7 July 2017
[17–17]

Supporting document 1

Risk and technical assessment report (at Approval) – Application A1125

Endo ß(1,4) Xylanase as a Processing Aid (Enzyme)

Executive summary

Application A1125 seeks approval to use the enzyme endo β(1,4) xylanase (EC 3.2.1.8), produced by genetically modified (GM) *Bacillus subtilis* that contains a *xylanase* gene sourced from *Pseudoalteromonas haloplanktis*, for use as a processing aid in the manufacture of cereal products.

The food enzyme endo ß(1,4) xylanase is used as a processing aid in the manufacture of certain cereal products. Cereals contain arabinoxylans (polysaccharides) which impart important functional properties in baking processes, such as bread-making, and other cereal-based processes, including the production of pasta, noodles and snacks. This is due to their ability to interact with gluten, bind water and provide dough viscosity.

Xylanases catalyse the conversion of arabinoxylan polysaccharides into constituent arabinoxylan oligosaccharides. While xylanases are naturally present in many cereals, the addition of further endo β(1,4) xylanase (in this case from a microbial source) during processing allows the solubilisation of the arabinoxylans, which in turn improves the functional properties of these polysaccharides.

The evidence presented to support the proposed uses of the enzyme preparation provides adequate assurance that the enzyme, in its commercial form and proposed levels of usage, is technologically justified to be effective in achieving its stated purpose. It was also concluded that the enzyme performs its technological purpose during processing and manufacture of food after which it is inactivated so does not perform any technological function in the final food. It is therefore appropriately categorised as a processing aid and not a food additive. The enzyme preparation meets international purity specifications for enzymes used in the production of food.

There are no public health and safety issues associated with the use of the endo β(1,4) xylanase enzyme preparation, produced by GM *B. subtilis* that contains a *xylanase* gene sourced from *P. haloplanktis,* as a food processing aid on the basis of the following considerations:

- The production organism is not toxigenic or pathogenic. Further, GM and non-GM *B. subtilis* have a history of safe use as the production organism for a number of enzyme processing aids already permitted in the *Australia New Zealand Standards Code* (the Code) (FSANZ 2016) and overseas.

- The food use of *P. haloplanktis* xylanase expressed in *B. subtilis* has been approved in other countries.

- If residual endo β(1,4) xylanase were to be present in the final food, it would, because of exposure to high temperatures during baking, be denatured and hence inactivated. In addition, it would be as susceptible to digestion as any other dietary protein.

- Bioinformatic analysis indicated that *P. haloplanktis*-derived endo β(1,4) xylanase has no biologically relevant homology to known food protein allergens.

- There were no treatment-related signs of toxicity in a 90-day repeat dose study in rats with endo β(1,4) xylanase concentrate at a dose of 13.94 mg total organic solids (TOS)/kg bw/day. This is orders of magnitude higher than the likely exposure to the enzyme preparation according to the proposed uses.

- The enzyme was not genotoxic or mutagenic *in vitro*.

Based on the reviewed toxicological data, it is concluded that, in the absence of any identifiable hazard, an Acceptable Daily Intake ‘not specified’ is appropriate. A dietary exposure assessment is therefore not required. It was therefore concluded that endo β(1,4) xylanase produced by GM *B. subtilis* that contains a *xylanase* gene sourced from *P. haloplanktis,* is considered unlikely to pose any health concern when used as a food processing aid.

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1 Introduction

FSANZ received an application from Puratos NV (Puratos), Belgium, seeking approval for the enzyme endo β(1,4) xylanase (EC 3.2.1.8) (also noted in the Application as endo β(1-4) xylanase and henceforth referred to as xylanase), as a processing aid. The Applicant states that this enzyme will be used in the manufacture of bakery products such as bread, biscuits and cakes, as well as other cereal-based products such as pasta, noodles and snacks.

The enzyme is derived from *Pseudoalteromonas haloplanktis* (an Antarctic bacterium), and produced by a GM *Bacillus subtilis.* Xylanase sourced from this bacterium is by means of a fermentation process. *B. subtilis* (both GM and non-GM) is the host microorganism for 11 other permitted enzymes in the Code.

