Puratos
Reliable partners in innovation

APPLICATION

DECEMBER 2015

TO:
FOOD STANDARDS AUSTRALIA NEW ZEALAND (FSANZ)

IN RELATION TO:
APPLICATION FOR APPROVAL OF ENDO β(1-4) XYLANASE FROM A GENETICALLY MODIFIED STRAIN OF BACILLUS SUBTILIS LMG S-24584 AS A PROCESSING AID

Puratos
Reliable partners in innovation

TABLE OF CONTENTS

TABLE OF CONTENTS		2
LIST OF APPENDICES, FIGURES AND TABLES		4
ADMINISTATIVE INFORMATION		8
1.	APPLICATION INFORMATION	9
2	PURPOSE OF THE APPLICATION	9
3	JUSTIFICATION FOR THE APPLICATION	10
3.1	NEED FOR THE PROPOSED CHANGE	10
3.2	ADVANTAGES OF THE PROPOSED CHANGE	13
3.3	DISADVANTAGES OF THE PROPOSED CHANGE	13
4	REGULATORY IMPACT INFORMATION	14
4.1	COSTS AND BENEFITS – CONSUMER	14
4.2	COSTS AND BENEFITS - INDUSTRY AND BUSINESS	14
4.3	COSTS AND BENEFITS – GOVERNMENT	14
4.4	IMPACT ON INTERNATIONAL TRADE	14
5	INFORMATION TO SUPPORT THE APPLICATION	15
5.1	DATA REQUIREMENTS	15
5.2	FSANZ ACT OBJECTIVES	15
5.3	PUBLIC HEALTH AND SAFETY ISSUES	15
5.4	CONSUMER CHOICE	15
5.5	SUPPORT FOR THE PROPOSED CHANGE	16
5.6	POLICY GUIDELINES	16
6	INTERNATIONAL AND OTHER NATIONAL STANDARDS	17
6.1	INTERNATIONAL STANDARDS	17
6.2	OTHER NATIONAL STANDARDS OR REGULATIONS	17

7 PROCESSING AID 21
7.1 TECHNICAL INFORMATION ON THE PROCESSING AID 21
7.2 INFORMATION ON THE SAFETY OF AN ENZYME PROCESSING AID 33
7.3 ADDITIONAL INFORMATION RELATED TO THE SAFETY OF AN ENZYME PROCESSING AID DERIVED FROM A MICROORGANISM 40
7.4 ADDITIONAL INFORMATION RELATED TO THE SAFETY OF AN ENZYME PROCESSING AID DERIVED FROM A GENETICALLY-MODIFIED MICROORGANISM 42
7.5 INFORMATION RELATED TO THE DIETARY EXPOSURE TO THE PROCESSING AID 64
REFERENCES 68

Confidential Commercial Information (CCI) Information

Additional material has been provided under CCI to support the Application for Sections 7.1.3; 7.1.4 and 7.4.1. This material has been provided separately to this Application document.

Puratos
Reliable partners in innovation

LIST OF APPENDICES, FIGURES AND TABLES

Appendices

Appendix	Title
1	Codex Committee on Food Additives (CCFA) 45 [h] session in 2013 (page 70) *Note: full copy provided with e-copy. Pages 1-4 & 70 provided in hard copy*
2	Authorisation of this xylanase produced by *Bacillus Subtilis* – France (afssa) *Note: provided in French and English*
3	Authorisation of this xylanase produced by *Bacillus Subtilis* – Brazil *Note: full copy provided with e-copy. Pages 1-3 provided in hard copy*
4	Authorisation of this xylanase produced by *Bacillus Subtilis* – USA
5	Authorisation of this xylanase produced by *Bacillus Subtilis* – Canada
6	Authorisation of this xylanase produced by *Bacillus Subtilis* – EU
7	JECFA • Authorized food enzymes (other than xylanase) produced by the same production organism, *Bacillus subtilis* • Authorized xylanase from production organisms other than *Bacillus subtilis*
8	France • Authorized food enzymes (other than xylanase) produced by the same production organism, *Bacillus subtilis* • Authorized xylanase from production organisms other than *Bacillus subtilis*
9	USA • Authorized food enzymes (other than xylanase) produced by the same production organism, *Bacillus subtilis* • Authorized xylanase from production organisms other than *Bacillus subtilis*
10	Denmark • Authorized food enzymes (other than xylanase) produced by the same production organism, *Bacillus subtilis*

Appendix	Title
	• Authorized xylanase from production organisms other than *Bacillus subtilis*
11	Technical Data Sheet Commercial endo β(1-4) xylanase (Premix X-608)
12	Certificate of Analysis (CoA) – Side Activities Methods (Table 4) provided as CCI
13	Analytical Method - Activity of the Enzyme (CCI)
14	Stability Tests – Xylanase Premix X-618
15	AMES Test
16	Chromosomal Aberration Test
17	90day Oral toxicity test
18	Acute Inhalation Toxicity
19	Dermal and Eye Irritation
20	Composition and Specification of the test material – CoA
21	Composition and Specification of the Commercial food enzyme – CoA
22	Allergenicity Sequence
23	Federal Register, Docket No. 84G-0257
24	Directive 2000/54/EC on the protection of workers from risks related to exposure to biological agent at work List of pathogens from Belgian (Biosafety Server, 2010)
25	NIH Guidelines (USA)
26	OECD (1992)
27	Report on cytotoxic assays for Giza7101 strain
28	Demonstration of the Absence of the GMM in the Product **(CCI)**

Appendix	Title
29	Demonstration of the Absence of the GMM in the Product – Sampling Wizard® Magnetic DNA Purification System for Food (Promega)
30	Euromonitor Bread Consumption Australian Health Survey: Nutrition First Results – Foods and Nutrients, 2011–12 — Australia - TABLE 6.3 Median amount of foods consumed (grams)(a)

Figures

Figure Number	Title	Page
1	The baking process	12
2	Relative xylanase activity vs pH and temperature	26
3	Relative xylanase activity at different temperatures	26
4	Schematic representation of the production process of the food enzyme	28
5	Description of *Pseudoalteromonas haloplanktis* as belonging to the risk 1 class of microorganism by the American Type Culture Collection	48
6	Description of *Pseudoalteromonas haloplanktis* as belonging to the risk 1 class of microorganism by the DSMZ.	49
7	A genetic map of the integration vector	50
8	DNA recombination after integration of the plasmid	51
9	Southern blot hybridization results	59
10	Genetic map of the expression vector and probe sequence	59

Tables

Table Number	Title	Page
1	Non-exhaustive list of authorisations of this xylanase produced by *Bacillus Subtilis*	17
2	Non-exhaustive list of authorized food enzymes (other than xylanase) produced by the same production organism, *Bacillus subtilis*	18-19
3	Non-exhaustive list of authorized xylanase from production organisms other than *Bacillus subtilis*	20
4	Protease and Amylase Activities	25
5	Stability Tests – Xylanase Premix X-618	27
6	Composition and Specification of the test material	36
7	Composition and Specification of the Commercial food enzyme	36
8	Recommended Enzyme Use Levels	64
9	TMDI Calculation	66
10	Foods Groups and Foods likely to contain the Enzyme	67

ADMINISTATIVE INFORMATION

Applicant Details

(As per section 3.1.2 of the Application Handbook 1 September 2013 - amended 1 June 2015)

Applicant: [redacted], Group Public and Environmental Affairs Director

Organization: Puratos NV (Puratos)

Address: Industrialaan 25, B-1702 Groot-Bijgaarden, Belgium

Telephone: [redacted]

Email address: [redacted]

Primary contact: Fiona Fleming [redacted]

Nature of Business

(As per section 3.1.2(f) of the Application Handbook 1 September 2013 - amended 1 June 2015)

Puratos is a company specialising in the development, production, distribution and marketing of high quality raw materials for the bakery, confectionery, chocolate and catering industry.

Details of other parties associated with the Application

(As per section 3.1.2(g) of the Application Handbook 1 September 2013 - amended 1 June 2015)

1. The following Scientific and Regulatory Consultants have been involved in the preparation, submission and stewardship of this application:
   - [redacted], Brooke-Taylor & Co Pty Ltd
   - [redacted] FJ Fleming Food Consulting Pty Ltd
2. Puratos Australia New Zealand Pty Ltd has an interest in approval of this application to enable them to market the enzyme in Australia and New Zealand.
3. The following **manufacturer** has an interest in the application:

   Company: Beldem – a division of Puratos NV

   Address: rue Bourrie 12, B-5300 Andenne, Belgium

   Tel. no: +32 (0)85 82 32 50

1. APPLICATION INFORMATION

Assessment Procedure

(As per section 3.1.6 of the Application Handbook 1 September 2013 - amended 1 June 2015)

Puratos seeks to proceed with an **unpaid** application for consideration as a General Procedure (maximum of 650 hours).

