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

SUPPORTING DOCUMENT 1

RISK ASSESSMENT REPORT

APPLICATION A1044
PULLULANASE AS A PROCESSING AID (ENZYME)

Summary

Background

The *Australia New Zealand Food Standards Code* (the Code) currently permits the use of numerous microbial enzymes as processing aids in the manufacture of food. Approval for pullulanase (EC 3.2.1.41) from a number of non-genetically modified sources already exists in the Code.

Application A1044 seeks approval for the use of pullulanase derived from a genetically modified (GM) *Bacillus subtilis* expressing the gene for pullulanase from *Bacillus acidopullulyticus*, as a processing aid.

The risk assessment has considered the technological suitability, the safety and identity of the donor and host microorganisms, and the safety of the pullulanase enzyme preparation. Based on the available data, no food safety concerns have been identified with the enzyme, or with the donor or host organisms used to produce the enzyme, which would preclude permitting its use as a food processing aid. The absence of any specific hazards being identified is consistent with pullulanase undergoing normal proteolytic digestion in the gastrointestinal tract.

The stated purpose for the use of this pullulanase is as a debranching enzyme used in the starch and alcohol industries for the saccharification of liquefied starch. The Application provides adequate assurance that the pullulanase is technologically justified and has been demonstrated to be effective in achieving its stated purpose.

The available data are considered sufficient to provide an acceptable level of confidence in the conclusions of this risk assessment in regard to the safety and suitability of this pullulanase for its stated purpose.

Conclusions

- The use of *B. subtilis* as the host organism is a well-characterised expression system for the production of enzymes, and has a long history of safe use
- There was no evidence of pullulanase toxicity at the highest dose tested in a 90-day repeat dose study. The No Observed Adverse Effect Level (NOAEL) for the pullulanase preparation was 938 mg pullulanase/kg bw/day. Consequently ‘an ADI not specified’ was established.
- There was no evidence of genotoxicity.

- Based on the available evidence, pullulanase produced in *B. subtilis* is considered safe for use in foods for human consumption.
- Pullulanase produced from the genetically modified *B. subtilis* described in this Application meets international specifications for identity and purity.
- The stated purpose for this pullulanase is as a debranching enzyme used in the saccharification of liquefied starch. When used as prescribed, the pullulanase is technologically justified and achieves its stated purpose.

Contents

1 Introduction

An application was received from Novozymes A/S on 16 March 2010 seeking approval to permit a pullulanase enzyme produced from *B. subtilis* expressing a gene for pullulanase from *Bacillus acidopullulyticus*, in the Table to Clause 17 of Standard 1.3.3 – Food Processing Aids of the *Australia New Zealand Food Standards Code.* Approval for pullulanase from a number of non-genetically modified sources already exists in the Code. The proposed use of the pullulanase is as a debranching enzyme in the starch and alcohol industries for saccharification of liquefied starch.

1.1 Objectives of the Assessment

In proposing to amend the Code to include a pullulanase derived from a genetically modified (GM) *B. subtilis* as a processing aid, a pre-market assessment is required. The objectives of this risk assessment are to determine:

- What are the potential public health and safety concerns associated with the use of this pullulanase as a processing aid?
- Is the proposed purpose clearly stated and does the enzyme achieve its technological function in the quantity and form to be added?

1.2 Risk Assessment Questions

The following risk assessment questions have been developed to address the objectives of the assessment.

- Does the enzyme preparation present any food safety issues?
- Does the enzyme achieve its stated technological purpose?

2 Characterisation of pullulanase

2.1 Identity of the enzyme

Systematic name:	Pullulan 6-α-glucanohydrolase
IUBMB Enzyme Nomenclature:	EC 3.2.1.41
C.A.S number:	9075-68-7
Common name:	Pullulanase
Other names:	Amylopectin 6-glucanohydrolase; α-dextrin endo-1,6-α-glucosidase; pullulan α-1,6-glucanohydrolase
Marketing Name:	Novozym® 26062

2.2 Chemical and physical properties

2.2.1 Enzymatic properties

Pullulanase hydrolyses (1→6)-α-D-glucosidic linkages in pullulan, amylopectin and glycogen, and in the α- and β-limit dextrins of amylopectin and glycogen.

The activity analysis exploits the ability of endo-pullulanases to hydrolyse α-1, 6-glycosidic bonds in red pullulan, releasing red substrate degradation products. The amount of colour liberated is measured spectrophotometrically and is proportional to the endo-pullulanase activity in the sample.

The enzyme preparation has a typical activity of 400 Pullulanase Units Novo (PUN)/g, using the Novozyme method contained in the dossier (refer to Appendix 1 in A1044).

