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

6 June 2019
[82–19]

Supporting document 1

Risk and technical assessment report – Application A1164
Pullulanase from *Bacillus licheniformis* as a processing aid (enzyme)

Executive summary

The purpose of the application is to seek amendment of Schedule 18 – Processing Aids of the Australia New Zealand Food Standards Code (the Code) to include the enzyme pullulanase (EC 3.2.1.41), from a genetically modified strain of *Bacillus licheniformis,* containing the pullulanase gene from *Bacillus deramificans* (BMP 139), for use in brewing and starch processing.

The evidence presented to support the proposed use of the enzyme provides adequate assurance that the enzyme, in the form and recommended amounts for particular applications, is technologically justified and has been demonstrated to be effective in achieving its stated purpose. The enzyme meets international purity specifications.

B. licheniformis is non-pathogenic and has a history of safe use in the production of enzyme processing aids, including several that are already permitted in the Code.

Pullulanase from *B. licheniformis* was not genotoxic *in vitro* or *in vivo*, and did not cause adverse effects in short-term toxicity studies in rats. The no observed adverse effect level (NOAEL) in a 13-week repeated dose oral toxicity study in rats was the highest dose tested, 2500 mg/kg bw/day or 246 mg/kg bw/day on a total organic solids (TOS) basis. The applicant's estimated theoretical maximal daily intake (TMDI) based on the proposed uses is 0.049 mg/kg bw/day TOS. A comparison of these values indicates that the Margin of Exposure (MOE) between the NOAEL and TMDI is more than 5000.

Bioinformatic analysis indicated that the pullulanase enzyme has no homology to known protein allergens or toxins and on this basis is unlikely to pose an allergenicity or toxicity concern. The enzyme product may contain traces of soy and wheat protein from the culture medium used to grow the production organism, however risk management measures exist to protect wheat-allergic or soy-allergic individuals. These involve the declaration of these substances on product labels.

Based on the reviewed toxicological data it is concluded that in the absence of any identifiable hazard, an Acceptable Daily Intake (ADI) of 'not specified' is appropriate. A dietary exposure assessment was therefore not required.

There are no public health and safety concerns associated with the use of pullulanase from *B. licheniformis* when used as a food processing aid.

Table of contents

1 Introduction

DuPont Australia Pty Ltd has made an application to FSANZ seeking permission to use the enzyme pullulanase (EC 3.2.1.41) from a genetically modified (GM) strain of *Bacillus licheniformis,* containing the pullulanase gene from *Bacillus deramificans,* as a processing aid in brewing and starch processing.

This pullulanase has already been authorised for use in France (2011)[1], Denmark and Japan (2001). The United States Food & Drug Administration (FDA) determined this enzyme to be GRAS[2] (US GRAS Notice No. GRN000072, 2001). In addition, this enzyme has been evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). Following the 74th meeting of JECFA, new specifications were prepared and published in FAO JECFA Monograph 11 (WHO 2011) for this enzyme, and JECFA established an ADI 'not specified'.

Currently, Schedule 18 of the Australia New Zealand Food Standards Code (the Code) includes permission for the use of pullulanase (EC 3.2.1.41) derived from a non-GM strain of *B. licheniformis,* as well as pullulanase derived from five other GM and non-GM organisms. *B. licheniformis* (both GM and non-GM) is the permitted production organism for seven other enzymes. However, there is currently no permission to use this pullulanase from a GM strain of *B. licheniformis,* containing a pullulanase gene from *B. deramificans.* Therefore, any application to amend the Code to permit the use of this particular enzyme as a food processing aid requires a pre-market assessment.

1.1 Objectives of the assessment

The objectives of this risk and technical assessment were to:

- determine whether the proposed purpose is clearly stated and that the enzyme achieves its technological function in the quantity and form proposed to be used as a food processing aid

- evaluate any potential public health and safety concerns that may arise from the use of this enzyme, produced by a GM organism, as a processing aid. Specifically by considering the:

  - history of use of the gene donor and production organisms
  - 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 applicant provided relevant information regarding the identity of the enzyme, and this has been verified using an appropriate enzyme nomenclature reference (IUBMB 2017).

[1] https://www.legifrance.gouv.fr/affichTexte.do?cidTexte=LEGITEXT000020667468
[2] Generally Recognized As Safe

Accepted IUBMB[3]/common name:	pullulanase
Systematic name:	pullulan 6-α-glucanohydrolase
IUBMB enzyme nomenclature:	EC 3.2.1.41
CAS[4] number:	9075-68-7
Other names:	limit dextrinase (erroneous); amylopectin 6-glucanohydrolase; bacterial debranching enzyme; debranching enzyme; α-dextrin endo-1,6-α-glucosidase; R-enzyme; pullulan α-1,6-glucanohydrolase.
Reaction:	Hydrolysis of (1→6)-α-D-glucosidic linkages in pullulan, amylopectin and glycogen, and in the α- and β-limit dextrins of amylopectin and glycogen.

2.1.2 Technological purpose of the enzyme

Pullulanase catalyses the hydrolysis of the α-1,6 glucosidic linkages in amylopectin and pullulan present in raw materials like starch, converting these branched polysaccharides into smaller fermentable sugars as part of the saccharification[5] process (Figure 1). It is this reaction that is of particular technological importance in brewing and starch processing.