Xylanases catalyse the hydrolysis of linear polysaccharides such as hemicellulose, one of the major components of plant cell walls. They are widely distributed, occurring in diverse genera of bacteria, actinomycetes and fungi (Beg et al. 2001). They have been used for several decades in the food industry (Pariza and Johnson 2001) including in baking (particularly bread-making) and fruit juice and beer clarification (Sharma and Kumar 2013).

Cereals contain arabinoxylans (polysaccharides) which impart important functional properties in baking and other cereal-based processes, due to their ability to interact with gluten, bind water and provide dough viscosity. The Applicant proposes to use xylanase to catalyse the conversion of arabinoxylan into constituent arabinoxylan oligosaccharides. While xylanase is naturally present in many cereals, the addition of further xylanase (in this case from a microbial source) during processing allows the solubilisation of the arabinoxylans, which in turn improves the functional properties of these polysaccharides, ultimately leading to better and/or more consistent product quality.

The enzyme preparation shows optimal activity at temperatures and pH typically used in dough proofing, resulting in increased efficiency during dough preparation and in batch to batch consistency.

1.1 Objectives of the assessment

Currently, there are no permissions for *P. haloplanktis* xylanase expressed in *B. subtilis* in the Code. Therefore, any application to amend the Code to permit the use of this enzyme as a food processing aid requires a pre-market assessment.

The objectives of this risk 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 the xylanase enzyme as a processing aid.

2 Food technology assessment

2.1 Characterisation of the enzyme

2.1.1 Identity of the enzyme

The host organism is *B. subtilis*, production strain Gizα7101 (accession number LMG S-24584, Belgian Co-ordinated Collection of Microorganisms), which was genetically modified from a strain derived from the type strain *B. subtilis* Marburg 168. *B. subtilis* Marburg 168 is considered the type strain of this species. The donor organism is the Antarctic bacterium *P. haloplanktis.*

Information regarding the identity of the enzyme that was taken from the Application has been verified using an appropriate enzyme nomenclature reference (IUBMB 2016). Additional information has also been included from this reference.

Generic common name:	Xylanase
Accepted IUBMB[1] name:	endo-1,4-β-xylanase
Systematic name:	4-β-D-xylan xylanohydrolase
IUBMB enzyme nomenclature:	EC 3.2.1.8
C.A.S. number:	9025-57-4
Other names:	endo-(1→4)-β-xylan 4-xylanohydrolase; endo-1,4-xylanase; xylanase; β-1,4-xylanase; endo-1,4-xylanase; endo-β-1,4-xylanase; endo-1,4-β-D-xylanase; 1,4-β-xylan xylanohydrolase; β-xylanase; β-1,4-xylan xylanohydrolase; endo-1,4-β-xylanase; β-D-xylanase
Reaction:	Endohydrolysis of (1→4)-β-D-xylosidic linkages in xylans (polysaccharides) It can use (arabino)xylans in wheat flour as a substrate
Commercial name:	Premix X-608 (also sold commercially as Premix X-618 and Bel'Ase B218)

2.1.2 Technological purpose

The enzyme xylanase, produced by GM *B. subtilis* that contains a *xylanase* gene sourced from *P. haloplanktis* is intended to be used as a processing aid in the manufacture of bakery products such as bread, biscuits and cakes, as well as other cereal-based products such as pasta, noodles and snacks.

[1] International Union of Biochemistry and Molecular Biology

The carrier of the commercial enzyme preparation is food grade wheat flour, which is compatible with its intended applications i.e. baking and other cereal-based processes. Specifically, the Applicant recommends the enzyme preparation be added to standard type flours/baking flours and dough conditioners. It can be used alone or in combination with other baking enzymes.

The enzyme catalyses the conversion of arabinoxylans (polysaccharides naturally present in cereals that impart important functional properties) to arabinoxylan oligosaccharides.

2.1.3 Technological justification

High molecular weight arabinoxylans can cause technical difficulties in the processing of raw materials in the baking industry due to their ability to increase viscosity of doughs. The technological justification for converting arabinoxylans to arabinoxylan oligosaccharides within the dough, leads to better and more consistent product quality during baking processing, due to facilitating:

- the handling of the dough (improved extensibility and stability; less stickiness leading to reduced loss of dough)
- improvement in the dough's structure and behaviour during the baking step
- a uniform volume and an improved crumb structure of the bakery product, which might otherwise be impaired by processing of the dough
- reduced batter viscosity; beneficial in the production process for e.g. waffles, pancakes and biscuits.

