of glucose syrups and other starch hydrolysates. The pullulanase is sourced from a genetically modified (GM) strain of *Bacillus subtilis* (*B. subtilis*) containing the pullulanase gene from *Bacillus deramificans*.

FSANZ has undertaken an assessment to determine whether the enzyme achieves its technological purpose in the quantity and form proposed, and to evaluate public health and safety concerns that may arise from the use of this enzyme.

Pullulanase is technologically justified when performing its technological purpose in the quantity and form proposed during the production of food and is therefore appropriately categorised as a processing aid. There are identity and purity specifications in the Code relevant to the enzyme. Evidence has been provided and assessed indicating that the enzyme preparation can comply with these specifications.

B. subtilis has a long history of safe use as a production microorganism of enzyme processing aids, including several that are already permitted in the Code. The production organism is neither pathogenic nor toxigenic.

No public health and safety concerns were identified in the assessment of the pullulanase enzyme under the proposed use conditions. Analysis of the genetically modified production strain confirmed the presence and stability of the inserted DNA.

Bioinformatics analysis found no significant homology of the pullulanase enzyme with any known toxins or food allergens. Pullulanase was not genotoxic *in vitro*, and no adverse effects were found in a subchronic oral toxicity study in rats. The no observed adverse effect level (NOAEL) in this study was 1285 mg total organic solids (TOS)/kg bw/day, the highest dose tested.

The theoretical maximum daily intake (TMDI) of the TOS from the pullulanase enzyme preparation was calculated to be 0.62 mg TOS/kg bw. A comparison of the NOAEL and the TMDI results in a Margin of Exposure (MOE) of approximately 2100.

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

Table of contents

1 Introduction

Novozymes Australia Pty Ltd (Novozymes) has applied to amend the Australia New Zealand Food Standards Code (the Code) to permit the use of the enzyme pullulanase (EC 3.2.1.41) as a processing aid in starch processing for the production of glucose syrups and other starch hydrolysates. This enzyme is from a newly developed source, a genetically modified (GM) strain of *Bacillus subtilis* containing the pullulanase gene from *Bacillus deramificans.* Therefore, a pre-market assessment is required before the enzyme can be used as a processing aid.

Objectives of the assessment

The objectives of this risk and technical assessment were to:

- determine whether the proposed purpose is a solely technological function 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, produced by a GM microorganism, as a processing aid by considering the:
  - history of use of the gene donor and production microorganisms
  - characterisation of the genetic modification(s), and
  - safety of the enzyme.

2 Food technology assessment

2.1 Characterisation of the enzyme

2.1.1 Identity of the enzyme

The production microorganism of the enzyme is a GM strain of *B. subtilis,* with the donor microorganism for the pullulanase gene being *B. deramificans*. The applicant provided relevant information regarding the identity of the enzyme which has been verified using the International Union of Biochemistry and Molecular Biology (IUBMB) enzyme nomenclature database (IUBMB, 2023). Details of the identity of the enzyme are provided in Table 1 below.

Table 1: Identity

Generic name	Pullulanase
IUBMB nomenclature	Pullulanase
Synonyms	Amylopectin 6-glucanohydrolase, alpha-dextrin endo-1,6-alpha-glucosidase, pullulan alpha-1,6-glucanohydrolase, pullulan 6-alpha-glucanohydrolase, limit dextrinase (erroneous), bacterial debranching enzyme, debranching enzyme, R-enzyme
IUBMB No.	EC 3.2.1.41
CAS No.	9075-68-7
Reaction	Hydrolysis of (1→6)-alpha-D-glucosidic linkages in pullulan, amylopectin and glycogen, and in the alpha- and beta-limit dextrins of amylopectins and glycogen

IUBMB: International Union of Biochemistry and Molecular Biology; CAS: Chemical Abstracts Service

2.2 Technological function of the enzyme

Pullulanase catalyses the hydrolysis of the α-1,6 glucosidic linkages in pullulan, amylopectin and glycogen, and in the alpha- and beta-limit dextrins of amylopectin present in raw materials like starch. This hydrolysis converts these branched polysaccharides into smaller fermentable sugars such as maltotriose and maltotetraose during the saccharification[1] process. It is this reaction that is of technological importance in starch processing.