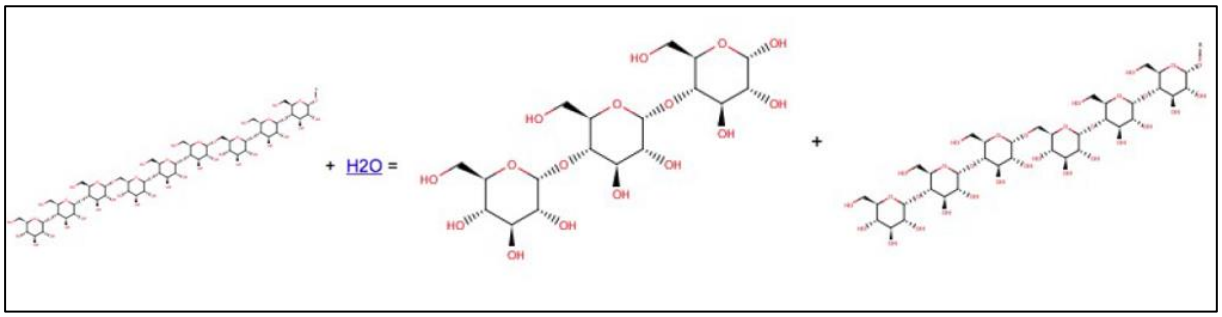

Figure 1 *Representation of hydrolysis reaction of pullulan catalysed by pullulanase*

The action of pullulanase on large, branched polysaccharides results in improved properties of the raw materials; better and/or more consistent product characteristics, and more effective production processes. Specifically, in brewing, the enzyme increases the amount of fermentable carbohydrates and the fermentation rate. In starch processing, the enzyme facilitates a higher glucose yield and less isomaltose[6] formation.

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 and brewing is supported by scientific literature (Hii et al. 2012; Huang & Tang 2017), and patent applications, providing adequate evidence that the technological purpose as claimed by the applicant is valid.

[3] International Union of Biochemistry and Molecular Biology.
[4] Chemical Abstracts Service.
[5] The process of breaking down complex carbohydrates into simple sugars.
[6] A disaccharide similar to maltose but with a α-1,6 linkage instead of the α-1,4 linkage.

The physical and chemical properties of the enzyme preparation are presented in Table 1.

Table 1 *Pullulanase enzyme preparation physical/chemical properties*

Physical/chemical properties of commercial enzyme preparation	
Enzyme activity	1200 ASPU[7]/g enzyme protein
Appearance	Amber coloured liquid
Temperature optimum Temperature range of activity	Approximately 52–62°C 30-80°C
Thermal stability	No residual activity after incubation at >67°C for 30 min
pH optimum	pH 4-5
Storage stability	>2 years at 4°C

Use of commercial enzyme preparations are typically in accordance with Good Manufacturing Practice (GMP), whereby use is at a level that is not higher than the level necessary to achieve the desired enzymatic reaction. The applicant provides efficacy examples for brewing and starch processing. In brewing, an increase in the real degree of fermentation (RDF) was observed when pullulanase was applied at 8 kg/t of grist[8], when compared to RDF in the control. In starch processing, trials involving the addition of pullulanase at 0.075 ASPU/g solids showed an increased glucose yield, compared to the control with glucoamylase but no pullulanase. Ultimately, individual manufacturers will fine-tune the enzyme use levels depending on their specific application, the type and quality of the raw materials used, and the process conditions.

2.1.3 Technological justification for the enzyme

As outlined above, the application suggests that the technological need for the enzymatic conversion of large, branched polysaccharides like amylopectin and pullulan, with the help of pullulanase, results in improved properties of the raw materials; better and/or more consistent product characteristics, and more effective production processes.

Specifically, the applicant stated that in brewing, the benefits of using pullulanase may include:

- Increased flexibility in the choice of raw materials
- An increase in the quantity of fermentable carbohydrates
- An increase to the rate of fermentation
- An improvement in the levels of attenuation (i.e. in the RDF)
- A more consistent production process.

In starch processing, the applicant stated that pullulanase may provide the following benefits:

- An increase in glucose yield
- A reduction in the saccharification time
- Saccharification at higher solid, thereby increasing production plant capacity

[7] The activity of pullulanase is defined in ASPU: acid stable pullulanase units. This unit cannot be defined in exact terms; rather, it relies on a specific assay and enzyme standard.
[8] Coarsely ground malt, primarily composed of starch.

- Reduced formation of reversion products (undesirable by-products of the manufacture) and isomaltose
- Increased flexibility around the manufacturing process.

In brewing, pullulanase is added at the mashing step, where it helps to break down the malt's starch into fermentable carbohydrates. It will be denatured in subsequent steps of lautering and boiling.

In starch processing, pullulanase is denatured after performing its technical function during saccharification. The syrup is heated during a dedicated inactivation step or removed during subsequent carbon or ion exchange treatments. As such, the enzyme does not have any residual enzymatic activity in the final syrups or the foods to which they are added.

2.2 Manufacturing process

2.2.1 Production of the enzyme

The enzyme is produced by submerged fed-batch pure culture fermentation of the GM strain of *B. licheniformis*.

The production process comprises three main steps: fermentation (growth of microorganism and production of enzyme), recovery (separation of cell mass from enzyme and concentration/purification of enzyme) and formulation (preparation of a stable final product).

Fermentation consists of three stages: laboratory propagation of the culture, seed fermentation, and primary fermentation. Fermentation is carried out in sealed vessels to prevent the release of the production organism and/or entry of foreign microorganisms and other contaminants. Biosynthesis of pullulanase occurs under well controlled process conditions governing the pH, temperature, air flow, agitation and oxygen content.

Once the fermentation is complete, the recovery process is initiated. The fermentation broth is transferred to processing tanks ready for separation and concentration steps. The separation step involves removing the cell debris from the liquid of the fermentation medium containing the desired enzyme. This may be achieved by filtration or centrifugation, or a combination of both. The remaining liquid containing the enzyme is filtered and concentrated. During several filtration steps, low molecular weight compounds, colour and other insoluble substances are removed. Diafiltration (a technique that uses ultrafiltration membranes) helps ensure that the enzyme solution achieves the desired enzyme activity prior to formulation.