Confidential commercial information (CCI)

(As per section 3.1.7 of the Application Handbook 1 September 2013 - amended 1 June 2015)

This application **does contain** information that is confidential commercial information (CCI).

Puratos has provided information to support this application which it considers to be CCI. This information is provided separately and clearly labelled as CCI.

Exclusive capturable commercial benefit

(As per section 3.1.8 of the Application Handbook 1 September 2013 - amended 1 June 2015)

This application will **not confer** an exclusive capturable commercial benefit for Puratos or any other individual company.

Status of Similar Applications

(As per Section 3.1.4 of the Application Handbook 1 September 2013 - amended 1 June 2015)

There are no similar current applications for approval of endo β(1,4)xylanase.

2 PURPOSE OF THE APPLICATION

(As per section 3.1.3 of the Application Handbook 1 September 2013 - amended 1 June 2015)

Puratos is making this application to amend Schedule 18 – Processing Aids, of the Australia New Zealand Food Standards Code (hereafter the Code) to include the food enzyme endo β(1,4) xylanase (EC 3.1.2.8) from *Bacillus subtilis* containing the gene for endo β(1,4) xylanase from *Pseudoalteromonas haloplanktis* in 18-4 Permitted Enzymes.

The food enzyme endo β(1,4) xylanase is used as a processing aid in the manufacture of baked cereal products. Arabinoxylans provide functional properties during bread making due to their ability to interact with gluten, bind water and provide dough viscosity. Limited hydrolysis of the water-unextractable arabinoxylans with the help of endo β(1-4) xylanase results in solubilized arabinoxylans with lower molecular weights, which improves the functional baking properties of these polysaccharides.

3 JUSTIFICATION FOR THE APPLICATION

(As per section 3.1.4 of the Application Handbook 1 September 2013 - amended 1 June 2015)

3.1 NEED FOR THE PROPOSED CHANGE

(As per section 3.14(a) of the Application Handbook 1 September 2013 - amended 1 June 2015)

Schedule 18 - Processing Aids contains a list of permitted enzymes of microbial origin (18-4 Permitted Enzymes). There is already approval for Hemicellulase endo β(1,4) xylanase (EC 3.2.1.8) from a range of source organisms including *Bacillus subtilis*.

The source microorganism for the applicant's endo β(1,4) xylanase is *Pseudoalteromonas haloplanktis* cloned in *Bacillus subtillis.*

Approval is required due to the use of a genetically modified source microorganism for the preparation of the enzyme.

This application will provide information to support the safety of the genetically modified *Bacillus subtillis* as a host organism.

3.1.1 Purpose of using the processing aid

In general, the technological need for the enzymatic conversion of (arabino)xylans with the help of endo β(1-4) xylanase can be described as the degradation of a component (the substrate arabinoxylan) which causes technical difficulties due to its high viscosity in processing of raw materials containing this component.

Endo β(1-4) xylanase is naturally present in many cereal raw materials, including wheat, barley and malt. The natural enzymatic conversion of (arabino)xylans in such materials is of technological benefit in several industrial food manufacturing processes. However, the levels of endogenous endo β(1-4) xylanase are often inadequate and vary from batch to batch of raw material, and the specificity of the enzyme may not be optimal to provide the desired process advantages. Therefore, microbial endo β(1-4) xylanase is used during baking.

Endo β(1-4) xylanase is acting on one family of the components of the plant cell wall, and is often used together with other enzymes (enzyme systems) which modify other components of the plant cell walls. In particular endo β(1-4) xylanase is often applied together with β-glucanase and cellulase (e.g. for brewing, grain processing, starch processing and beverage alcohol processing), or with amylase (baking processes and other cereal based processes).

The benefits of the use of microbial endo β(1-4) xylanase in typical food (ingredient) processes are described below. The beneficial effects mentioned in the various applications below are of value to the food industry because they lead to better and/or more consistent product quality. Moreover, the applications lead to more effective production processes, resulting in better production economy and environmental benefits such as the use of less raw materials and the production of less waste.

Baking processes and other cereal based processes

Endo β(1-4) xylanase has been used in baking and other cereal based products for over 25 years (Beg et al, 2001).

Endo β(1-4) xylanase can be used in the manufacture of bakery products such as bread, biscuits and cakes. Arabinoxylans are polysaccharides which provide functional properties during bread making due to their ability to interact with gluten, bind water and provide dough viscosity. Limited hydrolysis of the water-unextractable arabinoxylans by endo β(1-4) xylanase results in solubilized arabinoxylans with lower molecular weights, which improves the functional baking properties. The process flow of baking products is presented below in **Figure 1.**

Depending on the application, the conversion of arabinoxylans with the help of endo β(1-4) xylanase in baking can result in the following benefits:

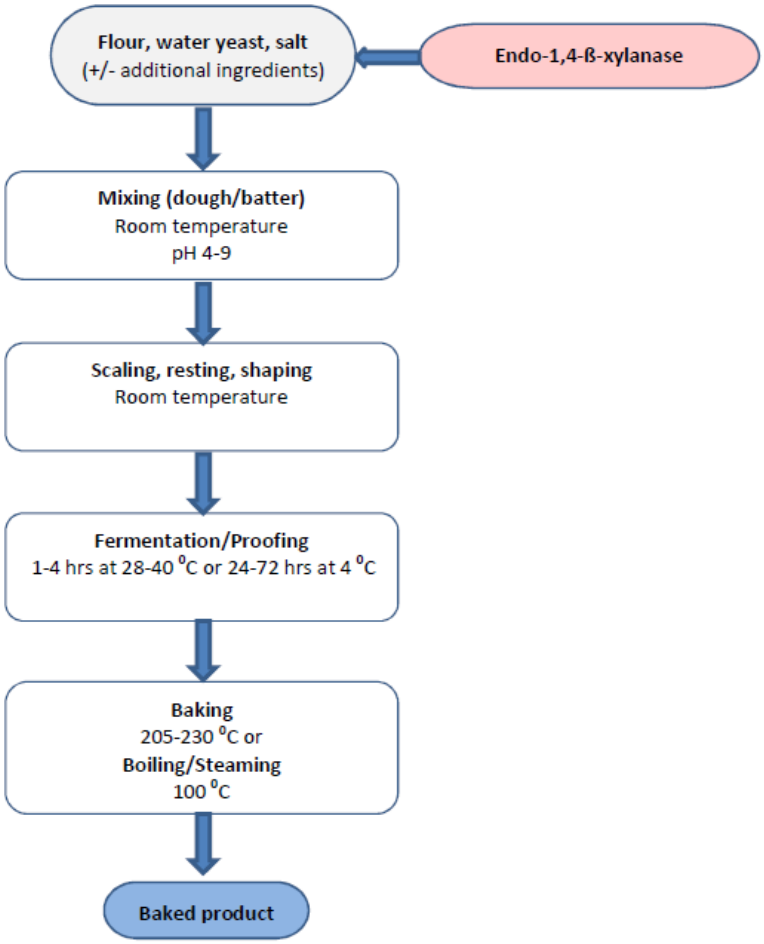
- Facilitate the handling of the dough (improved extensibility and stability; less stickiness leading to reduced loss of dough);
- Improve the dough structure and behaviour during the baking step;
- Ensure a uniform volume and an improved crumb structure of the bakery product, which might otherwise be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

Endo β(1-4) xylanase can also be used in the processing of other cereal based products such as, but not limited to, pasta, noodles and snacks, where they can improve the dough process ability and accelerate the drying step, thereby shortening the process time. Arabinoxylans provide functional properties during pasta, noodle and snack making due to their ability to interact with gluten, bind water and provide dough viscosity. Limited hydrolysis of arabinoxylans with the help of endo β(1-4) xylanase improves the functional properties of these polysaccharides.

Depending on the application, the conversion of arabinoxylans with the help of endo β(1-4) xylanase in other cereal based products can result in the following benefits:

- Facilitate the handling of the dough;
- Increase firmness and reduce oil absorption in instant noodles;
- Checking (formation of hair line cracks) is reduced; and
- Accelerate the drying step, thereby shortening the process time.

Figure 1: The baking process



3.2 ADVANTAGES OF THE PROPOSED CHANGE

(As per Section 3.1.4(b) of the Application Handbook 1 September 2013 - amended 1 June 2015)

The use of this endo β(1-4) xylanase from *Pseudoalteromonas haloplanktis* has following advantages for bakery manufacturers:

- Improved effectiveness under typical production conditions:
  a. optimal activity at temperatures around 25-40°C which corresponds to the dough proofing temperature range which is generally around 25-35°C. This psychrophilic xylanase retains 90% activity at 20°C.
  b. optimal pH range for this xylanase corresponds to the pH in the dough.

  This impacts the production process efficiency during dough preparation and proofing and the stability of the results from batch to batch.