2.2.2 Physical properties

The commercial enzyme preparation is a light brown water soluble liquid with a pH of 4.7. Typical composition is below:

Enzyme solids (TOS)[1]	approx 2%
Water:	approx 57%
Sucrose:	approx 41%
Potassium sorbate:	approx 0.3%
Sodium benzoate	approx 0.1%

2.3 Production of pullulanase

The manufacturing process is composed of a fermentation process, a purification process, a final product formulation process and then a quality control of the finished product, as outlined by Aunstrup (1979). The enzyme preparation is manufactured in accordance with current Good Manufacturing Practices and an ISO 9001 compliant quality management system.

The fermentation process is a submerged fed-batch pure culture fermentation of *B. subtilis* containing an adequate supply of carbon and nitrogen sources, as well as the minerals and vitamins necessary for growth. The pH and foam are controlled using appropriate compounds.

Recovery is a multi-step process designed to separate the exo-enzyme from the microbial biomass and to partially purify and concentrate the enzyme. Typical unit operations include primary separation, concentration and pre-filtration and germ filtration.

2.3.1 Description of the genetic modification

The production organism for the pullulanase is *Bacillus subtilis* strain A164Δ5. This strain has been genetically modified from the A164 strain to contain deleted versions of five genes (coding for sigma F, neutral protease, alkaline protease, amylase and surfactin C). As a result, A164Δ5 is non-sporulating, protease deficient, amylase negative and surfactin negative.

[1] TOS = Total Organic Solids, defined as: 100% - water – ash - diluents

The inserted gene is the pullulanase gene (*pulC*) from *Bacillus acidopullulyticus.* The expression of the pullulanase gene is under the regulation of a triple tandem promoter[2] and a serine protease terminator from *Bacillus clausii* at the *amyE* locus on the bacterial chromosome. An inactive fragment of the chloramphenicol resistance gene *cat* is also inserted at this site.

In order to construct the pullulanase-expressing strain, the *pulC* gene was ligated into a vector containing the triple tandem promoter, the *cat* gene and flanking regions of the *amyE* gene. The vector also contained genes for ampicillin and neomycin, as well as the pBR322 origin of replication. This vector was introduced into an intermediate host (A168Δ4) and integrated via homologous double cross-over recombination into the host genome. The resulting host strain thus contained the *pulC* and *cat* genes without ampicillin and neomycin resistance genes or an origin of replication.

Chromosomal DNA from transformants showing chloramphenicol resistance, neomycin sensitivity and pullulanase expression was used to transform A164Δ5. Chloramphenicol resistance was removed by a gene replacement event. Thus the full-length *cat* gene was replaced by a deleted version. This strain was tested for *cat* deletion by testing for growth on chloramphenicol-containing media and by polymerase chain reaction (PCR) analysis.

2.3.2 Identification of the donor and host organisms

a. Host strain

The safety of the production organism is an important consideration in the safety assessment for enzymes used as processing aids. The primary issue is the toxigenic potential of the production organism, that is, the possible synthesis of toxins by the production strain, and the potential for the carryover of these into the enzyme preparation (Pariza and Johnson, 2001).

The production organism for this pullulanase, *B. subtilis,* is widely distributed in the environment by virtue of its natural occurrence in soil and is also detectable in water, air and decaying plant material (US EPA, 1997). The bacterium is not pathogenic to humans or toxigenic (de Boer and Diderichsen, 1991; US EPA, 1997) and has been recommended for a qualified presumption of safety (QPS) by the Scientific Committee of the European Food Safety Authority (EFSA, 2007).

FSANZ has previously assessed the safety of *B. subtilis* as the production organism for a number of enzymes used as food processing aids. Standard 1.3.3 of the Code permits the use of the following enzymes derived from *B. subtilis*: α–acetolactate decarboxylase, α- and β–amylase, β–glucanase, hemicellulase endo-1,4- β-xylanase, hemicellulase multicomponent enzyme, maltogenic α –amylase, metalloproteinase, pullulanase and serine proteinase. In the US, several enzyme preparations from *B. subtilis* have Generally-Recognised-As-Safe (GRAS) status (FDA, 1999; FDA, 2003; FDA, 2006; FDA, 2009).

[2] This promoter contains modified versions of sequences upstream of the *Bacillus lichenformix* amylase gene, the *Bacillus amyloliquefaciens* amylase gene and *Bacillus thuringiensis* subsp. *Tenebrionis* cystal toxin gene.