The resultant ultrafiltered concentrate solution is ready for formulating into a dry food enzyme preparation using any one of the common drying methods, or is stabilised (using glycerol for example) to produce a liquid product. It can then be packaged and sold commercially.

The results of biological purity analytical tests provided by the applicant[9] were 'negative to test per gram of product' for the production strain, for a sample of concentrate used to make GC 797 (the test article).

The production of the enzyme is in accordance with GMP, as outlined in the United States Pharmacopeial Convention (USPC) Food Chemicals Codex 11th edition (USPC 2018) and the JECFA Compendium of Food Additive Specifications (JECFA 2017).

Depending on the intended use, the enzyme preparation may be sold under the commercial name Optimax® L-1000. The composition of this preparation has been provided by the

[9] These analytical results were provided as part of a file of confidential commercial information.

applicant, with the request that it be treated as confidential commercial information - CCI. FSANZ has assessed the formulation ingredients and can confirm that they are compatible with usage in food, particularly in their intended purpose. The main steps of the manufacturing process are shown in Figure 2 below taken from the application.

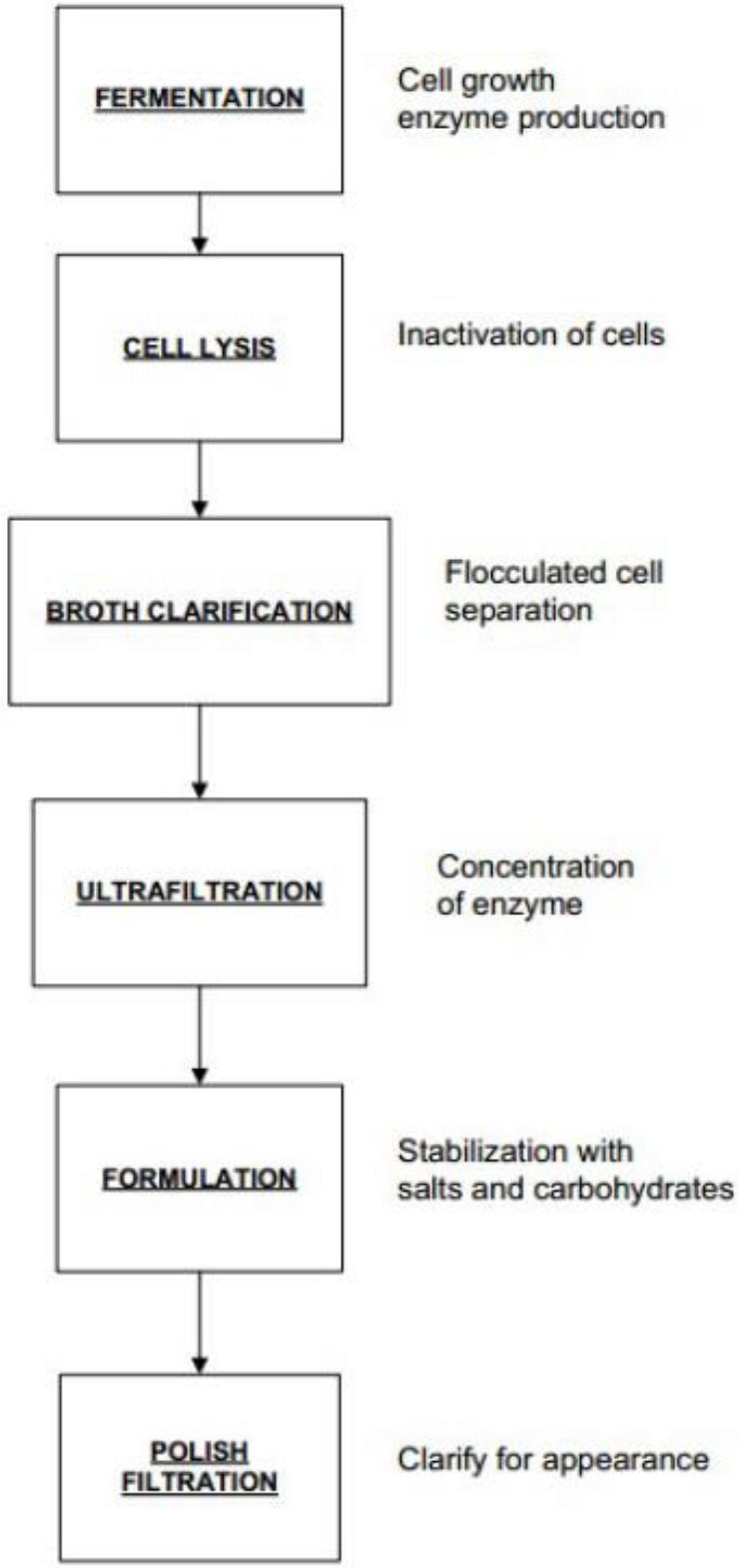

Figure 2 *Production process of pullulanase food enzyme from fermentation*

2.2.2 Allergen considerations

The application contained a list of raw materials used in the fermentation and recovery process (with the request that it be treated as CCI, as it is considered to be proprietary). However, the applicant has provided an allergen declaration of the enzyme concentrate in Appendix A of the application. Wheat (with glucose being the component) and soy are allergens (and products thereof) that have been used in the recovery process or in the formulation of the enzyme product and may be carried over in to the final enzyme preparation.

2.2.3 Specifications

The JECFA Compendium of Food Additive Specifications (2017) and the Food Chemicals Codex 11th edition are international specifications for enzymes used in the production of food. These are primary sources of specifications listed in Schedule S3—2 of the Code. Enzymes need to meet these specifications. Schedule 3 also includes specifications for heavy metals (section S3—4) if they are not already detailed within specifications in sections S3—2 or S3—3.