In addition, the enzyme preparation has benefits over other approved xylanases including:

- improved effectiveness under typical production conditions for temperature and pH:

  - optimal activity in temperatures ranging between 25–40°C, within which lies the temperature range for dough proofing which is generally between 25–35°C. *P. haloplanktis* is a psychrophilic[2] bacterium and the resultant enzyme preparation demonstrates good activity even at low temperatures, having 90% activity at 20°C.
  - optimal pH range for enzyme activity corresponds to the pH of dough

- increased production process efficiency compared with permitted xylanases of fungal origin, or mesophilic xylanases of bacterial origin. Two of the permitted bacterial xylanases have a higher optimal temperature of 50°C and have less activity at the preparation and proofing temperatures (25–35°C) so that they only retain 20% activity in the dough proofing temperature range

- highly purified state; apart from the main enzymatic activity, the enzyme preparation does not show significant levels of subsidiary/side activities compared to some other commercial xylanase preparations

- unaffected by certain xylanase inhibitors in cereals which are known to inhibit other microbial xylanases (more information is provided in the Application on this benefit)

- higher activity on insoluble arabinoxylans compared to some other xylanase enzyme preparations.

[2] Capable of growth and reproduction in cold temperatures.

The technological justification of using the xylanase of this Application is that it is able to convert large arabinoxylans polysaccharides from cereal raw materials into the smaller oligosaccharides useful in baking processes. There are a number of other xylanase enzymes that also perform this function but this endo β(1,4) xylanase has additional advantages over other enzymes that provide increased production efficiencies and better and more consistent final baked product.

2.2 Manufacturing process

2.2.1 Production of the enzyme

The enzyme preparation is produced by the fermentation of the production strain *B. subtilis* Gizα7101. The production process is a closed, pure culture, submerged fermentation. The production steps can be summarised as a fermentation process, filtration process, production of the concentrated enzyme, and standardisation of the final commercial enzyme preparation.

Cultures are started in a 1 L Erlenmeyer flask and then transferred to fermenters of increasing size with central stirrers. The xylanase produced by the *B. subtilis* Gizα7101 is secreted into the culture medium. Once the fermentation is complete, the biomass is separated from the enzyme-containing culture medium by microfiltration. The enzyme preparation is then concentrated by ultrafiltration, which is followed by sterile filtration. The resultant solution is therefore free of the production strain and any microorganisms. The concentrated liquid enzyme is then dried using a spray dryer – where it is sprayed on the carrier (wheat flour).

The final step is standardisation. This involves diluting the dried enzyme preparation with wheat flour to achieve the required concentration. The resultant commercial enzyme preparations are in a non-dusting, micro-granulate form, and light brown in colour.

The fermentation and downstream processes are schematically represented in Figure 1 below taken from the Application.