The bacterium itself is commercially available in many countries as a dietary probiotic intended to improve human health (Duc *et al*., 2004; Henriksson *et al*., 2005). It is also used as an animal feed additive (Klose *et al*., 2009; Lee *et al*., 2010), although its efficacy has been questioned (Arthur *et al*., 2010; Danicke and Doll, 2010) and growth promotant in aquaculture (Farzanfar, 2006). Strains of *B. subtilis* are used to make fermented soybean products such as thua nao (Thailand) and natto (Japan) (Hosoi and Kiuchi, 2008; Inatsu *et al*., 2006).

b. Donor strain

The pullulanase protein is encoded by the *pulC* gene from a species of *Bacillus* first described in 1984, although sold under the trade name PROMOZYME 200L in Japan and Europe since 1983 (Jensen and Norman, 1984). *B. acidopullulyticus* is a gram-positive, rod-shaped bacterium that is catalase positive and a strict aerobe. Taxonomic evaluation failed to assign the isolated bacterium to a particular *Bacillus* species, and so the name *Bacillus acidopullulyticus* was adopted.

According to a study by Jensen and Norman (1984), administration of *B. acidopullulyticus* to mice and rats demonstrated an LD_{50} of 10^{10}-10^{11} cells per kg body weight in mice, and greater than the highest dosage administered (10^{11}) for rats[3]. These data suggest that *B. acidopullulyticus* is non-pathogenic and does not produce toxins. *B. acidopullulyticus* was also shown to be unable to produce antibiotics.

Analysis of the pullulanase protein sequence via Blast protein sequence searching (Altschul *et al*., 1997; Altschul *et al*., 2005) reveals only distant homology to other members of the phylum Firmicutes (Figure 1). For example, the closest homology to the *B. acidopullulyticus* pullulanase is found in the pullulanase proteins from *Anaerobranca horikoshii* (50% identity) and *gottschalkii* (49% identity), *Bacillus thuringiensis* (44%) and *Bacillus cereus* (44%).

[3] Several reports were quoted in Jensen and Norman (1984) as being "available from the authors of this paper". These reports do not appear to have been subsequently published in the scientific literature.