Table 2 provides a comparison of the analysis of six batches of pullulanase enzyme product from the GM *B. licheniformis* with the international specifications established by JECFA and Food Chemicals Codex, as well as those detailed in the Code (as applicable). Based on the above analytical results, the enzyme preparation meets international and Code specifications for enzyme preparations used in the production of food.

Table 2 *Analysis[1] of DuPont enzyme pullulanase compared to JECFA, Food Chemicals Codex, and Code specifications for enzymes*

Analysis & batch number	DuPont analysis of individual batch	Specifications		
		JECFA	*Food Chemicals Codex*	*Australia New Zealand Food Standards Code (section S3—4)*
Lead (mg/kg)		≤ 5	≤ 5	≤2
1662762238	0.01			
1682653516	<0.12			
1683010202	<0.01			
Arsenic (mg/kg)		-	-	≤1
1662762238	0.02			
1682653516	<0.24			
1683010202	<0.01			
Cadmium		-	-	≤1
1662762238	<0.01			
1682653516	<0.05			
1683010202	0.001			
Mercury				≤1
1662762238	<0.01			
1682653516	<0.005			
1683010202	<0.005			

Analysis & batch number	DuPont analysis of individual batch	Specifications		
		JECFA	*Food Chemicals Codex*	*Australia New Zealand Food Standards Code (section S3—4)*
Coliforms (cfu/g)		≤30	≤30	-
F970695	Negative			
F970696	Negative			
F979012	Negative			
Salmonella (in 25 g)		Absent	Negative	-
F970695	Absent			
F970696	Absent			
F979012	Absent			
E. coli (in 25 g)		Absent	-	-
F970695	Absent			
F970696	Absent			
F979012	Absent			
Antimicrobial activity		Absent	-	-
F970695	Negative			
F970696	Negative			
F979012	Negative			

[1] three different batches were analysed for heavy metals and microbiological assays, respectively.

2.3 Food technology conclusion

The use of this pullulanase in brewing and starch processing is clearly articulated in the application. This enzyme will be used to hydrolyse linkages in branched polysaccharides to produce smaller fermentable sugars as part of the saccharification process.

DuPont's pullulanase is sourced from a GM strain of *B. licheniformis* and will provide food manufacturers with an alternative pullulanase product on the market.

The evidence presented to support the proposed use of the enzyme provides adequate assurance that the enzyme, in the form and recommended amounts for particular uses, is technologically justified and has been demonstrated to be effective in achieving its stated purpose. The enzyme meets international purity specifications.

3 Safety assessment

3.1 History of use

3.1.1 Host organism

B. licheniformis is widely distributed in the environment and is commonly found in soil. *B. licheniformis* is generally considered to be non-pathogenic to humans, as no cases suggest any invasive properties. There is a long history of safe industrial use of *B. licheniformis* for the production of food enzymes (de Boer et al 1994). The host strain is closely related to a type strain of *B. licheniformis*.

FSANZ has previously assessed the safety of *B. licheniformis* as the source organism for a number of enzymes used as processing aids. Schedule 18 to Standard 1.3.3 of the Code permits the use of the following enzymes derived from *B. licheniformis*: α-amylase, chymotrypsin, endo-1,4-beta-xylanase protein engineered variant, β-galactosidase, glycerophospholipid cholesterol acyltransferase protein engineered variant, maltotetraohydrolase protein engineered variant, pullulanase and serine proteinase.

3.1.2 Gene donor organisms

The gene sequence for pullulanase is from *B. deramificans* and has been shown to be non-pathogenic (Modderman and Foley 1995).

3.2 Characterisation of the genetic modification(s)

3.2.1 Description of DNA to be introduced and method of transformation

Recipient strain Bra7 is an industrial strain used in alpha-amylase production for over 20 years. It was modified through a series of targeted recombination events each of which resulted in the creation of intermediate strains until production strain BMP139 was generated. These events included deletion of the *spoIIAC* gene, essential for sporulation, and genes encoding several proteases and unwanted peptides, such as endogenous alpha-amylase. These modifications are designed to improve efficiency and safety (the modified strain has a limited survival capacity in the environment) and do not raise any safety concerns.

The expression plasmid used for the final transformation contained two expression cassettes that permitted integration at a specific locus of the BMP139 genome by homologous recombination. The first cassette comprised the unmodified pullulanase gene from *B. deramificans*, regulated by an endogenous (Bra7) promoter, signal sequence and terminator. The second cassette comprised an endogenous chloramphenicol acetyltransferase (*cat*) gene, with 5′ flanking sequences whose presence permitted the increase of pullulanase copy numbers during selection of the production strain. This gene also acted as a selectable marker. Chloramphenicol is not used In the fermentation medium during the production of the pullulanase so there is no selective amplification of chloramphenicol resistant bacteria. Further to this, data submitted by the applicant indicate that *B. licheniformis* BMP139 is not detected in the final enzyme preparation to be used as a food processing aid. A multi-step recovery of the purified enzyme following submerged fed-batch pure culture fermentation should remove the organism.

3.2.2 Characterisation of inserted DNA

Southern blot analysis of chromosomal DNA from BMP139, using probes for the vector backbone and for the pullulanase gene, confirmed the only DNA incorporated into the genome is from the expression cassettes.

Whole genome sequencing (WGS) of strain BMP139 was used to characterise the insert. Essentially, WGS is used to obtain sequence reads in a number of individual overlapping DNA segments called contigs. The reads in individual contigs are then assembled to provide full coverage of the whole genome. While this can be done using older technology such as Sanger sequencing, the more recently developed next generation sequencing (NGS) platforms, of which there are several (see e.g. Quainoo et al 2017) have revolutionised sequencing and enabled faster, more accurate, automated characterisation of DNA compared to these older methods. NGS has proven to be particularly useful in the area of

pathogen genotyping (Quainoo et al 2017).