- Six (6) of the approved xylanases listed in Schedule 18 are from fungal origin. It has been demonstrated that xylanases from fungal origin are less efficient in bakery applications.

- Two (2) of the listed bacterial xylanases are mesophilic with a higher optimal temperature (50°C) and less activity at the preparation and proofing temperature of the dough (25-35°C) as mesophilic xylanases only retain 20% at this temperature range.

- This endo xylanase preparation is highly purified and does not show any side activities. This is not the case for some other commercial preparations.

- Most xylanases belong to the glycoside hydrolase family 10 and 11. Cereals produce various metabolites and among others, xylanase inhibitors such as Titricum Aestivum endoXylanase Inhibitor (TAXI), endoXylanase Inhibiting Protein (XIP) and Thaumatine-Like Xylanase Inhibitor (TLXI):

  - TAXI inhibits family 11 microbial xylanases (eg. *Aspergillus niger, Tricoderma resei, Bacillus subtilis)* but not family 10 (eg. *Aspergillus oryzae* with a competitive type of inhibition.
  - XIP shows ability to inhibit different glycoside hydrolase families (10, 11 and 13 eg. from *Streptomyces olivaceoviridis*) and acts as a competitive inhibitor.
  - TLXI is a non-competitive inhibitor of a number of glycoside hydrolase family 11 xylanases but is inactive on family 10 xylanases.

- Xylanases of family 8 also have a higher activity on insoluble arabinoxylans.

3.3 DISADVANTAGES OF THE PROPOSED CHANGE

The Applicant is not aware of any disadvantages of the proposed change.

4 REGULATORY IMPACT INFORMATION

(As per section 3.1.4 A of the Application Handbook 1 September 2013 - amended 1 June 2015)

4.1 COSTS AND BENEFITS – CONSUMER

The potential benefit to consumers includes:

- choice of additional products which become available due to the availability of endo β(1-4) xylanase for Australian and New Zealand food manufactures, and
- access to food products containing endo β(1-4) xylanase that are currently manufactured overseas.

The proposed amendment places no additional economic cost on consumers.

4.2 COSTS AND BENEFITS - INDUSTRY AND BUSINESS

Approval of this endo β(1-4) xylanase will benefit manufacturers in that this will extend the options to source processing aids and offer alternative sources of endoxylanase with different properties - optimum activities under various conditions such as pH and temperatures, resistance to *in vivo* inhibitors present in wheat flour, reaction time in line with the process parameters for example

Use of endo β(1-4) xylanase will be at the discretion of business, therefore there are no direct costs imposed on industry.

4.3 COSTS AND BENEFITS – GOVERNMENT

The proposed amendment places no additional regulatory costs on government beyond the initial regulatory cost of approving endo β(1-4) xylanase as a processing aid.

4.4 IMPACT ON INTERNATIONAL TRADE

The Applicant notes that, in developing food standards, FSANZ must have regard to its WTO obligations; the promotion of consistency between domestic and international food standards; and the promotion of fair trading in food. These matters encompass consideration of international standards and trade issues.

This amendment would bring Australia and New Zealand into line with other countries where this endo β (1-4) xylanase is approved for use.

5 INFORMATION TO SUPPORT THE APPLICATION

(As per section 3.1.5 of the Application Handbook 1 September 2013 - amended 1 June 2015)

5.1 DATA REQUIREMENTS

Refer to **Section 7** for information about the processing aid.

5.2 FSANZ ACT OBJECTIVES

Information is provided in this application to enable the objectives specified in Section 18 of the FSANZ Act to be addressed as follows:

(a) The protection of public health and safety: information to support objective (a) is provided in the following sections:

- 7.2 - Information on the Safety of an Enzyme Processing Aid;
- 7.3 - Additional Information Related to the Safety of an Enzyme Processing Aid Derived from a Microorganism; and
- 7.4 - Additional Information Related to the Safety of an Enzyme Derived from a Genetically-Modified microorganism of the Application.

(b) The provision of adequate information relating to food to enable consumers to make informed choices.

Processing aids are not required to be labelled however consumers are able to contact manufactures to request information in relation to finished products if they have an interest or query.

(c) The prevention of misleading or deceptive conduct.

Processing aids are not required to be labelled however consumers are able to contact manufactures to request information in relation to finished products if they have an interest or query.

5.3 PUBLIC HEALTH AND SAFETY ISSUES

Refer **Section 7** for information about the processing aid.

5.4 CONSUMER CHOICE

No consumer choice issues related to the proposed change are foreseen.

Endo β(1-4) xylanase does not perform any technological function in the final foods containing ingredients prepared with this enzyme. Moreover, the food products prepared with endo β(1-4) xylanase do not have characteristics or nutritional value other than what is expected by the consumer.

5.5 SUPPORT FOR THE PROPOSED CHANGE

The Applicant does not have letters from potential customers however the Australia/New Zealand business (Puratos Australia-New Zealand Pty Ltd) intends to market the enzyme once it is approved.

5.6 POLICY GUIDELINES

(As per section 3.3.2 of the Application Handbook 1 September 2013 - amended 1 June 2015)

Information is provided in this application to address the Policy Guideline - Addition to Food of Substances other than Vitamins and Minerals[1].

Addition to Food of Substances other than Vitamins and Minerals

The addition of substances other than vitamins and minerals to food where the purpose of the addition is for other than to achieve a solely technological function should be permitted where:

Specific Order Policy Principles – Any Other Purpose	Section of Application
a) the purpose for adding the substance can be articulated clearly by the manufacturer (i.e. the 'stated purpose'); and	3
b) the addition of the substance to food is safe for human consumption; and	7
c) the substance is added in a quantity and a form which is consistent with delivering the stated purpose; and	7
d) the addition of the substance is not likely to create a significant negative public health impact to the general population or sub population; and	7
e) the presence of the substance does not mislead the consumer as to the nutritional quality of the food.	Not applicable

1 http://www.foodstandards.gov.au/code/fofr/fofrpolicy/documents/Addition%20to%20Food%20of%20Substances%20other%20than%20Vitamins%20and%20Minerals%20May%202008.pdf, accessed 17.12.2015

6 INTERNATIONAL AND OTHER NATIONAL STANDARDS

(As per section 3.1.9 of the Application Handbook 1 September 2013 - amended 1 June 2015)

The status of the processing aid with respect to other national standards or regulations is discussed under this section of the Application.

6.1 INTERNATIONAL STANDARDS

This endo-xylanase is listed on the updated inventory of substances used as processing aids prepared by New Zealand and presented to the Codex Committee on Food Additives (CCFA) 45th session in 2013 (refer to **Appendix 1)**.

6.2 OTHER NATIONAL STANDARDS OR REGULATIONS

6.2.1 Existing Authorisations and Evaluations

The food enzyme object of the present dossier has been evaluated and authorized in the following countries: France, Brazil, the USA and Canada as follows:

Table 1: Non-exhaustive list of authorisations of this xylanase produced by *Bacillus Subtilis*

Authority	Description	Reference
France	Xylanase de *Bacillus subtilis* Giza 3508 porteuse d'un gène codant une xylanase de *Pseudoalteromonas haloplankis*.	Appendix 2
Brazil	*Pseudoalteromonas haloplanktis* expresso em *Bacillus subtilis*	Appendix 3
USA	Use of the *Pseudoalteromonas haloplanktis* xylanase expressed in *Bacillus subtilis* for baking, fine bakery wares and similar applications - GRAS	Appendix 4
Canada	*Bacillus subtilis* Gizα3508 in bread; flour; whole wheat flour and unstandardized bakery products at GMP levels	Appendix 5
EU	Xylanase from *Bacillus subtilis* LMG S-24584	Appendix 6

6.2.2 Other Authorisations and Evaluations

Food enzymes are biological isolates of variable composition. Apart from the enzyme protein in question, microbial food enzymes will also contain some substances derived from the producing micro-organism and the fermentation medium. From a safety point of view, the similarity of the producing micro-organism is of higher importance than that of the enzyme protein in question. Therefore, the non-exhaustive lists below summarises not only authorized food enzymes with the same enzyme activity, but also authorised food enzymes from the same producing organism. As documented below, xylanase from various micro-organisms (including genetically modified ones) are widely accepted and *Bacillus subtilis* – whether or not genetically modified - is widely accepted as a safe production organism for a broad range of enzymes.