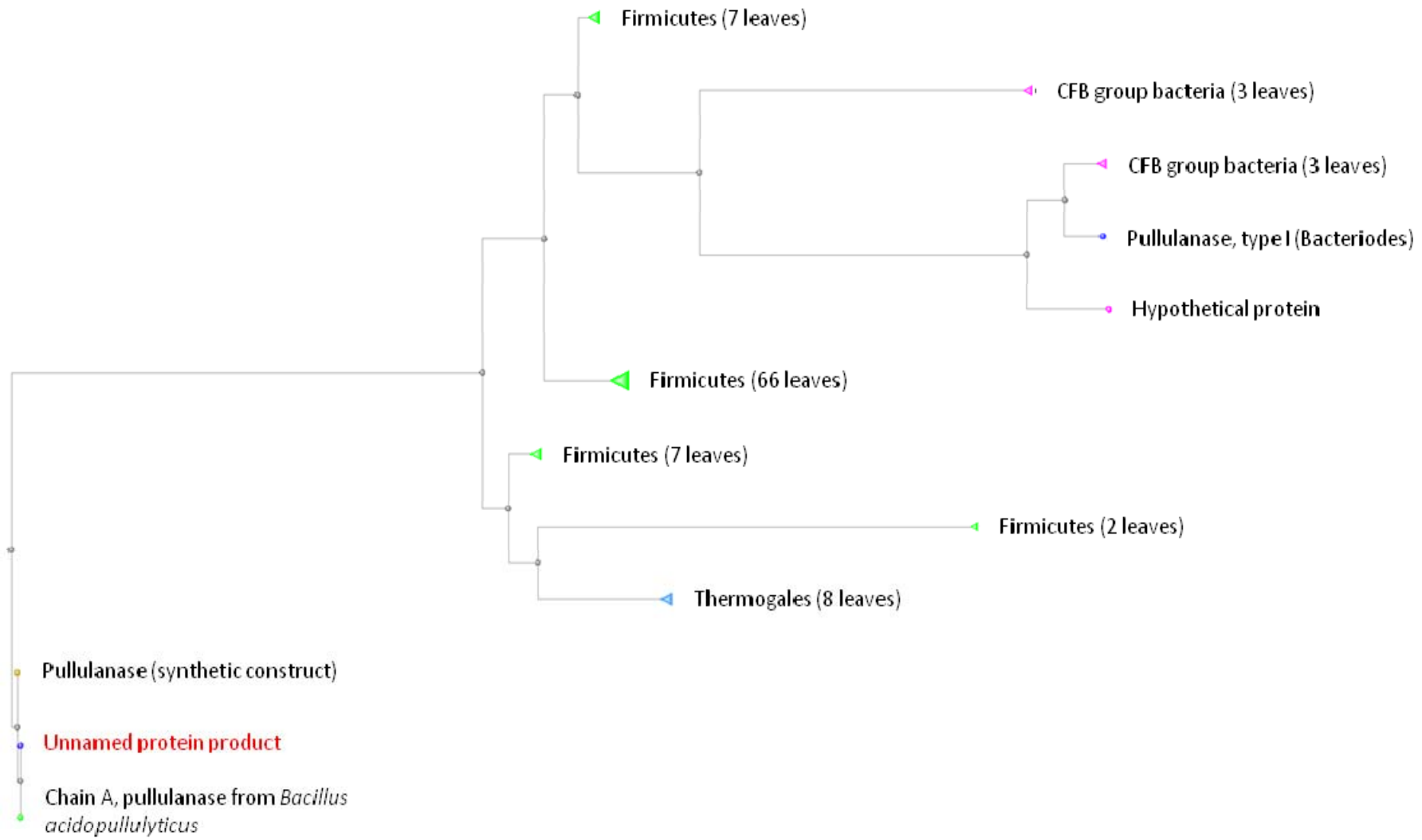

Figure 1: Distance tree results for the pullulanase protein of *Bacillus acidopullulyticus*. The pullulanase gene from *B. acidopullulyticus* is only distantly related to other members of the phylum Firmicutes.

2.4 Analysis and Specifications

2.4.1 Methods of analysis

A method for determining the activity of pullulanase during production and in the final enzyme preparation has been provided by the Applicant (refer to Appendix 1 in the application).

2.4.2 Specifications

Specifications for pullulanase comply with the international specifications for identity and purity relevant for enzymes prepared by the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2006). These specifications are primary reference sources listed in Clause 2 of Standard 1.3.4 - Identity and Purity, of the Code.

Specifications for an unstandardised commercial product comparative to the JECFA specifications has been provided by the Applicant and are detailed in the table below.

Table 1: Specifications for an unstandardised, representative batch of commercial enzyme product

	JECFA Specification	Sample
Pullulanase activity		812 PUN/g
Heavy Metals (as Pb)	Not more than 40 ppm	4.0
Lead	Not more than 5 mg/kg	< 1
Arsenic (as As)	Not more than 3 mg/kg	< 0.1
Cadmium		≤ 0.05
Mercury		≤ 0.03
Total Viable Count (cfu/g)	Not more than 5 x 10^4 cfu/ml	< 200
Total Coliforms	Not more than 30 cfu/ml	< 10
Enteropathogenic *E. coli*	Absent in 25 g	ND
Salmonella	Absent in 25 g	ND
Antibiotic activity	Negative by test	ND
Production Strain		ND

ND = Not Detected

Pullulanase produced from a genetically modified *B. subtilis*, meets international specifications for identity and purity.

3 Technological function of the enzyme

Pullulanase is an amylolytic exo-enzyme glucanase (debranching enzyme) that cleaves alpha-1,6 linkages in pullulan (alpha-glucan polysaccharides) to release maltotriose. Pullulanases used during starch processing can improve glucose yields, decrease reaction times and produce maltose syrups suitable for food applications (Jensen and Norman, 1986).

The proposed use of this pullulanase is in the starch and alcohol industries (beverage alcohol and brewing) for saccharification of liquefied starch, as outlined below:

- In dextrose (D-glucose) production, the pullulanase is used together with glucoamylase. The Applicant claims this increases the glucose yield by reducing the oligosaccharide content and allows a reduction in the glucoamylase dosage.
- In maltose production, saccharification is carried out with pullulanase, β-amylase and finally maltogenic α-amylase. Addition of the pullulanase is claimed to increase maltose yield and reduce the amount of branched oligosaccharides.
- For the alcohol and brewing industries, use of pullulanase in addition to glucoamylase enzymes, increases the amount of fermentable sugars and may facilitate filtration steps.

Saccharification is the near-total hydrolysis of starch to glucose. The majority of starches used in the manufacture of glucose syrups contain 75-85% amylopectin. Amylopectin is a highly branched polysaccharide consisting of linear chains of 1,4-alpha linked D-glucose residues, linked with α-1,6-glycosidic linkages every 20-25 D-glucose units. Amylopectin contains approximately 4-5% of α-1, 6-glycosidic linkages.

De-polymerisation occurs by the action of glucoamylase from the non-reducing chain ends in a stepwise manner. Having both exo-amylase and debranching activity, fungal glucoamylases efficiently hydrolyse 1,4-alpha links but slow upon reaching a 1,6-alpha link. Pullulanase specifically hydrolyses the branch points in the amylopectin residues so that the glucoamylase only has to hydrolyse the linear 1,4-alpha-glucosidic linkages. Some advantages of using synergistic enzymes such as a pullulanase and a glucoamylase are increased D-glucose yield, reduction in polymerisation of D-glucose to isomaltose, higher substrate concentration, reduced reaction times and a reduction in the glucoamylase requirement (Jensen and Norman, 1986).