Once the full genomic sequence of BMP139 was obtained by NGS, it was searched *in silico* with the entire expression plasmid sequence. This confirmed a) the absence of vector backbone, b) the inserted genetic material has the same sequence as that found in the two expression cassettes i.e. the cassettes have gone into the BMP139 genome intact and without rearrangement, c) the cassettes have inserted at the expected locus and d) there are multiple tandem copies of the cassettes.

In order to test whether the cassettes may have recombined at the locus where the promoter used to drive the pullulanase gene is located, a polymerase chain reaction (PCR) analysis was done of the BMP139 genome and a control (an unrelated strain known to have an intact locus), using primers that would amplify a particular sized fragment if no expression cassettes were integrated. The same sized fragment was obtained for BMP139 and the control.

3.2.3 Genetic stability of the inserted gene

The stability of the insert was demonstrated by NGS of samples grown in flasks mimicking commercial fermentation conditions over 60 generations. Genomic DNA was isolated and sequenced using Illumina MiSeq®[10] technology (see also e.g. Quainoo et al 2017 for a discussion of the Illumina platform) such that there was > 99.99% coverage of the genome and an average read depth of at least 93 (where read depth is the number of times any one base of the genome is expected to be independently sequenced). No change was observed, in the presence of the expression cassette at the expected locus, between samples at generation 0 and replicates taken from generation 60. Additionally, no change in flanking sequence was found over this time. These results demonstrate that the insertion cassettes are stably maintained through generations during the fermentation process.

3.3 Safety of pullulanase

3.3.1 History of safe use of the enzyme

Pullulanase derived from *B. deramificans* in *B. licheniformis* has been used in the USA since 1999 for saccharification in starch processing, and for similar purposes in Europe, Latin America, China and other Asian countries thereafter. No reports of adverse effects from the use of pullulanase in these countries have been identified.

3.3.2 Toxicology studies in animals

The applicant submitted one 13-week toxicity study in rats as part of the application. In addition, a published scientific paper reporting the results of a 28-day toxicity study in rats conducted with a pullulanase enzyme preparation from *B. licheniformis* containing the pullulanase gene from *B. deramificans* was included in the references listed in the application (Modderman and Foley 1995). Literature searches by FSANZ did not identify any additional relevant studies.

In the studies reported by Modderman and Foley the enzyme preparation was concentrated by ultrafiltration and freeze-dried. The enzyme preparation had an activity of 1300 ASPU/g, but the total organic solid (TOS) content was not reported.

The test item in the study submitted by the applicant was an ultrafiltrate concentrate with a TOS content of 9.82%, and an activity of 4251 ASPU/g. The test substance in the applicant's

[10] https://sapac.illumina.com/systems/sequencing-platforms/miseq/specifications.html

study was a more concentrated version of the final product, but without the other formulation ingredients. None of the other components of the formulation give cause for concern at the very low levels of exposure that would result from the use of the enzyme as a processing aid.

28-day repeated dose dietary toxicity study in rats (Modderman and Foley 1995) Regulatory status: GLP; Conducted according to the US Food and Drug Administration (FDA) draft guidelines for subacute toxicity testing

Groups of 10 male and 10 female Crl:CD BR rats aged 5 weeks were given pullulanase mixed with the feed at concentrations of 0, 0.2, 1 and 5% of the feed for 28 days. These doses were selected based on a previous 2-week range-finding study in which no adverse effects were observed at dietary pullulanase concentrations of up to 5%. The average daily dose of pullulanase in the treatment groups was calculated to be 191, 950 and 4773 mg/kg bw/day, respectively, in males and 209, 1011 and 5153 mg/kg bw/day, respectively in females. Clinical signs were observed daily and body weights and feed consumption were recorded weekly. A neurobehavioural evaluation (functional observation battery) was conducted during the last week of the study. Haematology and blood chemistry analyses were performed on separate groups of five animals before the start of the study and on all animals at study termination. All animals underwent ophthalmoscopic examinations and urinalysis pre-test and at the end of the study. All animals were necropsied at the end of the study and selected organs were weighed. Microscopic examinations were conducted on all control and high-dose animals. When tissues from high-dose animals were found to have lesions, microscopic evaluations were also performed on those tissues in the lower dose groups.

All rats survived to the end of the study, and no treatment-related effects on clinical signs, neurobehavioural observations, food consumption, body weight, ophthalmoscopy, haematological, clinical chemistry, urinalysis or histopathologic evaluations were observed. Absolute and relative thymus weights were increased in males in the two highest dose groups. These changes were not considered to be of toxicological relevance as there was no difference in thymus weight between the mid- and high-dose groups, similar changes were not observed in females, and no corresponding histopathological or haematological changes were observed.

The no observed adverse effect level (NOAEL) in this study was 4773 mg/kg bw/day in males and 5153 mg/kg bw/day in females, the highest dose tested.

90-day repeated dose oral toxicity study in rats (Boyer 1999) Regulatory status: GLP; Conducted according to OECD test guideline (TG) 408

Pullulanase was administered by oral gavage to Sprague Dawley Crl:CD(SD)BR rats (10/sex/group) at doses of 0, 625, 1250 and 2500 mg/kg bw/day for 13 weeks. The vehicle control was water. Clinical observations were performed daily and body weight and food consumption were recorded weekly. Ophthalmology was conducted prior to treatment and in Week 13, and urine and blood were sampled for urinalysis, haematology and clinical chemistry analyses in Week 13. A functional observational battery and assessment of motor activity were performed after 11 weeks of treatment. Gross pathology and measurement of organ weights was conducted on all animals at study termination, and a histopathological examination was conducted on selected organs and tissues from the control and high-dose group animals. Any gross lesions identified in rats from the low- and mid-dose groups also underwent histopathological examination.