The technological purpose of the enzyme, as proposed in the application, aligns well with that described in the FAO/WHO JECFA Monograph 11, which states that pullulanase will be used for the *hydrolysis of carbohydrates in the manufacture of starch hydrolysates (maltodextrins, maltose and glucose), high fructose corn syrup, beer and potable alcohol.* Further, its proposed technological purpose in starch processing is supported by scientific literature (Hii et al. 2012; Huang & Tang 2017) providing adequate evidence that the technological purpose as claimed by the applicant is valid.

The benefits of the action of the pullulanase in starch processing for glucose syrups production and other starch hydrolysates are:

- efficient degradation of starch
- increasing the substrate availability for other enzymes
- enabling higher yield of the substrate (dextrins) used for further processing and production of syrups.

The physical and chemical properties of the enzyme preparation are presented in Table 2.

Table 2 *Pullulanase enzyme preparation physical/chemical properties*

[1] The process of breaking down complex carbohydrates into simple sugars.

Physical/chemical properties of commercial enzyme preparation	
Enzyme activity (pullulanase units)	1350 NPUN/g
Appearance	The enzyme concentrate is formulated into a final enzyme preparation. The enzyme concentrate may be intended for a single enzyme preparation or a blend with other food enzymes and formulated as a yellow to light brown liquid product depending on the characteristics of the intended food process in which it will be used.
Temperature optimum	Pullulanase is active at temperatures up to approximately 60 °C with an optimum around 50 °C at pH 5.5, and within a pH range of 4 to 7 with an optimum of pH 6 at 30 °C.
Thermal stability	30 minutes incubation at pH 5, Pullulanase exhibited full activity up to 30 °C, above which the activity rapidly declines with no activity remaining at ≥70 °C.

2.3 Manufacturing process

2.3.1 Production of the enzyme

Microbial enzymes 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, may be produced as a liquid, semi-liquid or dried product. 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 2018).

Novozymes' pullulanase is produced by submerged fermentation the steps being, inoculum, seed fermentation, main fermentation followed by the recovery stage involving pre-treatment, primary separation, filtration, concentration, evaporation, preservation and stabilization. A detailed manufacturing process was provided within the application. The production is manufactured in accordance with current Good Manufacturing Practice and the quality management system used in the manufacturing process complies with ISO 9001:2015.

FSANZ concludes that sufficient information has been provided on the manufacturing process and the quality assurance system implemented by Novozymes to ensure there are no safety issues due the production process.

2.3.2 Specifications

Internationally recognised specifications are available for enzyme preparations used in food production. These have been established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2006) and the Food Chemicals Codex (FCC, 2022). These specifications

are included in the primary sources listed in section S3—2 of Schedule 3 of the Code and enzymes used as a processing aid must meet either of these specifications. 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.

Table 3 provides a comparison of the analysis of three different batches of Novozymes' pullulanase with international specifications established by JECFA and Food Chemicals Codex, as well as those in the Code where applicable. Based on these results, the enzyme meets all relevant specifications.

Evidence has been provided and assessed that the enzyme preparation does not contain any known food allergens.

Table 3: Comparison of Pullulanase to JECFA, Food Chemicals Codex, and Code specifications for enzymes

Analysis	*Analysis provided by manufacturer*	*Specifications*		
		JECFA (2006)	*Food Chemicals Codex (FCC, 2020)*	*Australia New Zealand Food Standards Code (section S3—4)*
Lead (mg/kg)	ND (LOD <0.5)	≤ 5	≤ 5	≤2
Arsenic (mg/kg)	0.13 (LOD < 0.1)	-	-	≤1
Cadmium (mg/kg)	ND (LOD < 0.05)	-	-	≤1
Mercury (mg/kg)	ND (LOD < 0.03)	-	-	≤1
Coliforms (cfu/g)	<10	≤30	≤30	-
***Salmonella* (in 25 g)**	ND	Absent	Negative	-
***E. coli* (in 25 g)**	ND	Absent	-	-
Antibiotic activity	ND	Absent	-	-

ND: Not detected; LOD: Limit of detection; CFU: Colony-forming unit

Note: Analysis was performed on three batches

2.4 Food technology conclusion

Analysis of the evidence provides adequate assurance that the proposed use of the enzyme, at a level not higher than necessary to achieve the desired enzyme reaction under GMP usage levels, is technologically justified.