Table 2: Non-exhaustive list of authorized food enzymes (other than xylanase) produced by the same production organism, *Bacillus subtilis*

Authority	Food enzyme	Reference
JECFA	α-acetolactate decarboxylase	Appendix 7
	α-amylase	
	branching glucosyltransferase	
	maltogenic amylase	
	mixed microbial carbohydrase and protease	
Australia / New Zealand	α-acetolactate decarboxylase	ANZ Food Standards Code Schedule 18 – Permitted Enzymes – 18-4 (Copy not provided with Application)
	α-amylase	
	β-amylase	
	β-glucanase	
	hemicellulase multicomponent enzyme	
	maltogenic α-amylase	
	Metalloproteinase	
	pullulanase	
	serine proteinase	
Canada	α-acetolactate decarboxylase	Appendix 5
	Amylase	
	amylase (maltogenic)	
	Glucanase	
	Protease	
	Pullulanase	

Table 2: Non-exhaustive list of authorized food enzymes (other than xylanase) produced by the same production organism, *Bacillus subtilis*		
Authority	**Food enzyme**	**Reference**
France	α-acetolactate decarboxylase	Appendix 8
	α-amylase	
	β-glucanase	
	exo-α-amylase maltogenic	
	branching glucosyltransferase	
	Protease	
	serine protease	
	(metallo) protease	
	Pullulanase	
USA	asparaginase	GRN476 **Appendix 9**
	1,4-α-glucan branching enzyme	GRN406
	branching glycosyltransferase	GRN274
	pectate lyase	GRN114
	Pullulanase	GRN205
	bacterially-derived carbohydrases enzyme	§184.1148
	bacterially-derived protease enzyme	§184.1150
Denmark	α -amylase	Appendix 10
	peptidase	
	Acetolactate decarboxylase	

Table 3: Non-exhaustive list of authorized xylanase from production organisms other than *Bacillus subtilis*		
Authority	**Production organism**	**Reference**
JECFA	*Humicola insolens*	Appendix 7
	Fusarium venenatum	
Australia / New Zealand	*Aspergillus niger*	ANZ Food standards Code Schedule 18 – Permitted Enzymes – 18-4 (Copy not provided with Application)
	Aspergillus oryzae	
	Bacillus amyloliquefaciens	
	Humicola insolens	
	Trichoderma reesei	
Canada	*Aspergillus niger*	Appendix 5
	Aspergillus oryzae	
	Trichoderma reesei	
France	*Aspergillus niger*	Appendix 8
	Aspergillus oryzae	
	Humicola insolens	
	Trichoderma longibrachiatum	
USA	*Aspergillus niger (as carbohydrase)*	GRN89 **Appendix 9**
	Aspergillus oryzae (as carbohydrase)	GRN90
	Bacillus amyloliquefaciens (as carbohydrase)	§184.1148
	Bacillus licheniformis (pending)	GRN472
	Bacillus licheniformis (as mixed carbohydrase and protease)	§184.1027
	Fusarium venenatum	GRN54
	Humicola insolens	GRN195
	Talaromyces emersonii (pending)	GRN479
Denmark	*Aspergillus niger var. awamori*	Appendix 10
	Aspergillus oryzae	
	Aspergillus niger	

7 PROCESSING AID

(As per section 3.3.2 of the Application Handbook 1 September 2013 - amended 1 June 2015)

7.1 TECHNICAL INFORMATION ON THE PROCESSING AID

(As per section 3.3.2A of the Application Handbook 1 September 2013 - amended 1 June 2015)

The material described in this section is representative of the commercial product.

7.1.1 Information on the type of processing aid

The endo β (1-4) xylanase enzyme preparation belongs to the category of processing aids described in Schedule 18-4 Permitted Enzymes.

Endo β(1-4) xylanase hydrolyses xylan.

The enzyme catalyses the following reaction:

Hydrolysis of (1→4)-β-D-xylosidic linkages

It can use (arabino)xylans in wheat flour as a substrate.

Evidence that the form and the amount of the processing aid performs the intended function

In 2002 at a scientific convention the Applicant published a poster (Collins et al, 2002) on the use of the psychrophilic xylanase in bakery applications. The Applicant has shown that an amount in the range of 25 to 200 SXU/100kg flour gave a significant impact on the volume of hard rolls. In other unpublished studies the Applicant could demonstrate that the optimal range could slightly vary depending on the type of bread and bakery process.

It is important to note that activities in SXU were measured at pH 4.5 and 30°C where the actual activity measurements are expressed in GDXU at pH 6.5 and 25°C. Looking at **Figure 2** under 7.1.3.4 the activity is much higher with a factor of 70 at pH 6.5 which is also closer to the pH of dough in most bakery products.

GDXU is the unit system used today for the activity of this xylanase. Consequently the range 25-200 SXU becomes 1750-14,000 per 100 kg of flour or 17.5 to 140 GDXU/kg of flour.

The Applicant therefore recommends a range from 10 to 150 GDXU/kg of flour, depending on the applications and processes. It is the responsibility of the user to define the level of use (functional vs financial).

7.1.2 Information on the identity of the processing aid

Enzyme

Name of the enzyme protein: endo β(1-4) xylanase

Synonyms:

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

Abbreviations: None

EC (IUBMB) number: EC 3.2.1.8

The **classification of the enzyme** according to the IUBMB is as follows:

EC 3	hydrolases
EC 3.2	glycosylases
EC 3.2.1	glycosidases
EC 3.2.1.8	endo β(1-4) xylanase

CAS No: Not applicable

Enzyme Preparation

Commercial Name:

This endo β(1-4) xylanase is already sold under different commercial names:

- Premix X-608
- Premix X-618
- Bel'Ase B218

A Technical Data Sheet for Premix X-608 is provided as **Appendix 11.**

Host Organism

Name: Bacillus subtilis Gizα7101.

The organism is deposited under the number LMG S-24584 at the node of the Belgian Co-ordinated Collection of Microorganisms located at the University of Gent.

The address of the Culture Collection is:

LMG – Culture Collection, R.U.G., Ledeganckstraat 35, 9000 Gent, Belgium

Donor Organism

Name: *Pseudoalteromonas haloplanktis*
The characteristics of the *Pseudoalteromonas haloplanktis* xylanase have been described in Collins et al (2002).

"This xylanase, isolated from the Antarctic bacterium Pseudoalteromonas haloplanktis, is not homologous to family 10 or 11 enzymes but has 20–30% identity with family 8 members. NMR analysis shows that this enzyme hydrolyzes with inversion of anomeric configuration, in contrast to other known xylanases which are retaining. No cellulase, chitosanase or lichenase activity was detected. It appears to be functionally similar to family 11 xylanases. It hydrolyzes xylan to principally xylotriose and xylotetraose and is most active on long chain xylo-oligosaccharides. Kinetic studies indicate that it has a large substrate binding cleft, containing at least six xylose-binding subsites. Typical psychrophilic characteristics of a high catalytic activity at low temperatures and low thermal stability are observed. An evolutionary tree of family 8 enzymes revealed the presence of six distinct clusters. Indeed classification in family 8 would suggest an (α/α)6 fold, distinct from that of other currently known xylanases."

7.1.3 Information on the chemical and physical properties of the processing aid

The enzyme is a biological isolate of variable composition, containing the enzyme protein, as well as organic and inorganic material derived from the microorganism and fermentation process.

Apart from the main enzymatic activity, the food enzyme does not contain significant levels of subsidiary/side activities.

7.1.3.1 Molecular Mass, Subunit Structure and Amino Acid Sequence of the Enzyme Protein

The endoβ(1-4) xylanase protein from *Pseudoalteromonas haloplanktis* expressed in *Bacillus subtilis* consists of a monomer with 185 amino acids for the mature peptide.

AFNNNPSSVGAYSSGTYRNLAQEMGKTNIQQKVNSTFDNMFGYNNTQQLYYPYTENGVYKAHYIKAIN
PDEGDDIRTEGQSWGMTAAVMLNKQEEFDNLWRFAKAYQKNPDNHPDAKKQGVYAWKLKLNQNGF
VYKVDEGPAPDGEEYFAFALLNASARWGNSGEFNYYNDAITMLNTIKNKLMENQIIRFSPYIDNLTDPS
YHIPAFYDYFANNVTNQADKNYWRQVATKSRTLLKNHFTKVSGSPHWNLPTFLSRLDGSPVIGYIFNG
QANPGQWYEFDAWRVIMNVGLDAHLMGAQAWHKSAVNKALGFLSYAKTNNSKNCYEQVYSYGGAQ
NRGCAGEGQKAANAVALLASTNAGQANEFFNEFWSLSQPTGDYRYYNGSLYMLAMLHVSGNFKFYN
NTFN

A signal peptide sequence is used for the secretion of the enzyme. Its Molecular Mass is 45.982 Da.

Further detail is provided under **Confidential Commercial Information** (**7.1.3)**

7.1.3.2 Information on post translational modification of the enzyme protein

The only post-translational modification that can be considered here is the cleavage of the signal peptide during the secretion of the enzyme.

7.1.3.3 Information and rationale on protein engineering of the enzyme protein

Not applicable. Protein engineering has not taken place.