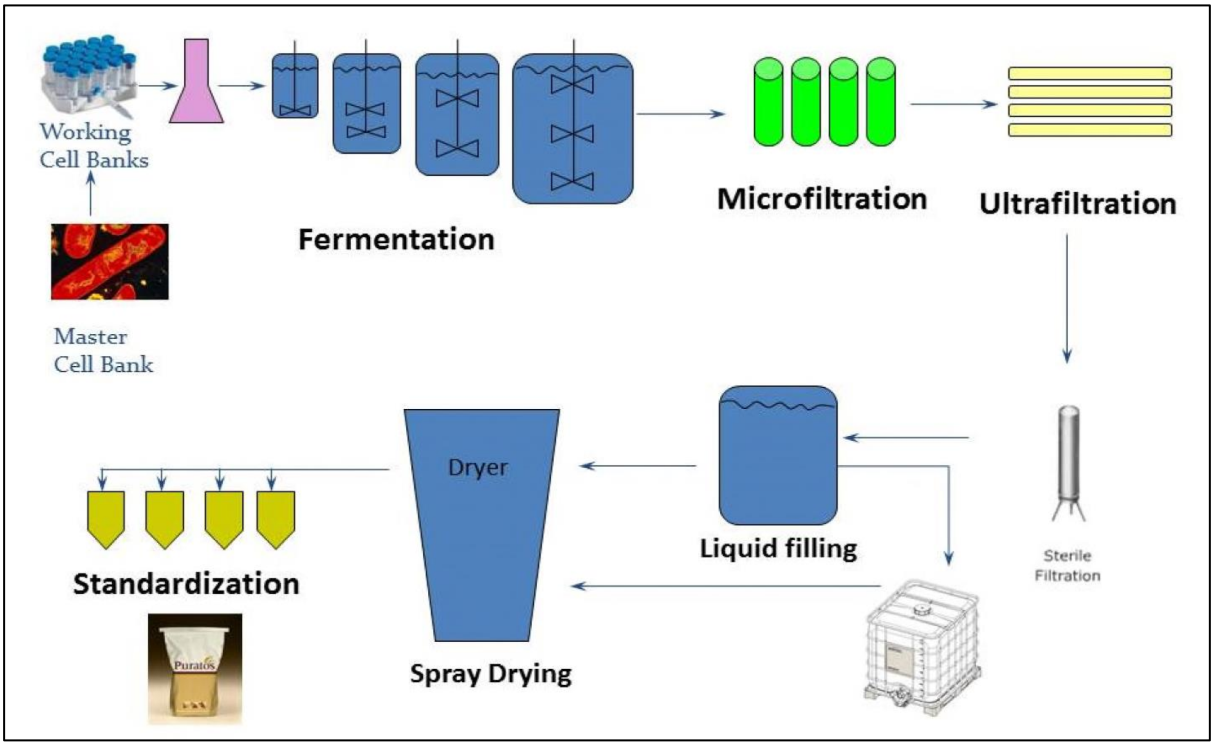

Figure 1: Schematic representation of the production process for xylanase

The raw materials used in the fermentation and recovery process are of food grade quality, in line with the EU Food Hygiene Regulation (852/2004) (European Parliament 2004) and meet the requirements of the Code. The enzyme preparation is reported to be made according to Good Manufacturing Practices (GMP) and the principles of HACCP. A HACCP plan was provided with the Applicant's Confidential Commercial Information (CCI) material

2.2.2 Allergen considerations

The commercial enzyme preparation is granulated and fixed to a food grade wheat carrier. As well, maltodextrin and starch (which may be produced from wheat starch), may be among the raw materials used as fermentation media in the production of the enzyme. The presence of wheat or products derived from wheat such as maltodextrin and starch could be of concern to people with wheat allergies or intolerances.

The Application states the commercial product does not contain any other identified allergens that require declaration as per section 1.2.3—4 in Standard 1.2.3 – Information requirements – warning statements, advisory statements and declarations, neither as ingredients nor through possible cross contamination.

2.2.3 Specifications

There are international specifications for enzyme preparations used in the production of food. These have been established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) Compendium of Food Additive Specifications (JECFA 2016) and the Food Chemicals Codex (Food Chemicals Codex 2014). These primary sources of specifications are listed in section S3—2. Enzyme preparations need to meet these enzyme specifications. Schedule 3 also includes specifications for heavy metals (section S3—4) if they are not specified within specifications in sections S3—2 or S3—3.

Table 2.1 provides a comparison of the product specifications with the international specifications established by JECFA as well as those detailed in the Code (as applicable).

Table 2.1: Product specifications for commercial enzyme preparation compared to JECFA and Code specifications for enzymes

Analysis	Specifications		
	Enzyme preparation	JECFA	*Australia New Zealand Food Standards Code (section S3—4)*
Lead (mg/kg)	<0.10	≤ 5	≤2
Arsenic (mg/kg)	<0.10	-	≤1
Mercury (mg/kg)	<0.010	-	≤1
Cadmium (mg/kg)	<0.010	-	≤1
Coliforms (cfu/g)	≤10	≤30	-
Salmonella (in 25 g)	Absent	Absent	-
E. coli (in 25 g)	Absent	Absent	-

Based on the above product specifications, the final enzyme preparation meets international and Code specifications for enzyme preparations used in the production of food.