No mortality occurred during the study, and no treatment-related clinical signs were observed. Administration of pullulanase did not result in any adverse effects on body weight, body weight gain, food consumption, ophthalmology, functional observational battery and

motor activity parameters, haematology and biochemistry measurements. A statistically significant increase in urine sodium levels was found in males in the high dose group, and significant decreases in urinary calcium and phosphorus levels were seen in females administered 1250 (phosphorus only) and 2500 mg/kg bw/day. These changes were not considered to be toxicologically relevant in the absence of any correlating histopathological lesions. No treatment-related adverse effects on organ weights, gross pathology and histopathology were observed.

It was concluded that the NOAEL in this study was 2500 mg/kg bw/day, the highest dose tested. Based on a TOS content of 9.82% in the enzyme preparation tested, the NOAEL corresponds to 246 mg/kg bw/day TOS.

3.3.3 Genotoxicity assays

Two genotoxicity assays were submitted as part of the application. In addition, the references cited in the application included a published scientific paper reporting the results of *in vitro* and *in vivo* genotoxicity studies with an alternative pullulanase enzyme preparation (Modderman and Foley 1995). Literature searches by FSANZ did not identify any additional relevant studies.

The test item in the studies submitted by the applicant was the same as that used in the 13-week oral toxicity study in rats. The enzyme preparation used in the studies reported by Modderman and Foley was the same as that used in the 28-day toxicity study reported by these authors (see section 3.3.2).

Bacterial reverse mutation assay (Modderman and Foley 1995) Regulatory status: GLP; conducted according to the US FDA draft guidelines for genotoxicity testing

The potential mutagenicity of pullulanase was evaluated in *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and TA1538 with and without metabolic activation. Based on a preliminary range-finding step, the enzyme preparation (vehicle unspecified) was tested at concentrations up to 5000 µg/plate. The positive control for all strains in the presence of metabolic activation was 2-anthramine. In the absence of metabolic activation, positive controls were sodium azide for TA100 and TA1535, 4-nitro-o-phenylenediamine for TA98 and TA1538 and 9-aminoacridine for TA1537. The assay was repeated to provide confirmatory data.

No concentration-related increases in revertant colonies were observed in cultures treated with pullulanase in the first assay, with or without metabolic activation. In the confirmatory assay, a concentration-related increase in histidine revertant colonies/plate was observed for the TA1538 strain in the presence of metabolic activation. However, the increase in revertant colonies was less than three-fold higher than the solvent control value, and therefore did not meet the criteria for a positive response (≥ 3-fold increase). No other concentration-related or significant increases in mutation frequency were observed. Results with the positive controls were not reported.

It was concluded that pullulanase was not mutagenic in *S. typhimurium* under the conditions of this study.

Bacterial reverse mutation assay (Wagner and Walton 1999) Regulatory status: GLP; conducted according to OECD TG 471

The potential for pullulanase to induce mutations in bacteria was assessed in *S. typhimurium* strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* strain WP2*uvr*A. Following a dose range finding study in which no signs of toxicity or precipitation were observed at

concentrations up to 5000 µg/plate, concentrations of 100, 333, 1000, 3333 and 5000 µg/plate were tested in the presence and absence of a metabolic activation system (rat liver S9 mix) using the plate incorporation method. The vehicle and negative control was water. In the presence of S9 the positive control for all strains was 2-aminoanthracene, while in the absence of metabolic activation positive controls were 2-nitrofluorene for TA98, sodium azide for TA100 and TA1535, 9-aminoacridine for TA1537 and methyl methanesulfonate for WP2*uvr*A. All doses of the test item and controls were plated in triplicate, and the assay was performed twice in order to confirm the results of the first test.

No increases in the number of revertant colonies were observed in any of the test strains after treatment with pullulanase in the presence or absence of metabolic activation. Increases in the number of revertant colonies were found following treatment with the positive controls, confirming the validity of the test system.

It was concluded that pullulanase showed no evidence of mutagenic activity under the conditions of this assay.

Forward mutation assay in mouse lymphoma cells (Modderman and Foley 1995) Regulatory status: GLP; conducted according to the US FDA draft guidelines for genotoxicity testing

The ability of pulllulanase to induce gene and chromosomal mutations at the thymyidine kinase (*tk*) locus was tested using L5178Y mouse lymphoma cells from clone 3.7.2C. Following a range-finding test, the assay was conducted using pullulanase concentrations of 1-4, 4.4 and 5.0 mg/mL in the absence of metabolic activation, and at 4.0, 4.2, 4.6, 4.8 and 5.0 mg/mL in the presence of activation. Positive controls were cyclophosphamide and hycanthone in the presence and absence of metabolic activation, respectively. The solvent used was not specified. A portion of cells from each plate was used to determine cloning efficiency, and the remainder was treated with trifluorothymidine and cloned to determine mutant frequency and size. The assay was repeated to determine reproducibility using eight pullulanase concentrations ranging from 4.0 – 5.0 mg/mL, with and without metabolic activation.

In both assays, treatment with pullulanase did not result in an induced mutation frequency ≥ 70 X 10^{-6} in the presence or absence of metabolic activation. Results with the positive controls were not reported.

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

In vitro chromosomal aberration test in cultured human lymphocytes (Gudi and Schadly 1999) Regulatory status: GLP; conducted according to OECD TG 473

Pullulanase was tested for its ability to cause structural chromosomal aberrations in cultured mammalian cells using human peripheral blood lymphocytes. The assay was conducted in the presence and absence of metabolic activation (rat liver S9 mix). The vehicle and negative control was water, and positive controls were cyclophosphamide and mitomycin C in the presence or absence of S9, respectively.