Pullulanase performs its technological purpose during the production of food and is not performing a technological purpose in the final food. It is therefore appropriately categorised as a processing aid as defined in the Code.

There are relevant identity and purity specifications in the Code that enzyme preparations containing this enzyme would need to meet if its use is approved. Analytical data for three representative batches of the enzyme was provided in the application, showing compliance with the purity criteria of the specification.

3 Safety assessment

The objectives of this safety assessment are to evaluate any potential public health and safety concerns that may arise from the use of this enzyme, produced by this microorganism, as a processing aid.

Some information relevant to this section is Confidential Commercial Information (CCI), so full details cannot be provided in this public report.

3.1 History of use

3.1.1 Host organism

Bacillus subtilis

There is a long history of safe industrial use of *B. subtilis* as a microorganism to produce enzymes for food processing in many countries including Australia. Schedule 18 to Standard 1.3.3 of the Code currently permits the enzyme pullulanase derived from *B. subtilis* and *B. subtilis* containing the gene for pullulanase from *B. acidopullulyticus*.

B. subtilis is a class 1 organism and is generally considered to be non-pathogenic and non-toxigenic and one of ten host organisms for Tier 1 exemption under US Environmental Protection Agency regulations (EPA, 1996). It has been affirmed as GRAS by US FDA and covered in the regulations under 21 CFR 184.1148 and 21 CFR 184.1150. JECFA has conducted a technical review of *B. subtilis* as a safe strain for enzyme production which confirms its safety (FAO/WHO, 2006).

Data provided with the application confirmed the identity of the production strain as *B. subtilis* which was derived from the parent strain *B. subtilis* A164 obtained from The American Type Culture Collection. The production organism is eligible for the determination as a safe strain under the safe strain lineage concept of Pariza and Johnson (2001). The applicant has previously used the same parent strain for enzyme production that have been approved by FSANZ.

The microbial quality of the final enzyme preparation meets the specifications required by JECFA (2006) and there is no antimicrobial activity detected for the enzyme. The stability of the production strain is demonstrated phenotypically through consistent batch parameters and application of suitable microbiological controls through production. The organism was not detected in final product in three independent fermentation batches.

3.1.2 Gene donor organisms

Bacillus deramificans

The application dossier includes reference to one donor species, namely *Bacillus deramificans* where the gene to produce pullulanase enzyme was derived.

3.2 Characterisation of the genetic modification(s)

3.2.1 Description of DNA to be introduced and method of transformation

An expression cassette containing the pullulanase gene was introduced into the *B. subtilis* host strain's genome, producing the production strain. The pullulanase gene including the ribosome binding site and signal peptide sequence was derived from *B. deramificans* and

was placed under the control of an engineered promotor and the native pullulanase terminator (Figure 1). Data provided by Novozymes and analysed by FSANZ confirmed the expected pullulanase amino acid sequence.

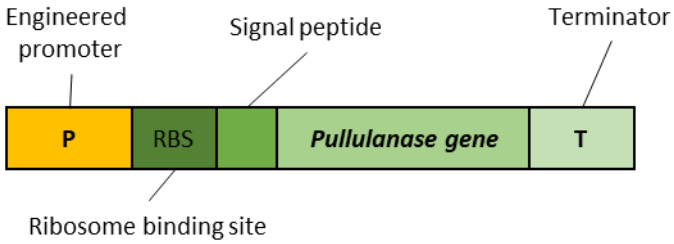

Figure 1: Pullulanase gene expression cassette.

A vector contaning the pullulanase expression cassette and antibiotic resistance gene was used to transform the host strain. These sequences were inserted into the host's genome at specific integration sites. A second vector was then used to remove the antibiotic resistance gene introduced during the integration of the pullulanase expression cassette. The final production strain was selected based on rapid growth and high pullulanase activity.

3.2.2 Characterisation of the inserted DNA

Data provided by Novozymes confirmed the presence of the inserted DNA in the production strain. The applicant also provided the results of genome sequencing which confirmed the absence of antibiotic resistance genes in the production strain.