7.1.3.4 Information on side activities of the enzyme protein which might cause adverse effects

As far as the Applicant is aware, the endo β(1-4) xylanase described in this dossier does not possess any enzymatic side activities which might cause adverse effects.

Microbial food enzymes are concentrates typically containing minor amounts of other enzyme activities (side activities) naturally produced by the microorganism. However, these activities are not relevant from an application or safety point of view.

Food enzymes are biological concentrates containing – apart from the desired enzyme protein (expressing the activity intended to perform a technological purpose in a certain food process, also called 'main enzyme activity') - also some other substances. This is the reason why JECFA developed the TOS concept for food enzymes and why it is important that the source of a food enzyme is safe.

These other substances may include various enzyme activities (defined as 'side activities') derived from the producing microorganism and the fermentation medium. Like all living cells, microorganisms produce a variety of enzymes responsible for the hundreds of metabolic processes that sustain their life. As microorganisms do not possess a digestive system, many enzymes are excreted to digest the material on which the microorganisms grow. Most of these enzymes are hydrolases that digest carbohydrates, proteins and lipids. These are the very same activities that play a role in the digestion of food by – amongst others – the intestinal micro flora in the human body and in the production of fermented food.

In addition, if a food raw material contains a certain substrate (e.g. carbohydrate, protein or lipid) then by nature it also contains the very same enzymatic activities that break down such a substrate, (e.g. to avoid its accumulation). Consequently, the presence in food of such enzyme activities and of the potential reaction products is not new and should not be of any safety concern. In addition, it is generally accepted that the enzyme proteins themselves do not pose any safety concern either.

During the production of food enzymes, the main enzyme activity is normally not separated from the other substances present. Consequently, the food enzyme may contain a number of other enzymes excreted by the microbial cells or derived from the fermentation medium. Other strains of *Bacillus Subtilis*, selected to produce other main enzyme activities, will produce and excrete the same set of enzymatic activities, albeit in various amounts. Consequently, the food enzymes from *Bacillus Subtilis* which are approved and used in food processes already for many years (Section 6.2.3), will also contain these activities. These activities are of no safety concern and their fate in the final food will be

the same as that of the main enzyme activity. If they also do not break down the main enzyme protein and do not play a role in the intended technological function in food processing, these side activities are not considered as 'significant' enzyme activities, and there is no reason to specifically investigate their nature.

'Significant' enzyme activities are considered those that do play a role in the intended food processes. In those cases, where unwanted side activities are produced (e.g. that break down the main enzyme protein or that could create an unwanted technological effect during food processing), care is taken that such activities are not present in the food enzyme – either by genetic modification of the producing organism, or by specific purification steps.

In the case the food enzyme endo β(1-4) xylanase described in this Application, no other significant enzymatic activities have been identified.

Protease and alpha-amylase activities could be relevant in bakery processes. Therefore, the activities have been measured showing no or negligible measures. Values under 10 are considered without any technological interest.

Table 4: Protease and Amylase Activities

Type	Activity			Method
	GIZ - 1401	GIZ - 1402	GIZ - 1403	
Protease (mU/ml)	0	0	0	AZO-Caséine (Megazyme) Provided in **Appendix 12** (CCI)
Amylase (skb/ml)	0	0	0.05	Arena20 (reagents Roche) Provided in **Appendix 12** (CCI)

A Certificate of Analysis (CoA) is provided in **Appendix 12** (non CCI).

Performances assays run with various enzymes batches in bread making never suggest that, even when measurable, the enzymatic side activities (also called secondary activities) have any technical impact in the application.

7.1.3.5 Information on the activity of the food enzyme under various reaction conditions

The activity of the food enzyme endo β(1-4) xylanase from *Bacillus Subtilis* was measured under various pH and temperature conditions using the analytical method provided in **Appendix 13**. The results are presented in the figures below.

Figure 2: Relative xylanase activity vs pH and temperature

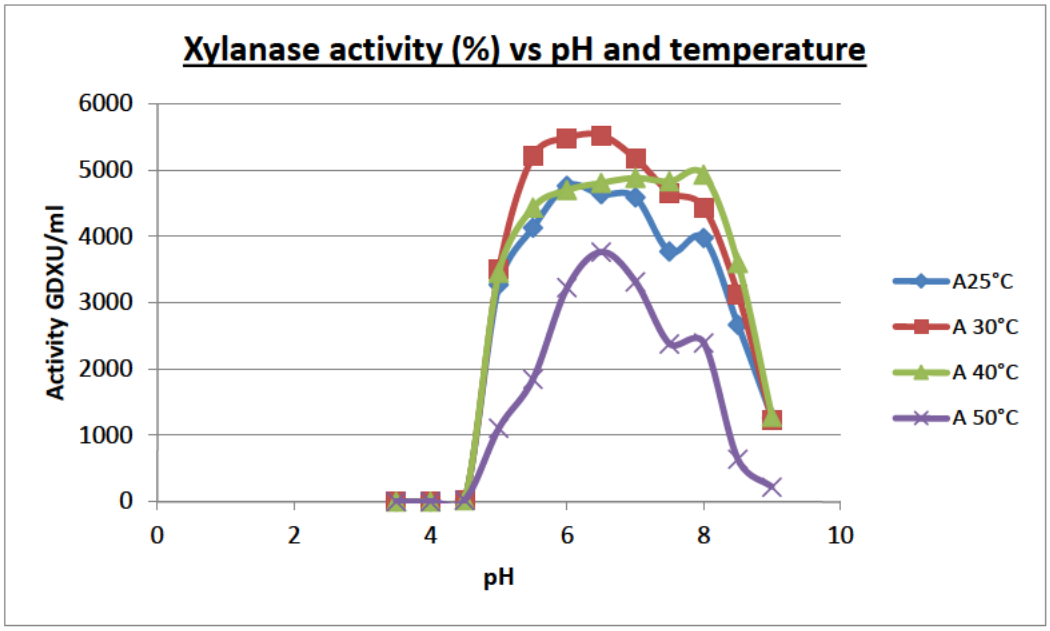
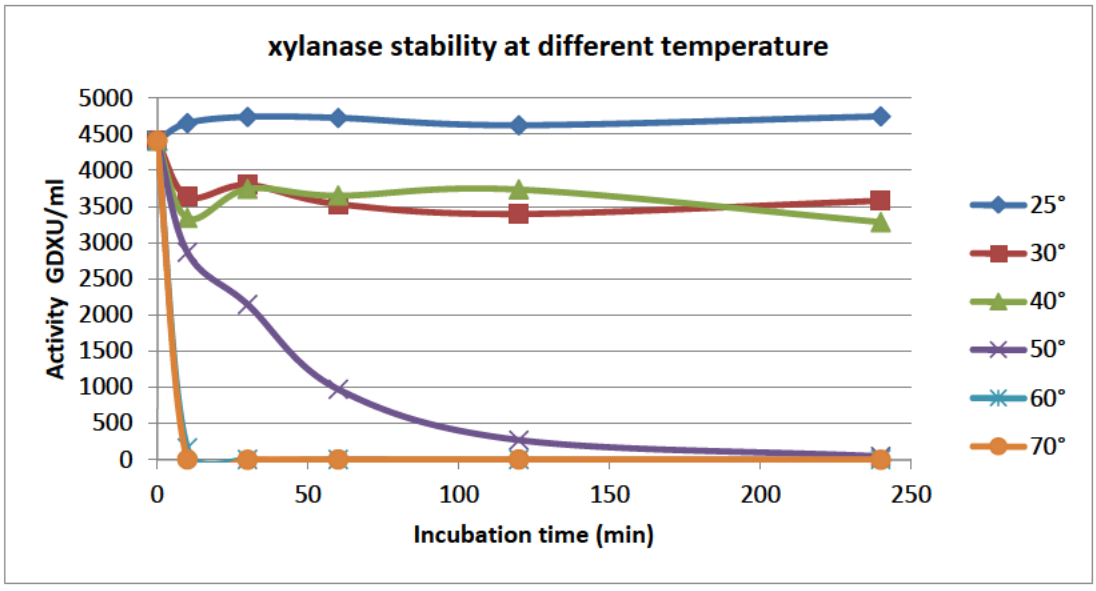


Figure 3: Relative xylanase activity at different temperatures



Figures 2 and 3 show the food enzyme endo β(1-4) xylanase from *Bacillus Subtilis* exhibits activity from pH 5 to pH 8 and from 25°C to 50°C. The optimum pH range lies between pH 6 and 7, whereas the optimum temperature is around 30°C. No enzyme activity is left at temperatures above 60°C.

7.1.3.6 Data on the stability of the food enzyme during storage and before use

Food enzymes are not sold as such, but formulated into various enzyme preparations in order to obtain standardized and stable products. The stability thus depends on the type of formulation, not on the food enzyme as such.