Although the action of glucoamylase slows on reaching a branch point, the action of maltogenic exo-amylases ceases. When starch or liquefied starch is hydrolysed with a maltogenic exo-amylase such as a soybean β-amylase, the branch points are only partially hydrolysed with the maximum maltose yield of approximately 60% possible. Considerably higher yield is possible when a pullulanase is used along with an α-amylase free β-amylase. A study conducted by Jensen and Norman (1984) demonstrated a yield of around 80% maltose using a 30% Dry Solids (DS), Dextrose Equivalent (DE) 5 enzyme liquefied corn starch substrate, at 60^{o}C.

During the early phases of brewing, the mashing step involves the liberation of fermentable sugar from starch. Worts for ‘low-carb’ beers normally contain 4% (w/v) of their carbohydrates as un-fermentable dextrins (Bigelis, 1993). Use of pullulanase and fungal alpha-amylase together can effectively hydrolyse wort dextrins to fermentable sugars (Bigelis, 1993). Data provided by the Applicant show that the pullulanase when used in combination with a glucoamylase yielded a higher amount of fermentable sugars than glucoamylase used alone: 84% compared with approximately 81%.

The Applicant claims this pullulanase will provide the starch and brewing industries faster and more efficient processing. Data presented in support of this claim show the ability of this pullulanase (Pullulanase 3) compared to two different pullulanases, to reduce the amount of non-fermentable carbohydrates in wort. Mashing was undertaken at 46^{o}C for 26 minutes, followed by a 1^{o}C/min increase until 64^{o}C and then held constant. Glucoamylase and α-amylase were added at 1000 AGU/kg DS and 250 AFAU/kg DS respectively. The samples were boiled (10 min), filtered, analysed by HPLC and then the proportion of non-fermentable carbohydrate was calculated. Samples were taken at 98, 128 and 158 minutes.

After 98 minutes mashing time, 3.38 mg and 27.33 mg of pullulanase 1 and 2 respectively were required to achieve the same effect as with 2.74 mg of this pullulanase. The effects are even greater with increased mashing times (158 min: 4.56 mg and 40.37 mg), indicating that this pullulanase has improved yield, can be used at lower dosages and is more stable in application than other pullulanases.

Usage levels for the enzyme are according to the requirements for normal production (Good Manufacturing Practice). The optimum dosage depends on the desired effect, the specific process conditions and the combination of enzymes used. A recommended dosage range used in the starch industry is between 1-25 kg per ton of starch dry substance. For the brewing industry, a recommended dosage range is from 40 – 650 PUN per kilogram of starch. Attenuation control is noted by the Applicant as the most important application for this enzyme.

3.1 Conclusion

The stated purpose for this pullulanase, namely for saccharification of liquefied starch, is clearly articulated in the Application and the evidence presented in support of the Application provides adequate assurance that the pullulanase is technologically justified and has been demonstrated to be effective in achieving its stated purpose.

4 Safety Assessment

The following studies were evaluated as part of the hazard assessment:

- Bioinformatic analysis of pullulanase
- 14-day dose range-finding oral toxicity study in rats
- 90 day oral (diet) toxicity study in rats
- *In vitro* cytotoxicity test
- Bacterial reverse mutation assay (Ames test)
- *In vitro* mammalian chromosomal aberration test with human lymphocytes
- Biochemical characterization of the pullulanase enzyme

4.1 Bioinformatic analysis

4.1.1 Bioinformatic analysis of pullulanase homology to known toxins

The applicant presented an *in silico* analysis of the homology of pullulanase with known protein toxins. They compared the amino acid sequence of the pullulanase enzyme with that of known toxins contained in the UNIPROT database. The highest degree of homology to a known protein toxin in this database was 15.1%. This level of homology is not indicative of a true homologue, and as such, pullulanase does not share biologically significant homology to known toxins.

4.2 Toxicity studies

4.2.1 Cytotoxicity

J. Lichtenberg (2006). Pullulanase, batch PPY 25645. *In vitro* cytotoxicity test: neutral red uptake in L929 monolayer culture. Novozymes, Bagsværd, Denmark.

The test material used in all the toxicity studies came from two batches, PPY 25645 and PPY 24933. The liquid pullulanase preparation had an enzyme activity of 1561 PUN-RPA[4]/mL, and total organic solids (TOS) contents of 9.6% (PPY 25645) and 812 PUN-RPA/g and 7.2% TOS (PPY 24933).