3.2.3 Stability of the production organism and inheritance of the introduced DNA

The assessment confirmed the inserted gene is 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 introduced pullulanase gene, the applicant provided enzyme activity and protein synthesis data from large-scale fermentation of the production strain. These data confirmed that the pullulanase gene is expressed over multiple generations and is stable.

3.3 Safety of pullulanase

3.3.1 History of safe use

Multiple pullulanase enzymes from other microbial sources are currently permitted as processing aids in Schedule 18 of the Code. The Applicant has indicated that their pullulanase enzyme is used as a processing aid during the processing of starch-containing raw materials in a range of countries where there is a general approval of enzymes for food manufacture, although no information on the length of use was provided.

3.3.2 Bioinformatic assessment of enzyme toxicity

The Applicant performed a search for sequence homology of the pullulanase enzyme to known toxins using the UniProt[2] database. The search was performed in 2021. No significant homologies were found.

3.3.3 Evaluation of toxicity studies

Toxicity studies were performed on two representative pullulanase liquid enzyme concentrate batches (PPY27880 and PPY6454).

Animal studies

13-week oral toxicity study in rats (Scantox 2000) Regulatory status: GLP; conducted in accordance with OECD Test Guideline (TG) 408

Pullulanase (Batch No. PPY6454; 11.9% TOS) was administered to Sprague Dawley rats (10/sex/group) by oral gavage at doses of 0, 128.5, 387 or 1285 mg TOS/kg bw/day for 13 weeks. Water was used as the vehicle control. Animals were observed daily. Body weight, food consumption and detailed clinical examinations for signs of toxicity were recorded weekly. Ophthalmological examination was conducted on all test animals prior to treatment and on animals in the control and high dose groups at study termination. Animals were assessed for sensory reactivity to various stimuli, grip strength and motor activity in week 11 of treatment. Samples were collected for haematology, clinical chemistry and urinalysis at the end of the study. Gross pathology, measurement of organ weights and histopathological examination was conducted on all animals at termination.

No treatment-related deaths occurred during the study. One female in the high dose group died due to a dosing error (intratracheal administration of the test item). There were no treatment-related clinical signs and no differences were observed in the stimulus-induced reaction and open field tests. No treatment-related adverse effects on body weight, body weight gain, food consumption, ophthalmology, haematology, clinical chemistry or urinalysis parameters were observed. A dose-dependent increase in specific gravity was observed in the urine of males, but was not considered to be of significance as there were no other kidney-related findings. The urinary nitrite concentration was significantly higher in high dose females compared with controls. This was considered incidental as it only occurred in one sex, and potentially due to excretion of degradation products of the test item which contained nitrogen. There were no treatment-related changes in organ weights, gross or microscopic pathology.

It was concluded that the no observed adverse effect level (NOAEL) in this study was 1285 mg TOS/kg bw/day, the highest dose tested.

[2] UniProt database: https://www.uniprot.org/

Genotoxicity

Bacterial reverse mutation test (Novozymes 2008) Regulatory status: GLP; conducted in accordance with OECD TG 471

The potential mutagenicity of pullulanase (Batch No. PPY27880; 11.9% TOS) was evaluated in *Salmonella enterica* ser. Typhimurium strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* WP2uvrA. Water was used as the vehicle control. Test concentrations ranged from 156 – 5000 µg/plate. The study was conducted by the plate incorporation method with and without metabolic activation using rat liver homogenate (S9). Positive controls in the absence of S9 were sodium azide (TA1535 and TA100), 2-nitrofluorene (TA98), 9-aminoacridine (TA1537) and 1-methyl-N′-nitro-N-nitrosoguanidine (WP2uvrA). In the presence of S9 2-aminoanthracene was used as the positive control. Two independent experiments were conducted.

No cytotoxicity was observed following treatment with pullulanase. In the first experiment no increases in the number of revertant colonies compared with negative controls were observed following treatment with the test item. In the second experiment, small but significant increases in revertant colony counts were observed at the highest dose level in tests with the strain TA1535 with and without S-9, TA98 and the *E. coli* strain without S-9. Tests with these conditions were repeated a third time and all results were clearly negative. The study authors noted that crude fermentation preparations such as that of the test item contain low concentrations of free amino acids such as histidine and tryptophan, and suggested the increases in revertant colony counts in the second experiment may have been due to a feeding effect from the free amino acids present in the test item. All positive controls treatments showed the anticipated increases in mutagenic activity demonstrating the validity of the assay.