Tests of stability were performed on products at end of shelf-life. The results are summarized in **Table 4** and full details provided in **Appendix 14**.

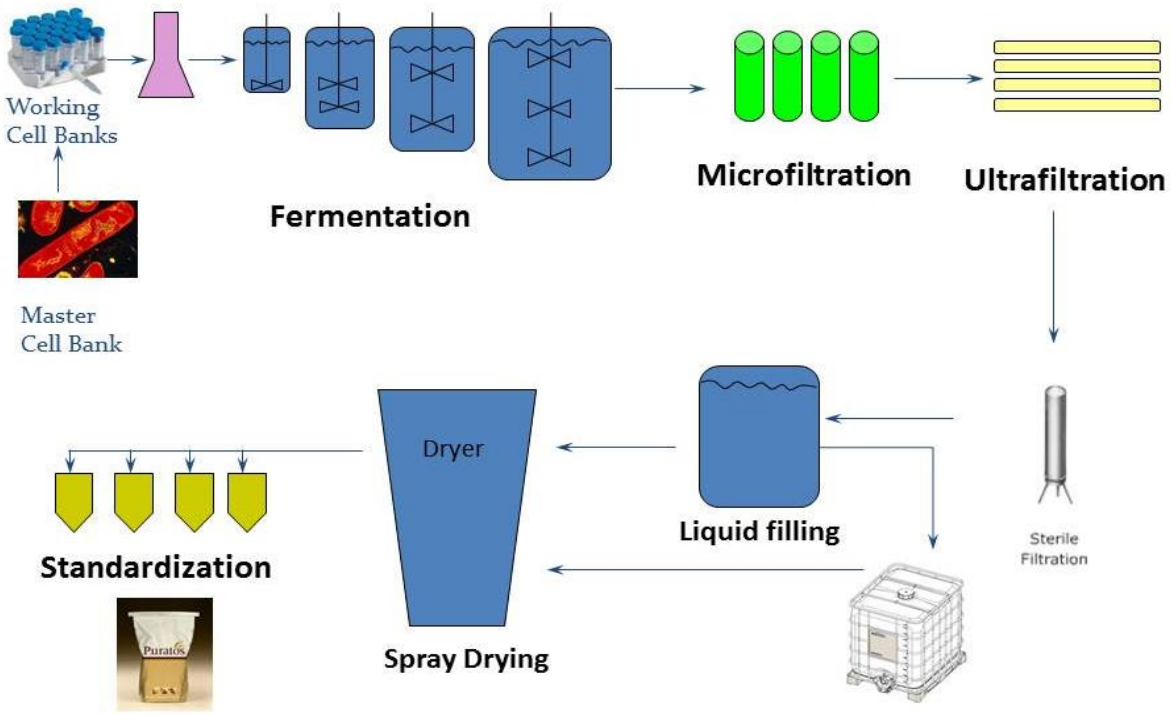
Table 5: Stability Tests – Xylanase Premix X-618

Batch	Activity just after production (IU/g)	Activity end of shelf life (IU/g)
Premix X618 Batch: 0000075555	999	1150
Premix X618 Batch: 0000075558	992	1092
Premix X618 Batch: 0000117908	1006	922

7.1.4 Manufacturing process

The fermentation and downstream processes are schematically represented in **Figure 4.** The production process is completely closed until the formulation of the commercial product. Each fermentation run is started from pure starter cultures.

Figure 4: Schematic representation of the production process of the food enzyme



7.1.4.1 Description of the Process

Cultures are started in a 1L Erlenmeyer flask and then transferred to fermenters of increased volume up to the production fermenter of 30 m³. The fermenters used are designed for submerged culture with central stirrer. The carbon source for the *Bacillus subtilis* fermentation is chosen among the following: sucrose, maltose, glucose, maltodextrins and starch.

The nitrogen source is chosen among the following: peptones, protein hydrolysates, yeast extracts, glutamate and urea.

The medium is usually supplemented with various inorganic salts. A feed batch is used to provide additional nutrients all along the fermentation. Chemicals used in the fermentation medium are all certified food grade by the suppliers. As the production of food enzymes fall under the EU Food Hygiene Regulation, all raw materials used during fermentation and recovery are of food grade quality.

Specific process parameters are applied and controlled throughout the whole fermentation.

The plant is automated and is managed from the control room by an operator. All the crucial parameters are stored in “production recipes” and their actual values are recorded on-line and displayed on a screen.

The production strain is kept as pure culture in Master Cells Bank (MCB) with sterile glycerol and stored at -70°C. “Security” stocks of MCB are kept in different locations in the company. Based on those MCB, Working cells Bank (WCB) are prepared. The purity is verified before use in production.

During fermentation samples are taken on a regular basis and analysed.

During production, the operator has the opportunity to adjust the parameters ensuring an optimal fermentation. The fermentation process is completely closed to avoid any contamination from outside (and to prevent any leakage from the vessels).

The fermentation ends when the optimal requested level of biomass and enzymatic activity is obtained.

At the end of the fermentation, samples are taken and analyzed for:

- total cell and viability count; and
- check of contaminant.

The xylanase produced by *Bacillus subtilis* described above is secreted in the culture medium. After fermentation and heat treatment, the biomass is separated from the enzyme containing culture medium by microfiltration.

At the end of the microfiltration process, the biomass is destroyed by an alkaline treatment (pH 12).The biomass can be then disposed in the waste water treatment station without any environmental risk.

The enzyme preparation is concentrated by ultrafiltration before sterile filtration. The obtained solution is free from any microorganisms.

The concentrated liquid enzyme is dried using a spray-dryer where it is sprayed on a carrier (wheat flour). The enzyme preparation is thus granulated, reducing its dustiness.

The carrier of the commercial enzyme preparation is food grade wheat flour. This ingredient is totally compatible with the usage in food, and in particular in the intended applications, i.e. baking. For standardization of the product, the dried enzyme preparation is diluted with the same ingredient, i.e. wheat flour.

The food enzyme does not contain any material as referred to in the opinion of EFSA’s scientific committee on “The potential risks arising from nanoscience and nanotechnologies on food and feed safety” (EFSA, 2009a).

7.1.4.2 Good Manufacturing Practice and HACCP

The enzyme is manufactured according to good manufacturing practices (GMP) and the principals of HACCP. When manufactured in the EU, it is also subject to Regulation (EC) No 852/2004 of the European Parliament and of the Council of 29 April 2004 on the hygiene of foodstuffs (Food Hygiene Regulation (852/2004)).

A HACCP plan is applied to the production of the xylanase to manage all potential risk that may come from fermentation**.**

The HACCP plan is provided with the CCI material (7.1.4 Manufacturing Process).

Potential Hazards

In order to comply with current GMP and HACCP principles for food production, the following potential hazards in food enzyme production are taken into account:

Identity and Purity of the Producing Microorganism

The assurance that the production microorganism efficiently produces the desired enzyme protein is of utmost importance to the food enzyme producer. Therefore, it is essential that the identity and purity of the microorganism are controlled.

Microbiological Hygiene

For optimal enzyme production, it is important that hygienic conditions during the whole fermentation process are controlled. Microbial contamination would immediately result in less growth of the production organism and consequently in a low yield of the required enzyme protein and eventually a rejected product.

Chemical Contaminants

It is also important that the raw materials used during fermentation are of suitable quality and do not contain contaminants which might affect the product safety of the food enzyme and/or the optimal growth of the production organism and thus enzyme yield.

Control Measures

The main measures to control the hazards identified above are:

Identity and Purity of the Producing Microorganism

Production of the required enzyme protein is based on a well-defined Master (MCB) and Working Cell Bank (WCB). A Cell Bank is a collection of ampoules containing a pure culture. The cell line history and the production of a Cell Bank, propagation, preservation and storage are monitored and controlled. The MCB is prepared from a selected strain. The WCB is derived by sub-culturing of one or more ampoules of the MCB. A WCB is only accepted for production runs if its quality meets the required standards. This is determined by checking identity, viability, microbial purity and productivity of the WCB. The accepted WCB is used as seed material for the inoculum.

To validate a WCB, different tests are performed:

- Blood agar to check the absence of toxic contaminant.
- Growing on Q-Tray to check the presence of another micro-organism than Bacillus.
- Microscopic visualisation
- Growing of 460 clones on LB with xylene agar to check the capacity of the clones to produce xylanase (100% is required to validate the WCB).
- Measurement of absorbance at 600 nm for the turbidity to follow the speed of growing of the biomass.

Microbiological Hygiene

Measures to guarantee microbiological hygiene and prevent contamination with microorganisms ubiquitously present in the environment (water, air, raw materials) are:

- Hygienic design of equipment
- Cleaning and sterilization:
  - Validated standard cleaning and sterilization procedures of the process area and equipment
  - Sterilization of all fermentation media
  - Use of sterile air for aeration of the fermenter
- Hygienic processing:
  - Aseptic transfer of the content of the WCB ampoule, inoculum flask or seed fermenter
  - Maintaining a positive pressure in the fermenter
- sterilizing filtration

Chemical Contaminants

It is ensured that all raw materials used in production of food enzymes are of food grade quality or have been assessed to be fit for their intended use and comply with agreed specifications.