This *in vitro* study examined the cytotoxic potential of the pullulanase preparation. The Neutral Red Uptake assay uses L929 mouse fibroblast cells to quantify the amount of neutral red taken up by cells in the presence or absence of the test substance. As the cells multiply over time, they take up the neutral red from the medium. Toxicity of a test substance will interfere with cell division, thus reducing the uptake of neutral red. This study was GLP compliant (OECD, 1998).

Pullulanase was tested at 30, 10, 3, 1 and 0.3 mg/mL in growth medium in quadruplicate. The pullulanase preparation in growth medium was added to near-confluent L929 mouse fibroblasts and incubated for 24 hours. The concentration of test substance required to reduce neutral red uptake by 50% is the endpoint of this experiment.

The concentration of pullulanase preparation required to reduce neutral red uptake by 50% was 18 mg/mL. This result indicates that pullulanase is not cytotoxic in this assay system.

4.2.2 Short-term toxicity

N. Hughes & A. Broadmeadow (2006). Pullulanase, PPY 25645. Acute oral toxicity study to the rat (highest non-lethal/lowest lethal dose). Huntingdon Life Sciences ltd. Huntingdon, England.

This preliminary and limited study examined the acute toxicity of a single dose of the pullulanase preparation. The study was conducted according to the requirements of the Guidance for Industry, Single Dose Acute Toxicity Testing for Pharmaceuticals, FDA/CDER (1996) and Yakushinyaku No 88, Single Dose Toxicity Study, Pharmaceuticals Affairs Bureau, Japanese Ministry for Health and Welfare (1993).

The study consisted of two parts; a preliminary, seven day, dose-finding study involved one male and one female Crl:CD® (SD)IGS BR rat; the main part of the study involved five male and five female Crl:CD® (SD)IGS BR rats over a 14 day period. Food and water were supplied *ad libitum*. In both studies, rats were delivered a single 20 mL/kg bodyweight (bw) dose (the maximum practical volume-dosage for oral administration to rats) of the pullulanase preparation by oral gavage.

Clinical observations were made once per day in the acclimatisation period (5 days) and twice daily following pullulanase administration. Bodyweights were recorded on Day 1 and once per week thereafter. Animals were killed at the end of the observation period and subjected to gross necropsy.

In the preliminary study, no animals died and none showed signs of toxicity or ill health. Both animals gained a satisfactory amount of weight. There were no macroscopic abnormalities seen at necroscopy.

In the main study, no deaths were recorded and there were no clinical signs. All the animals showed normal bodyweight gain and no treatment-related abnormalities were noted at necropsy.

[4] PUN-RPA = Pullulanase Unit Novozymes, Red pullulan Promozyme Activated

The conclusion of the study was that a single dose of pullulanase at 20 mL/kg bw (equivalent to 1.92g TOS/kg bw) was well-tolerated by the rats. Therefore the highest non-lethal dose is greater than 1.92g TOS/kg bw.

N. Hughes, B.S. Pedersen, M.J. Collier, P. Lee, P. Travis, D.J. Bell, G.F. Healey (2007). Pullulanase, PPY 25645 toxicity study by oral gavage administration to CD rats for 13 weeks. Huntingdon Life Sciences Ltd. Huntingdon, England

A GLP compliant study was conducted according to the UK Good Laboratory Practice Regulations[5]. The study consisted of four groups of ten male and ten female Crl:CD® (SD)IGS BR rats. The control group received dionised water while the three test groups received 96,317 or 960 mg Total Organic Solids (TOS)/kg bw/day respectively of the pullulanase preparation. All doses were administered daily by oral gavage for 13 weeks.

Clinical observations were made twice daily. Bodyweights were recorded on day 1 and once per week thereafter. Food consumption was measured weekly. Water consumption was measured over a three day period each week. Neurobehavioural testing (functional observational battery tests, including approach response, grip strength testing, auditory startle reflex, tail pinch response and touch response) was conducted in week twelve. Motor activity was also measured in week twelve. Ophthalmoscopic observations were made prior to treatment and then in week 13 of treatment in the control and high-dose groups only. Haematology was conducted during week 13 of treatment, as was clinical chemistry and urinalysis. At the conclusion of the treatment period, all animals were killed. All animals were subjected to detailed necroscopy, including a full macroscopic examination of the tissues. External features, orifices, brain, pituitary gland and cranial nerves were examined visually, as were the neck, the thoracic, abdominal and pelvic cavities and their viscera. Organs were weighed and examined visually and microscopically along with other tissues.