Based on the findings of a preliminary toxicity assay in which doses of pullulanase up to 5000 µg/mL were tested, doses selected for the chromosomal aberration assay ranged from 25 – 500 µg/mL for the 4 hour exposure tests and from 7 – 200 µg/mL for the 20 hour exposure test. In the absence of metabolic activation, cells were treated for 4, 20 or 44 hours. In the presence of metabolism, cells were treated for 4 hours. Following the 4 hour exposure periods, cells were washed, suspended in fresh medium and incubated for an additional 16 or 40 hours. Colcemid was added to all cultures 2 hours prior to the scheduled

harvest. After harvesting, cells were fixed on slides and scored for cytotoxicity and chromosomal aberrations. A minimum of 200 metaphase spreads (100 per duplicate treatment condition) were examined and scored for chromatid-type and chromosome-type aberrations.

For all test conditions, three concentrations were evaluated for chromosomal aberrations. For the tests with a 20-hour culture period, the highest test concentration evaluated in the 4-hour exposure group without S9 was 100 µg/mL (54% mitotic inhibition), while in the 4-hour exposure with S9 the highest concentration evaluated was 200 µg/mL (59% mitotic inhibition). The highest concentration evaluated in the 20-hour non-activated exposure group was 50 µg/mL (55% mitotic inhibition). In the tests with a 44-hour culture period, the highest concentration evaluated in the 44-hour non-activated exposure group was 100 µg/mL (62% mitotic inhibition) and the highest concentration in the S9-activated 4-hour exposure period was 150 µg/mL (55% mitotic inhibition).

In all cultures treated with pullulanase there were no significant increases in the percentage of cells with structural or numerical chromosomal aberrations compared with the vehicle controls. The positive controls induced the expected significant increases in the number of cells with structural aberrations.

It was concluded that pullulanase did not induce chromosomal aberrations under the conditions of this assay.

Mouse bone marrow micronucleus and chromosomal aberration test (Modderman and Foley 1995) Regulatory status: GLP; conducted according to the US FDA draft guidelines for genotoxicity testing

The ability of pullulanase to induce chromosomal aberrations and/or micronucleus formation *in vivo* was assessed in Swiss-Webster mice. Groups of 15 male and 15 female mice were administered single i.p. doses of 500, 889, 1581, 2811 and 5000 mg/kg bw pullulanase, based on the results of range-finding studies. Vehicle (not specified) was administered to control animals (5/sex/group) and trimethylamine was administered as a positive control to mice (5/sex/group) at doses of 0.4 mg/kg bw (micronucleus test) and 0.2 mg/kg bw (chromosomal aberration study). Animals were observed twice daily. In each study, groups of 5 mice/sex/pullulanase dose group were killed at intervals of 12, 24 and 48 hours after treatment. Positive and vehicle controls were killed 24 hours after treatment. Slides were prepared from bone marrow cells obtained immediately after the animals were killed, stained with a Giemsa staining solution and evaluated for signs of toxicity and the presence of micronuclei or other chromosomal aberrations. In the micronucleus assay, the ratios of polychromatic erythrocytes (PCEs) per 5000 erythrocytes and the number of micronucleated cells per 1000 PCEs and per 1000 normochromatic erythrocytes (NCEs) were calculated for each treatment group. In the chromosomal aberration study, 500 cells/sex/dose were assessed for chromosomal aberrations.

In the micronucleus study, there was no significant decrease in the PCE/erythrocyte ratio at any dose of pullulanase. As a result, the three highest dose groups were selected for analysis of micronuclei. No significant increases in the number of micronucleated PCEs was observed compared with controls. The positive control resulted in a decreased PCE/erythrocyte ratio and a clear increase in the number of micronucleated PCEs.

In the chromosome aberration study, one male mouse dosed with 2811 mg/kg pullulanase and one female treated with 5000 mg/kg were found dead within the first 12 hours, and one male exposed to 5000 mg/kg bw died before the 24 hour termination of test animals. The deaths of these mice were attributed to dosing errors as none of the surviving animals showed any signs of toxicity and no deaths had occurred in the dose range-finding study.

Due to these deaths, bone marrow cells from only four mice in each of these dose groups were evaluated. Mitotic indices and chromosomal aberration frequencies in the vehicle and positive controls were consistent with values reported in the literature. No significant effects on mitotic indices, or the number of cells with aberrations or chromosome breaks were found in cells from mice treated with pullulanase.

It was concluded that pullulanase did not induce micronucleus formation or chromosomal aberrations in mouse bone marrow under the conditions of this study.

3.3.4 Potential for toxicity

The applicant provided the results of a BLAST search for homology of the mature pullulanase sequence against the complete UniProt annotated Protein Knowledge database. The search was performed with a threshold E-value of 0.1. The vast majority of database hits were pullulanases, and none of the top 1000 hits were annotated as a toxin or venom.

A BLAST search for homology was also performed against the UniProt animal toxin database. No matches were identified.

The results of these searches indicate that the pullulanase sequence does not share homology with a known toxin or venom sequence.

3.3.5 Potential for allergenicity

The applicant provided the results of searches for sequence homology of pullulanase with known allergens conducted with the AllergenOnline database. A FASTA alignment of the full sequence using an E-value of <0.1 as a cutoff found no matches to sequences in the database. Searches for 80-amino acid stretches in the sequence with greater than 35% identity to known allergens and for exact matches of 8 contiguous amino acids also found no matches with known allergens.

The results of the bioinformatics analysis indicate that pullulanase is unlikely to pose an allergenicity concern.

Nutrient raw materials used in the bacterial fermentation process to produce pullulanase include soy protein and glucose derived from wheat. Therefore the enzyme preparation may contain traces of wheat or soy. The applicant has estimated that the highest amount of soy protein or wheat protein in the final food would be 2-3 ppb and 5 ppb, respectively.