In-Process Testing and Monitoring

In addition to the above mentioned control measures, in-process testing and monitoring is performed to guarantee an optimal and efficient enzyme production process and a high quality product (GMP).

These in-process controls comprise:

Microbial Controls

Absence of significant microbial contamination is analysed by microscopy or plate counts before inoculation of both the seed and main fermentation and at regular intervals and at critical process steps during fermentation and recovery.

Monitoring Of Fermentation Parameters
Monitoring of fermentation parameters may include:

- pH
- Temperature
- Dissolved oxygen content
- CO_2

The measured values of these parameters are constantly monitored during the fermentation process. The values indicate whether sufficient biomass or enzyme protein has been developed and the fermentation process evolves according to plan.

Deviations from the pre-defined values lead to adjustment, ensuring an optimal and consistent process.

Enzyme Activity and Other Relevant Analyses (Like Dry Matter, Refraction Index or Viscosity)
This is monitored at regular intervals and at critical steps during the whole food enzyme production process.

7.1.5 Specification for identity and purity

The commercial enzyme product also complies with Standard 1.1.1 Structure of the Code and general provisions – 1.1.1 – 15 Identity and Purity and Schedule – Identity and Purity - S3-2 - Substances with specifications in primary sources - the product complies with current versions of Food Chemicals Codex (9th ed) and JECFA specifications for chemical and microbiological purity of food enzymes (FAO/WHO, 2006).

Evidence that the food grade enzyme endo β(1-4) xylanase complies with these specifications is shown by the analyses on various different batches **(Appendix 12).**

7.1.6 Analytical method for detection

The endo β(1-4) xylanase enzyme preparation is to be used in the food industry as a processing aid. The Application handbook does not require this information in the case of an enzymatic processing aid.

7.2 INFORMATION ON THE SAFETY OF AN ENZYME PROCESSING AID

(As per section 3.3.2C of the Application Handbook 1 September 2013 - amended 1 June 2015)

7.2.1 General information on the use of the enzyme as a food processing aid in other countries

Refer to overseas approvals above **Sections 6.1 and 6.2**

7.2.2 Information on the potential toxicity of the enzyme processing aid

7.2.2.1 Information on the Enzyme's prior history of human consumption

Refer to overseas approvals above **Sections 6.1 and 6.2**

7.2.2.2 Information on any significant similarity between the amino acid sequence of the enzyme and that of known protein toxins

The Applicant considers that approval of this enzyme in other countries as outlined in Sections 6.1 and 6.2 demonstrates a history of safe human consumption.

7.2.2.3 Assessment of genotoxicity

AMES test

A reverse mutation assay (AMES test) using *Salmonella thyphimurium* has been performed with the enzyme preparation.

The method was designed to meet the requirements of the OECD Guidelines Testing of Chemicals N°471 "bacterial reverse mutation test" method B13/14 of Commission Directive 2000/32/EC and the USA, EPA (TSCA) OPPTS harmonized guidelines.

The study report and results are presented in **Appendix 15.**

The assay was performed with strains TA1537, TA98, TA100, TA 102 and TA1535 with and without metabolic activation using an S9 activation system. The sample, dissolved and diluted in sterile water, was tested at the following concentration: 0, 0.15, 0.5, 1.5, 5, 15, 50, 150, 500, 1500 and 5000 µg/plate. An aliquot of the sterile water was used as negative control.

The test material caused no visible reduction in the growth of the bacterial background lawn at any dose level (even at the maximum recommended dose level of µg/plate). No test material precipitate was observed on the plates at any of the doses tested in either the presence or absence of S9-mix.

No significant increases in the frequency of revertant colonies were recorded for any of the strains of Salmonella, at any dose level either with or without metabolic activation.

The tested material was considered to be non-mutagenic under the conditions of the test.

Chromosomal aberration test

A Chromosome aberration test in Human lymphocytes has been performed with the enzyme preparation.

The method was designed to meet the requirements of the OECD Guidelines Testing of Chemicals N°473 “Genetic Toxicology: Chromosome Aberration Test” method B10 of Commission Directive 2000/32/EC.

The study report and results are presented in **Appendix 16**.

Duplicate cultures of human lymphocytes, treated with the test material, were evaluated for chromosome aberrations at up to three dose levels, together with vehicle and positive controls. Four treatment conditions were used for the study in two experiments:

Experiment 1:

4 hours in the presence of an induced rat liver homogenate metabolising system (S9), at a 2% final concentration with cell harvest after a 20-hour expression period and a 4 hours exposure in the absence of metabolic activation (S9) with a 20-hour expression period.

Experiment 2:

The 4 hours exposure with addition of S9 was repeated (using a 1% final S9 concentration) whilst in the absence of metabolic activation the exposure time was increased to 24 hours.

All vehicle (solvent) controls had frequencies of cells with aberrations within the range expected for normal human lymphocytes.

All the positive control materials induced statistically significant increases in the frequency of cells with aberrations indicating the satisfactory performance of the test and of the activity of the metabolising system.

The test material was non-toxic and did not induce any statistically significant increases in the frequency of cells with aberrations, in either of two separate experiments, using a dose range that included the maximum recommended dose Level.

The test material was considered to be non-clastogenic to human lymphocytes in vitro.

7.2.2.4 Sub-chronic toxicity

A sub chronic oral toxicity test was performed in order to evaluate the potential toxicity of the food enzyme following daily oral administration to rats for 13 weeks. The study design was designed to meet the requirements of OECD Guidelines No 408, 1998. A 2 week, range finding test was not considered necessary because it was decided to perform a limit test with one dose level.

The report of the study and results are presented under **Appendix 17.**

Rats were divided as follows: Test groups 12 male & 12 female; control groups 10 male & 10 female; satellite groups: 10 male & 10 female. Groups were dosed with 0.5 ml/100 g bodyweight/day by gastric tub. Both the test and satellite groups received the test material, whilst the control group received the vehicle (water) alone. The enzyme concentration of the test preparation was 290 DXU/ml, giving a daily dose of 1450 DXU / kg bodyweight. The dose was the highest possible level due to the physical-chemical nature of the test substance. The satellite group were kept top screen for possible long term toxicity.

Mortality, morbidity, weight gains and feed consumption was observed during the study. At termination, complete blood analysis and histological analysis of each organ of each animal (control and treated) was undertaken. There was an absence of any toxicity by repeated oral administration.

7.2.2.5 Respiratory Toxicity

The acute inhalation study was been performed (**Appendix 18**) in compliance with OECD guidelines for testing chemicals N° 403 (OECD, 2009) EC Council Directive 87/18 EEC.

The inhalation toxicity was performed on 10 rats exposed during 4 hours to an aerosol of endo β(1-4) xylanase. The animals were kept under observation for 14 days.

There were no treatment-related signs during the observation period. There were no macroscopic findings at *post-mortem*. No acute inhalation toxicity was detected after a 4-hour exposure.

The LC 50 (4-hour) for endo β(1-4) xylanase is in excess of 4.74 mg/l of air.

7.2.2.6 Dermal irritation

An acute dermal irritation test was been performed (**Appendix 19**) in compliance with OECD guidelines for testing chemicals N° 404 (OECD, 2002) EC Council Directive 87/18 EEC.

This acute dermal irritation test in the rabbit was performed with the food enzyme endo β(1-4) xylanase on three animals. The potential for inflammatory or corrosive activity of the enzymes to skin was assessed by a single exposure to 0.5-ml material for four hours. Responses were noticed after 1, 24, 4, 72 hours and reported. No dermal reaction at the test site of any animal was detected.

Endo β(1-4) xylanase from *Bacillus subtilis* can be classified as non-irritant to skin.

7.2.2.7 Eye irritation

An acute eye irritation test was been performed **(Appendix 19)** in compliance with OECD guidelines for testing chemicals N° 405 (OECD, 2012) EC Council Directive 87/18 EEC.

This acute eye irritation in the rabbit was performed with 0.1 ml of food enzyme endo β(1-4) xylanase on three animals. The consequences on the eye were looked at 1, 24, 48 and 72 hours after treatment.

Endo β(1-4) xylanase from *Bacillus subtilis* can be classified as non-irritant to the eye.

7.2.2.8 Test material used in genotoxicity and sub-chronic toxicity studies

The composition and specifications of the test material used in the genotoxicity and sub-chronic toxicity studies are given in the Table below.