Three rats died during the study, and one was euthanased because of gavaging accidents. There were no treatment-related differences in appearance or general behaviour of the animals. Sensory reactivity and grip strength were unaffected by treatment. Bodyweight and food consumption were unaffected in all treatment groups. There were no treatment-related ophthalmic changes. A few transient changes in measured clinical signs, blood chemistry, urinalysis and organ weights achieved statistical significance, such as lower platelet counts in female rats receiving the highest dose and slightly low total protein in male rats receiving the lowest dose. However, these were considered to be of no toxicological significance because they were not dose related and present in only one sex. The higher absolute and adjusted (to body weight) adrenal and liver weights in male rats at the highest dose were not associated with changes in the tissues at a macroscopic or histopathological level, and were therefore also considered to have arisen by chance.

Increased water intake, slightly low urinary pH and high urinary chloride concentrations were dose-related, statistically significant and, in the case of the highest dose, found in both sexes. These findings are therefore considered to be related to treatment but not adverse. As discussed in the oral toxicity study report, as well as in the cytotoxicity study, the formulation used in the experiments was hyperosmotic due to a high concentration of electrolytes. Control rats received only dionised water whereas the rats on test received a dose-related increase in electrolytes as the quantity of water used to suspend the pullulanase preparation was reduced. The increased chloride concentration observed in the urine of treated rats is also consistent with a higher electrolyte intake relative to controls.

[5] (Statutory Instrument 1999 no. 3106, as amended by Statutory Instrument 2004 no 994), OECD Principles of Good Laboratory Practice (as revised in 1997) ENV/MC/CHEM (98)17 and EC Directive 2004/10/EC of 11 Februrary 2004 (Official Journal no L 50/44.

Overall, administration of up to 960 mg TOS/kg bw/day of the pullulanase preparation was well tolerated. The No Observed Adverse Effect Level (NOAEL) was considered to be at the highest tested dose of 938 mg pullulanase/kg bw/day. This corresponds to 960 mg TOS/kg bw/day assuming an enzyme activity of 1561 PUN-RPA/mL,

4.2.3 Genotoxicity

The results of two *in vitro* genotoxicity studies with the pullulanase preparation are summarised below. Both studies were GLP compliant and conducted in accordance with OECD guidelines. Neither test revealed any genotoxic potential associated with the pullulanase preparation.

4.2.3.1 Reverse mutation test (Ames test)

P.B. Pedersen (2006). Pullulanase, PPY 25645: Test for mutagenic activity with strains of *Salmonella typhimurium* and *Escherichia coli*. Novozymes, Bagsværd, Denmark.

A bacterial reverse mutation test (Ames test) was conducted with the same preparation of enzyme as described above. This test was done to determine if the pullulanase preparation as prepared from *B. subtilis* has mutagenic potential. This test was done in compliance with the OECD Good Laboratory Practice regulation ENV/MC/CHEM(98)17 (1998).

This test was done using four strains of *Salmonella typhimurium* (TA 1535, TA 1537, TA 98 and TA 100) as well as the *Escherichia coli* mutant WP2 *uvrA*. The tests were carried out in the presence or absence of rat hepatic microsomal fraction S9. Two independent experiments were carried out both with and without S9 (i.e. four experiments in total).

Six concentrations of pullulanase were analysed: 5,000 µg, 2,500 µg, 1,250 µg, 625 µg, 313 µg and 156 µg TOS per plate using the plate incorporation assay technique. All positive and negative controls were as expected. All concentrations of pullulanase were non-toxic as judged microscopically. All treatments, either in the presence or absence of S9 mix resulted in a negative response. That is, there is no indication that the pullulanase preparation is mutagenic, when tested under the conditions in the study.

4.2.3.2 Chromosomal aberration test

C.N. Edwards (2006). Pullulanase: *in vitro* mammalian chromosome aberration test performed with human lymphocytes. Scantox, Lille Skensved, Denmark

The enzyme preparation containing *B. subtilis*-derived pullulanase was tested for its potential to induce chromosomal aberrations in cultured human lymphocytes. This was done in the presence or absence of S9-mix.

Two tests were conducted, each with lymphocytes cultured from a different donor. In the first test, the lymphocytes were incubated with 0, 156, 313, 625, 1,250, 2,500 and 5,000 µg TOS/ml of the enzyme preparation for three hours in the presence or absence of S9-mix, before being incubated for an additional 17 hours in fresh medium before being harvested. In the second test, cells were treated for 20 hours before harvest.

After fixation, 200 cells in metaphase per concentration were analysed microscopically for polyploidy and endoreduplicated metaphases. A further 100 metaphases were examined for aberrations. At the observed concentrations, no statistically significant increase in the number of aberrant cells was observed at any concentration.

The conclusion drawn from the study was that the enzyme preparation from *B. subtilis* was not clastogenic to cultured human lymphocytes.