2.2.4 Stability

Results of stability analyses provided by the Applicant for three samples indicate that xylanase activity at the end of the recommended shelf-life of 24 months had not reduced. The storage conditions of the enzyme preparations were not reported though the company's technical data sheet indicates the enzyme preparations should be stored between 5–20°, less than 65 % relative humidity but stored in the original tightly sealed polyethylene multilayer paper bags (25 kg).

2.3 Food technology conclusion

The stated purpose of this endo β(1,4) xylanase preparation namely, for use as a processing aid is clearly articulated in the Application. The Applicant states that this enzyme will be used in the manufacture of bakery and other cereal-based products by solubilisation of the arabinoxylans derived from cereal raw materials. These smaller oligosaccharides have improved functional properties during the baking process which ultimately leads to increased efficiency and improved batch to batch consistency. The evidence presented to support the proposed uses provides adequate assurance that the enzyme, in its commercial form and proposed usage levels, is technologically justified and has been demonstrated to be effective in achieving its stated purpose. That is, it performs its technological purpose during processing and manufacture of food after which it is inactivated so does not perform any technological function in the final food. It is therefore appropriately categorised as a processing aid and not a food additive. The enzyme preparation meets international purity specifications.

3 Hazard assessment

3.1 Background

3.1.1 Chemistry

Details of the chemistry of the *P. haloplanktis*-derived xylanase enzyme produced by *B. subtilis* including relevant physicochemical and enzymatic properties, and specifications of Premix X-608, are provided in the Food Technology Assessment (Section 2).

3.1.2 Description of the genetic modification

The genetic modification involved the integration of the *endo β(1,4) xylanase* gene (designated *exp* for the purposes of this report) from *P. haloplanktis* into *B. subtilis* strain DB105 to give a production strain designated Gizα7101.

An expression cassette was constructed comprising:

- part of a promoter from *B. subtilis*
- the coding sequence of the exp gene
- a signal peptide from *B. subtilis*
- a terminator sequence from *B. subtilis*.

The cassette was part of a pBR322 plasmid vector to allow cloning in *E. coli*. The plasmid also contains a gene for chloramphenicol resistance which allows for selection of the transformed *B. subtilis* and two host strain DNA fragments (derived from *B. subtilis*) which flank the expression cassette and are targets for sequence integration (fragments A and B).

B. subtilis containing the integrated plasmid vector was selected and maintained on a medium containing chloramphenicol. *B. subtilis* could not replicate in the presence of chloramphenicol unless the plasmid was integrated into the chromosome. Omission of chloramphenicol from the medium resulted in homologous recombination (*i.e.* between identical A or B fragments). Homologous recombination between B fragments resulted in integration of the expression cassette only (*i.e*. none of the rest of the plasmid is integrated). Confirmation of this event was established through loss of chloramphenicol resistance and Southern blotting using the resistance gene as a probe.

The above process of integration was repeated a number of times so that there are multiple copies of the *exp* gene in Gizα7101.

Following transformation, 16S analysis was performed on the parental strain and Gizα7101. The phylogenetic comparison, showing 100% identity, indicates that Gizα7101 is indeed *B. subtilis*.

To test the stability of the insert in the production strain, Southern blot analysis, using a probe derived from part of the introduced *exp* gene, was done on DNA from cells from three separate end-of-production batches and was compared to reference genomic DNA of the production strain. The band patterns (number and size) obtained for all the production batches were identical to the band pattern of the reference production strain, thus indicating stability of the insert.

3.1.3 Scope of the hazard assessment

The hazard of xylanase derived from *P. haloplanktis* was evaluated by considering:

- the hazard of the production organism, including any history of safe use in food production processes
- the hazard of the encoded protein, including potential allergenicity
- toxicity studies on the enzyme preparation intended for commercial use.

3.2 Hazard of the production organism – *B. subtilis* strain Gizα7101

The production strain, Gizα7101, containing multiple copies of the *exp* gene was genetically modified from a strain, DB105, derived from the type strain *B. subtilis* Marburg 168. DB105 was developed through classical congression which introduced four auxotrophic mutations (i.e. a mutant strain requiring specific media supplements not required by the wild type strain). DB105 was altered further through the deletion of a xylanase gene and the addition of a mutation in a second xylanase gene to reduce resident xylanase activity. Targeted recombination was used to inactivate genes encoding extracellular alkaline and neutral protease, as well as a gene essential for sporulation, with the aim of enhancing the safety and stability of the final xylanase product. A Vero cell cytotoxicity assay performed according to an adapted protocol (EFSA 2014) indicated Gizα7101 does not produce toxic compounds.