3.3.6 Approvals or safety assessment reports prepared by international agencies or other national government agencies

JECFA reviewed the safety of pullulanase from *B. deramificans* expressed in *B. lichenifiormis* in 2011. The Committee established an ADI not specified for the enzyme when used in the applications specified and in accordance with GMP (WHO 2011).

In 2001 the US FDA responded to a GRAS notification for Pullulanase derived from *B. licheniformis* carrying a gene encoding pullulanase from *B. deramificans* for use as a processing aid in the manufacture of starch hydrolysates and high fructose corn syrup, indicating that the Agency had no questions (GRN No. 72).

The enzyme is also approved for the production of glucose syrup and potable alcohol in France and Denmark, and is listed as a permitted food additive for bakery, brewing and other applications. In Canada, the enzyme is listed as a permitted food enzyme in the production of

several foods including bread, bakery products and brewers mash[11].

4 Conclusions

There are no public health and safety concerns associated with the use of pullulanase from *B. licheniformis* when used as a food processing aid.

No extraneous coding genetic material is carried across from the donor organism or through the large number of steps leading to the final genetic modification. The modification involving the insertion of the pullulanase gene has been shown to be stably inherited.

Pullulanase showed no evidence of mutagenicity in a bacterial reverse mutation assay or a forward mutation assay in mouse lymphoma cells, and was not clastogenic in a chromosomal aberration assay in human lymphocytes *in vitro*. Pullulanase also showed no evidence of genotoxicity in an *in vivo* mouse bone marrow micronucleus and chromosomal aberration assay. Pullulanase did not cause adverse effects in short-term toxicity studies in rats. The NOAEL in a 13-week repeated dose oral toxicity study in rats was the highest dose tested, 2500 mg/kg bw/day or 246 mg/kg bw/day on a TOS basis. The applicant's estimated theoretical maximal daily intake (TMDI) based on the proposed uses is 0.049 mg/kg bw/day TOS. A comparison of these values indicates that the Margin of Exposure is more than 5000.

Bioinformatic analysis indicated that the pullulanase enzyme has no homology to known protein allergens or toxins and is unlikely to pose an allergenicity or toxicity concern. The enzyme product may contain traces of soy and wheat protein, however.

Based on the reviewed toxicological data it is concluded that, in the absence of any identifiable hazard, an acceptable daily intake (ADI) 'not specified' is appropriate. A dietary exposure assessment was therefore not required.

5 References

Boyer M (1999). A 13-week oral gavage toxicity study of GC797 (Pullulanase) in the albino rat. ClinTrials BioResearch Ltd, 87 Senneville Road, Senneville, Quebec, Canada H9X 3R3. Project Number: 88873.

Codex Alimentarius (2003). Guideline for the Conduct of Food Safety Assessment of Foods Produced Using Recombinant-DNA Microorganisms. (CAC/GL 46-2003).

De Boer A.S., Priest F, Diderichsen B (1994) On the industrial use of *Bacillus licheniformis*: A review. Appl Microbiol Biotechnol 40:595-598

JECFA (2017). Combined compendium of food additive specifications (FAO JECFA Monograph 1) http://www.fao.org/docrep/009/a0691e/A0691E03.htm

Gudi R and Schadly EH (1999). In vitro mammalian chromosome aberration test: GC 797 (Pullulanase). BioReliance, 9630 Medical Center Drive, Rockville, Maryland 20850, Laboratory Study Number AA16GE.341.BTL.

Hii AL, Tan JS, Ling TC and Ariff AB (2012). Pullulanase: Role in starch hydrolysis and potential industrial applications. Enzyme Research 2012.

Huang W and Tang I (2007). Bacterial and yeast cultures – process characteristics, products and applications. Bioprocessing for value-added products from renewable resources 2007.

IUBMB (2017). EC 3.2.1.41. https://www.qmul.ac.uk/sbcs/iubmb/enzyme/EC3/2/1/41.html

Ministry of Health Labour and Welfare (MHLW) Japan (2001) Foods and food additives produced by recombinant DNA techniques. https://www.mhlw.go.jp/english/topics/food/

Modderman JP and Foley HF (1995). Safety evaluation of pullulanase enzyme preparation derived from *Bacillus licheniformis* containing the pullulanase gene from *Bacillus deramificans*. Regulatory

[11] https://www.canada.ca/en/health-canada/services/food-nutrition/food-safety/food-additives/lists-permitted/5-enzymes.html

Toxicology and Pharmacology 21: 375-381.
Quainoo SC, Coolen JP, van Hijum SAFT, Huynen MA, Melchers WJG, van Schaik W, Wertheim HFL (2017). Whole-genome sequencing of bacterial pathogens: the future of nosocomial outbreak analysis. Clinical Microbiology Reviews 30: 1015-1063. doi: 10.1128/CMR.00016-17
USPC (2018) Food Chemicals Codex 11th Edition, United States Pharmacopeial Convention, Rockville, MD. http://publications.usp.org/
US Food and Drug Administration (2001) GRAS notices – GRN000072. https://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&id=72&sort=GRN_No&order=DESC&startrow=1&type=basic&search=pullulanase
Wagner VO and Walton EW (1999). Bacterial reverse mutation assay with an independent repeat assay: GC 797 (Pulllulanase). BioReliance, 9630 Medical Center Drive, Rockville, Maryland 20850, Laboratory Study Number AA16GE.507.BTL
WHO (2011). Evaluation of certain food additives and contaminants. Seventy-fourth report of the Joint FAO/WHO Expert Committee on Food Additives. WHO Technical Report Series No. 966. World Health Organization, Geneva. http://whqlibdoc.who.int/trs/WHO_TRS_966_eng.pdf#page=51 Accessed 13/02/19