4.3 Production strain analyses

4.3.1 Analysis for toxin production

As discussed above (section 2.3.2), *Bacillus subtilis* is consumed widely. It is therefore unlikely that this bacterium produces proteins that are harmful to human health. Nevertheless, the potential for *Bacillus subtilis* to produce toxins was investigated recently. Pedersen *et al* (2002) assessed the toxigenic potential of *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, and *Bacillus subtilis* strains currently used for industrial purposes.

A cytotoxicity study on Chinese Hamster Ovary (CHO-K1) cells *in vitro* was performed. An MTT assay (previously used to detect toxigenic strains of *B. cereus* and other *Bacillus* species was employed, along with ELISAs reactive to haemolytic enterotoxin and non-haemolytic enterotoxin (Beattie and Williams, 1999)). The *B. subtilis* strain tested (MB 1252) was not cytotoxic and did not show cross-reactivity with either haemolytic or non-haemolytic enterotoxins (Pedersen *et al*., 2002). This strain is the one used in the current enzyme preparation.

4.4 Conclusion

Following the safety assessment of pullulanase from *B. subtilis*, it is concluded that:

- There was no evidence of any toxicity in a 13-week oral toxicity study in rats. Based on food consumption, the NOAEL was 960mg TOS/kg bw (13,9809 DLU).
- There was no evidence of any genotoxicity;
- Pullulanase from *B. acidopullulyticus* shares no amino acid sequence homology with known toxins.

4.5 Potential allergenicity

4.5.1 History of use

Enzymes from a variety of bacterial sources have a long history of safe use in food. They are rarely, if ever, known to be allergens (Bindslev-Jensen *et al*., 2006). This pullulanase, and pullulanase enzymes from four other bacterial species, have been approved for use as processing aids in the Code. None of these pullulanases has proven to be allergenic.

4.5.2 Homology to known allergens

The Applicant presented the results of a bioinformatic assessment of the pullulanase protein. In the first analysis, the pullulanase amino acid sequence was compared with the Allermatch database to identify sequences of 35% or greater homology with known allergens. No matches were found between this pullulanase and known allergens.

In a second analysis, the pullulanase sequence was compared with the Allermatch database to identify sequences of 8 contiguous amino acids with 100% identity to known allergens. No matches were found between this pullulanase and known allergens.

4.5.3 Ingredients used in manufacture

Soybean meal is utilised as a nitrogen source during the fermentation process in the production of the enzyme preparation. However, the Applicant states that no known allergens as listed in Standard 1.2.3 of the Code are present in the final enzyme preparation.

4.6 JECFA consideration

JECFA has not considered enzymes from *B. acidopullulyticus.*

5 Dietary Exposure

Processing aids perform their technological function during the manufacture of food and are either not present in the final food or present only at very low levels.

For the intended use of this pullulanase in the beverage alcohol application, the Applicant claims no enzyme or enzyme residues are present in the final food, while for the brewing and starch applications there is expected to be minimal carry-over of enzyme into the final food.

This pullulanase is expected to be inactivated and removed during the manufacturing and production stages. Any residual enzyme in the final food would be present as denatured protein and would undergo normal proteolytic digestion in the gastrointestinal tract.

The applicant provided a theoretical worst-case scenario of dietary exposure for the pullulanase using the Budget method. Using extremely conservative assumptions the calculated margin of safety in processed foods and drinks was 358. This calculated margin of safety is theoretical only, because pullulanase showed no toxicity even at the highest dose. This, together with 'an ADI not specified' supports the conclusion that another dietary exposure calculation is unnecessary.

6 Response to Risk Assessment Questions

Does the enzyme preparation present any food safety issues?

The Safety Assessment reviewed evidence examining the potential toxicity of the pullulanase enzyme preparation. Based on the results of *in vitro* and animal toxicity studies, bioinformatic analysis and a known history of use, no hazards were identified, which would preclude permitting use of the enzyme as a food processing aid. The absence of any specific hazards being identified is consistent with the pullulanase being safe for use in food.

Does the enzyme achieve its stated technological purpose?

The Application clearly articulates the stated purpose for this enzyme, namely as a debranching enzyme used in the starch and alcohol industries for the saccharification of liquefied starch. The evidence submitted in support of the Application provides adequate assurance that the pullulanase, in the form and amounts added, is technologically justified and has been demonstrated to be effective in achieving its stated purpose.

7 Conclusion

The evidence presented was sufficient to determine that no toxicological or hazard-related concerns with the enzyme or the donor or host microorganisms exist. The absence of any specific hazards being identified is consistent with the pullulanase undergoing normal proteolytic digestion in the gastrointestinal tract. Pullulanase from the identified bacterial source is considered safe for use as a food processing aid.

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