B. subtilis is a ubiquitous soil microorganism that contributes to nutrient cycling through production of proteases and other enzymes that degrade natural substrates. It is a gram-positive bacterium which multiplies and disseminates by asexual processes. Wild type strains produce endospores that allow for endurance of extreme environmental conditions such as heat and desiccation.

The bacterium has been used for decades for production of food enzymes with no known reports of adverse effects on human health or the environment (de Boer and Diderichsen 1991).

It has been used in the commercial production of α-amylase since 1929 (Reed 1966). Considering its long history of commercial use and following an extensive risk assessment, the US Environmental Protection Agency, in a report (US EPA 1997), concluded that *B. subtilis* is neither a human pathogen nor toxigenic. Additionally, a review of the literature by the US EPA in the same report did not reveal the production of any secondary metabolites of toxicological concern by *B. subtilis*. The bacterium 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 source organism of food processing aids and the following enzymes derived from *B. subtilis* (both GM and non-GM) are listed as permitted in Schedule 18: α-acetolactate decarboxylase, α-amylase, β-amylase, asparaginase, endo-1,4-beta-xylanase, β-Glucanase, hemicellulase multicomponent enzyme, maltogenic α-amylase, metalloproteinase, pullulanase, and serine protease. In the US, several enzyme preparations from *B. subtilis* have GRAS status (FDA 1999; FDA 2003; FDA 2006; FDA 2009).

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) (Inatsu et al. 2006; Hosoi and Kiuchi 2008).

Data submitted with the application indicate that the *B. subtilis* production strain is not detectable in the final enzyme preparation to be used as a food processing aid. The organism is removed during a multi-step recovery of the purified enzyme following closed pure culture submerged fermentation. The microfiltration stage towards the end of the manufacturing process involves filtration at alkaline pH and is followed by ultrafiltration and sterile filtration steps that result in an enzyme concentrate solution free of the production strain.

The Applicant has indicated that, if present, secondary activities from enzymes such as protease and α-amylase could be relevant in bakery processes. Assays of three xylanase batches showed no protease activity and only one batch showed negligible amylase activity. Performance assays run with various enzyme batches indicated that enzymatic secondary activities (even when measurable) did not have any technical impact on bread making. As noted above, α-amylase derived from *B. subtilis* is listed as a permitted processing aid in Schedule 18.

3.3 Hazard of the enzyme xylanase

It is noted that the signal peptide is cleaved off the mature xylanase (designated EXP for the purposes of this report) produced by *B. subtilis* Gizα7101 during its secretion. The protein is identical in sequence to the native xylanase and there is no post-translational modification.

The intention is that the final enzyme preparation (designated Premix X-608 and used in microgranulate form – see Table 3.1) is added to baking ingredients. The enzyme is active during dough preparation and leavening but is inactivated within 15 minutes at temperatures of 60^{o} C and above. This regime is well below the high temperatures used during baking. It is likely that any residual enzyme in the final food would therefore be present as denatured protein and undergo normal proteolytic digestion in the gastrointestinal tract. To confirm the digestibility of EXP, potential cleavage sites were investigated by FSANZ using the amino acid sequence of EXP and the PeptideCutter tool in the ExPASy Proteomics Site.

EXP has multiple cleavage sites for pepsin (79 sites at pH 1.3 and 133 sites at pH >2), trypsin (35 sites), chymotrypsin (62 high-specificity sites, 103 low-specificity sites) and endopeptidases (69 sites). On this basis, EXP is considered likely to be as susceptible to digestion as the vast majority of dietary proteins.