It was concluded that pullulanase was not mutagenic under the conditions of this assay.

In vitro mammalian micronucleus test in human lymphocytes (Covance 2008) Regulatory status: GLP; conducted in accordance with OECD draft TG 487

The potential of pullulanase (Batch No. PPY27880; 11.9% TOS) to induce micronuclei formation in mammalian cells was tested using human peripheral blood lymphocytes. Cells were exposed to pullulanase for 24 hours without metabolic activation (S9), or for 3 hours in the presence or absence of S9 followed by culture for a further 21 hours. Pullulanase concentrations up to 5000 µg/mL were evaluated for all conditions. The vehicle control was water. Mitomycin C and vinblastine were used as clastogenic and aneugenic positive controls, respectively, in the absence of S9. Cyclophosphamide was the clastogenic positive control in the presence of S9.

No increases in the frequency of micronuclueated binucleated cells (MNBN) compared with vehicle controls were observed following treatment with pullulanase in the presence or absence of S9. The positive controls produced significant increases in MNBN cells, validating the sensitivity of the test system.

It was concluded that pullulanase was not clastogenic or aneugenic under the conditions of this study.

3.3.4 Potential for allergenicity

The applicant conducted searches in October 2021 for sequence homology assessment of the pullulanase enzyme to known allergens in the Food Allergy Research and Resource Program (FARRP) allergen protein database[3] using the following search criteria:

- 35% identity over 80 amino acids
- 35% identity over 80 amino acids (scaled search)
- Full length alignment
- 100% identity over 8 amino acids

No matches to known allergens were identified in any of these searches.

3.3.5 Assessments by other regulatory agencies

The pullulanase enzyme is approved for use in Denmark. The safety assessment undertaken by the Danish authorities is not available to FSANZ.

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 pullulanase 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 or a NOAEL to estimate a margin of exposure (MOE) for risk characterisation purposes. Whilst the budget method 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 international regulatory bodies and the FAO/WHO Joint 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 bw/day
- 50% of solid food is processed
- all processed foods contain 25% starch (or starch-derived) dry matter
- the maximum physiological requirement for liquid is 100 mL/kg bw/day (the standard level used in a budget method calculation for non-milk beverages)
- 25% of non-milk beverages are processed
- all processed beverages contain 12% starch (or starch-derived) dry matter

[3] AllergenOnline: http://www.allergenonline.org/

- the densities of non-milk beverages are ~ 1
- all solid foods and non-milk beverages contain the highest use level of 67 mg TOS/kg starch (or starch-derived) dry matter
- 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 0.41 mg TOS/kg bw/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 bw/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 2020). 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 0.62 mg TOS/kg bw/day.

Both the FSANZ and applicant's estimates of the TMDI will be overestimates of the dietary exposure given the conservatisms in the budget method. This includes the assumption that all of the TOS from the enzyme preparation remains in the final foods and beverages whereas the applicant has stated that the enzyme is likely to either be inactivated or removed during processing. If any inactivated enzyme remained after processing, it would be present in insignificant quantities and perform no function in the final food to which the ingredient is added.

4 Discussion

No public health and safety concerns were identified in the assessment of pullulanase from GM *Bacillus subtilis* (gene donor: *Bacillus deramificans*). The production organism is neither pathogenic nor toxigenic.

Analysis of the GM production strain confirmed the presence and stability of the inserted DNA.

Bioinformatics analysis found no significant homology of the pullulanase enzyme with any known toxins or food allergens. Pullulanase was not genotoxic *in vitro*, and no adverse effects were found in a sub-chronic oral toxicity study in rats. The NOAEL in this study was 1285 mg TOS/kg bw/day, the highest dose tested.

The TMDI of the TOS from the pullulanase enzyme preparation was calculated to be 0.62 mg TOS/kg bw. A comparison of the NOAEL and the TMDI results in a Margin of Exposure (MOE) of approximately 2100.

5 Conclusion

Based on the reviewed data, it is concluded that in the absence of any identifiable hazard, an acceptable daily intake 'not specified' is appropriate.

6 References

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