3.3.1 Bioinformatic analysis for potential allergenicity

An *in silico* analysis compared the *P. haloplanktis*-derived xylanase amino acid sequence (not including the signal peptide) with known allergens in the FARRP (Food Allergy Research and Resource Program) dataset which is available through AllergenOnline. The FASTA bioinformatics tool (Pearson and Lipman 1988) version 35.04 was used for three analyses.

a) A full FASTA search which provides per cent identity and an E-score[3] to indicate whether there are any alignments between the query protein and sequences within the allergen database.
b) The sliding 80mer sliding window search in order to find any matches that meet or exceed the Codex Alimentarius (Codex 2003) FASTA alignment threshold (at least 35% identity over 80 amino acids) for potential allergenicity. This threshold aims to detect potential conformational IgE-epitopes.

c) An 8mer identity match which may be indicative of cross reactive proteins[4].

No significant homology with any known allergens was determined. The conclusion from these bioinformatic analyses is that the *P. haloplanktis*-derived xylanase does not show biologically relevant homology to any known allergen and on this basis is unlikely to be allergenic.

3.4 Evaluation of toxicity studies of the enzyme product

Unpublished toxicity studies on a representative xylanase preparation were submitted by the Applicant and independently evaluated by FSANZ. These studies comprised:

- two genotoxicity tests - an Ames test conducted in accordance with OECD test Guideline 471 (OECD 1997a) and a chromosome aberration test conducted in accordance with OECD test Guideline 473 (OECD 1997b)
- a 90-day oral toxicity study in rats conducted in accordance with OECD Test Guideline 408 (OECD 1998).

The test substance was purified xylanase designated as toxbatch 05/78 that was dissolved in sterile distilled water at the maximum concentration possible. This test substance differed from the commercial final product, Premix X-608 that is in micro-granulated form and is mixed with a wheat flower carrier. It is noted that toxbatch 05/78 was produced early in the development of Premix X-608 and the current efficiency of production yields an enzyme with approximately 10 x the activity of toxbatch 05/78.

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[3] Comparisons between highly homologous proteins yield E-values approaching zero, indicating the very low probability that such matches would occur by chance. A larger E-value indicates a lower degree of similarity. Commonly, for protein-based searches, hits with E-values of 10^{-3} or less and sequence identity of 25% or more are considered significant although any conclusions reached need to be tempered by an investigation of the biology behind the putative homology (Baxevanis 2005).

[4] It is considered unlikely that isolated identity matches of 6 or 8 amino acids would be found between cross-reactive proteins unless there was at least a 35% identity match over 80 amino acids (Goodman et al. 2008).

The activity of xylanase in current Premix X-608 is set at 540 GDXU[5]/g thereby diluting the enzyme by several factors. In addition, since the Premix X-608 is used at a maximum of 110 ppm the xylanase is further diluted > x9,000 for actual use in the bakery.

Table 3.1: Comparison of xylanase toxbatch 05/78 and Premix X-608

Characteristic	toxbatch 05/78	Premix X-608
Appearance	brown solution	brown micro-granules
Approx. Xylanase Activity	300 GDXU/ml	540 GDXU/g
Total Organic Solids (TOS) (approx. % w/w)	2.49	Not determined

3.4.1 Sub-chronic toxicity

Individual unpublished study

Thisse, V. (2005). Determination of repeated dose 90-day oral toxicity of enzyme preparation X608 in the rat. SGS Lab. Simon Study Report: S105705. Beldem (unpublished)

Male and female Sprague Dawley albino rats (Elevage Janvier, Belgium) were assigned to three groups: 12 animals per sex for the treatment group, 10 animals per sex in the control group, and 10 animals per sex in a satellite (recovery) group (maintained for 14 days post-treatment). Food (SAFE A04C from UAR, France) and tap water were provided *ad libitum.* The test material (toxbatch 05/78 xylanase) was administered via oral gavage at a dose of 1450 GDXU/kg bw/day, five days per week, over 90 days. Control animals received the water vehicle only. Animals were observed for mortality, body weight and feed consumption and blood was taken on day 90 for haematological examination. Necropsy was carried out at the end of the treatment period.

No treatment-related mortality, clinical signs, effects on body weight or haematology were observed. Necropsy findings were unremarkable. The No Observed Adverse Effect Level (NOAEL) for the xylanase concentrate was 1450 GDXU/kg bw/day, or 13.94 mg TOS/kg bw/day.

3.4.2 Genotoxicity

Individual unpublished studies

Thompson, P.W. (2005) X608: Reverse mutation assay “Ames Test” using *Salmonella typhimurium* SPL Project Number: 1869/0005. Puratos Group (unpublished).

Wright, N.P. and Pickard, F.E. (2006). X608: Chromosome aberration test in human lymphocytes *in vitro*. SPL Project Number: 1869/0006. Puratos Group (unpublished).

The results of these unpublished *in vitro* studies are summarised in Table 3.2. There was no biologically or stastically significant increase in the number of reverting colonies observed in a bacterial reverse mutation test at concentrations of up to 5000 µg/plate, either in the absence or presence of metabolic activation. No significant differences between the treated and the negative control in the frequency of cells with aberrations or polyploidy were observed in a chromosomal aberration test, in the absence or presence of metabolic activation (S9 mix).

[5] GDXU is the standard unit of measurement of xylanase activity developed by the Applicant (definition is Confidential Commercial Information).

Table 3.2: Summary of genotoxicity studies

Test	Test system	Test article	Concentration or dose range	Result
Bacterial reverse mutation (Ames test)	*Salmonella typhimurium* strains TA 98, 100, 102, 1535 & 1537	Xylanase derived from GM *B. subtilis* (Batch No. 05/78, 100% purity) Water vehicle Positive controls: • N-ethyl-N′-nitro-N-nitrosoguanidine (for TA100 & TA1535) • 9-Aminoacridine (for TA 1537) • Mitomycin C (for TA102) • 4-Nitroquinoline-1-oxide (for TA98)	Preliminary test (strainTA100 only): plate incorporation method (0-5000 µg/plate) Tests 1 & 2: plate incorporation method (50-5000 µg/plate)	Negative (<u>+</u>S9)
Chromosomal aberration test	Human lymphocytes	Xylanase derived from GM *B. subtilis* (Batch No. 05/78, 100% purity) Water vehicle Positive controls: • For minus S9 – Mitomycin C • For plus S9 - Cyclophosphamide	Preliminary tests: 4(20)hour<u>+</u>S9 19.53-5000 µg/mL 24hour-S9 312.5-2500 µg/mL Test 1: 4(20)hour<u>+</u>S9 156.25-5000 µg/mL Test 2: 4(20)hour<u>+</u>S9 156.25-5000 µg/mL 24-hour –S9 312.5-5000 µg/mL	Negative (<u>+</u>S9)

3.5 Risk assessment conclusions

There are no public health and safety concerns associated with the use of the product Premix X-608 containing xylanase produced by GM *B. subtilis* that contains a *xylanase* gene sourced from *P. haloplanktis* as a food processing aid on the basis of the following considerations:

- The production organism is not toxigenic or pathogenic. Further, GM and non-GM *B. subtilis* have a history of safe use as the production organism for a number of enzyme processing aids already permitted in the Code and overseas.

- The food use of P*. haloplanktis* xylanase expressed in *B. subtilis* has been approved in other countries.

- If residual xylanase were to be present in the final food, it would, because of exposure to high temperatures during baking, be denatured and hence inactivated. In addition, it would be as susceptible to digestion as any other dietary protein.

- Bioinformatic analysis indicated that *P. haloplanktis*-derived xylanase has no biologically relevant homology to known food protein allergens.

- There were no signs of toxicity in a 90-day repeat dose study in rats with the endo β(1,4) xylanase concentrate. The NOAEL was 1450 GDXU/kg bw/day or 13.94 mg Total Organic Solids (TOS)/kg bw/day. This is orders of magnitude higher than the likely human exposure to the enzyme preparation according to the proposed uses.

- The enzyme was not genotoxic or mutagenic *in vitro*.

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 is therefore not required.

4 Conclusion

This risk and technical assessment considered the technological suitability, potential hazard of the GM production microorganism and the encoded protein, including potential allergenicity.

It is concluded that the proposed use of the enzyme is technologically justified in its commercial form and proposed usage levels, and has been demonstrated to be effective. The evidence presented did not demonstrate any safety concerns associated with the production microorganism or the enzyme. Thus xylanase produced by GM *B. subtilis* that contains a *xylanase* gene sourced from *P. haloplanktis* is considered unlikely to pose any health concern when used as a food processing aid.

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