Table 6: Composition and Specification of the test material

Batch no	05/78
Activity (GDXU/ml)	289
TOS (%)	2,49%
Unit/ mg TOS	12

Certificate of Analysis in **Appendix 20**

In comparison with the more recent commercial batches as presented in the first table below**,** the test material (second table) can be regarded as representative for the commercial food enzyme.

Table 7: Composition and Specification of the Commercial food enzyme

Batch no	GIZ1401	GIZ1402	GIZ1403	Mean	St dev
Ash (%)	4.99	3.78	4.86	4.54	0.54
Water (%)	91.22	91.53	91.97	91.57	0.31
TOS (%)	3.79	3.78	3.16	3.58	0.29
Activity (GDXU/ml)	4284	4004	2946	3745	576.20
Units/mg TOS	113	106	93.2	104	8.20
Protein (g/l)	18.65	21.32	16.54	18.84	1.96

The certificate of analysis corresponding to those batches can be found in **Appendix 21.**

The "activity/mg TOS" ratio of the enzyme preparations is considerably improved. The preparation used in the toxicological studies had only 1/10 of the activity per mg TOS in comparison with actual production batches.

The TOS level of 2.49% of the tested substance indicates the presence of a higher amount of unknown variable compounds of biological origin (UVCB) with the enzymatic protein.

The consumption of 1450 GDXU/kg bw/day of test material means a higher consumption of UVCB (in comparison with actual preparations) which did not show any health effect.

The maximal amount of enzyme recommended for bakery applications is 150 GDXU/kg of flour, ie. addition of almost 10 times less UVCB.

Applying a 100 fold safety factor to the NOAEL from sub-chronic toxicity test would give an Acceptable Daily Intake (ADI) for human of 14.5 GDXU/kg/day or 0.1394 mg TOS/kg body weight/day (instead of 1.2 mg TOS/kg body weight for the tested preparation).

The Margin of Safety (MoS) for human consumption is calculated by dividing the NOAEL by the Total Theoretical Maximal Daily Intake (TMDI). As was shown in 7.5.1, the Total TMDI of the food enzyme is 0.0047 mg TOS/kg body weight/day. Consequently, the MoS is:

MoS = 13.94/0.0047 = 2966.45.

Applying the common safety factor of 100, the MoS would be 29.66.

As explained in 7.5.1, the Total TMDI is highly exaggerated; therefore, the MoS will be some magnitudes higher.

Consequently, there is no safety reason for determining a maximum level of use.

Summary of the toxicological data

Summarizing the results obtained from the several toxicity studies the following conclusions can be drawn:

- No mutagenic or clastogenic activity under the given test conditions was observed;
- The sub-chronic oral toxicity study showed a No Observed Adverse Effect Level (NOAEL) of at least 1450 IU/kg/day or 13.94 mg TOS/kg body weight/day and
- Non-irritant in respiratory, eye and dermal studies.

7.2.3 Information on the potential allergenicity of the enzyme processing aid

The amino-acid sequence for the endo β(1-4) xylanase enzyme protein has been determined, as described in section 7.1.3 (CCI). At present, validated testing methods to predict the allergenicity of an enzyme protein or its breakdown products after oral intake are not available. However, some information on the potential allergenicity of food enzymes can be obtained by applying the integrated, stepwise case-by-case approach used in the safety evaluation of the newly expressed proteins in genetically modified plants (EFSA, 2006; FAO/WHO, 2001). The allergenicity of the source of the food enzyme should be considered and a search for amino acid sequence and/or structural similarities between the expressed protein and known allergens should be undertaken where possible. As proposed in the FAO/WHO Report, cross-reactivity between a query protein and a known allergen has to be considered when there is: (a) more than 35% identity in the amino acid sequence of the expressed protein, using a window of 80 amino acids and a suitable gap penalty or: (b) identity of 6 contiguous amino acids. The online tool used to search allergens database was http://www.allergenonline.org/ and the sequence without signal peptide as query was used (**Appendix 22)**. Based on Full Fasta search method, Sliding 80mer Window search method or 8mer Extract Match search method, it can be concluded that endo β(1-4) xylanase from *Bacillus subtilis* does not present significant similarities with sequences of known allergenic proteins.

There are no scientific indications that small amounts of enzymes in food can sensitize or induce allergic reactions in consumers.

Respiratory Allergies

No allergenicity has been identified during the research and development work, or during pre-industrial trials and industrial up scaling, nor during downstream processing.

Enzyme preparations are regarded as respiratory sensitisers (R42). As such, measures should be taken to minimise the inhalation exposure of workers and inhalation toxicity studies are thus normally not required.

The powdered enzyme preparation is granulated and fixed on a carrier to avoid dust formation (see **Section 7.1.4)**. Therefore with a limited working protection the handling of the powdered enzyme preparation can be considered as safe.

No dust formation can occur with the liquid enzyme preparation and based on the inhalation study on rats with a liquid sample of the food enzyme endo β(1-4) xylanase it can be considered safe under its normal condition of use.

The only allergen present in the commercial enzyme preparation is the food grade wheat carrier. As the enzyme is exclusively intended to be used in bakery products it is not an issue as the main ingredient of all bakery products is wheat flour or other gluten containing cereals.

7.2.4 Safety assessment reports prepared by international agencies or other national government agencies, if available

Appendix 2 provides the scientific report from afssa (now called Anses) that has been used by the French authorities.

Furthermore, the FDA has given to enzymes preparations obtained from *Bacillus subtilis* the **GRAS** status (**Appendix 23**).

7.3 ADDITIONAL INFORMATION RELATED TO THE SAFETY OF AN ENZYME PROCESSING AID DERIVED FROM A MICROORGANISM

(As per section 3.3.2D of the Application Handbook 1 September 2013 - amended 1 June 2015)

7.3.1 Information on the source microorganism

The microorganism that is used for the production of endoβ(1-4) xylanase, is the bacterium *Bacillus Subtilis*. According to the current state of the art, the taxonomic classification of this microorganism is as follows:

Genus:	Bacillus
Sub genus:	-
Species group:	*Bacillus subtilis* group…
Species:	*Bacillus subtilis*
Subspecies/varieties:	subsp. Subtilis
Synonyms:	*Vibrio subtilis, Bacillus uniflagellatus, Bacillus natto, Bacillus globigii*

The source material for the food enzyme is *Bacillus subtilis LMG S-24584*. The organism has been genetically modified to produce the enzyme. Information regarding the construction is provided in **Sections 7.1.2 and 7.1.3.** Absence of antibiotic resistance genes and absence of genetically modified material (GMM) in the final product are demonstrated. rDNA was detected in the final product but a test of transformability demonstrated the absence of biological impact of this rDNA. There's no cytotoxicity on Vero-cells.

Bacillus subtilis as a species is one of the most widely used bacteria for the production of enzymes and specialty chemicals. *Bacillus subtilis* occurs ubiquitously in the environment and as a result can be also found in food. The bacterium has already been used for decades for the production of food enzymes with no known reports of adverse effects to human health or the environment.

7.3.2 Information on the pathogenicity and toxicity of the source microorganism

The production organism for this xylanase, *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. Schedule 18 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, hemicellulasemulticomponent 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 as covered in Section 6.

7.3.3 Information on the genetic stability of the source organism

This information is provided in Sections 7.4.1.3 and 7.4.1.4.

7.4 ADDITIONAL INFORMATION RELATED TO THE SAFETY OF AN ENZYME PROCESSING AID DERIVED FROM A GENETICALLY-MODIFIED MICROORGANISM

(As per section 3.3.2E of the Application Handbook 1 September 2013 - amended 1 June 2015)

7.4.1 Information on the methods used in the genetic modification of the source organism

7.4.1.1 Characteristics of the Recipient or (when appropriate) Parental Organism

Phenotypic and Genetic Markers

Bacilli form a group of rod shaped, endospore forming aerobic or facultative anaerobic, mostly Gram-positive, motile bacteria, chemo-organotrophic using a variety of organic acids and amino-acids as carbon sources. Most strains are common, apparently saprophytic inhabitants of soil and water which occupy a variety of ecological niches around the world. The genus comprises mesophilic, thermophilic, acidophilic and alkalophilic species. *Bacillus subtilis* is the type species of the genus (Claus and Berkeley, 1986).

Phenotypic grouping of the closely related species *Bacillus subtilis*, *Bacillus amyloliquefaciens*, and *Bacillus licheniformis* based on colony morphology, fatty acid composition and physiological characteristics such as carbohydrate is very often misleading in particular in efforts to distinguish *B. subtilis* and *B. amyloliquefaciens* (Coorevitis et al., 2008; Logan and Berkeley, 1984).

The genome of *B. subtilis* 168 was the first bacterial genome to be sequenced in 1997 (Kunst *et al*., 1997). Since then, a number of *B. amyloliquefaciens* genomes (Chen *et al*., 2007) and *B. licheniformis* genomes (Rey *et al*., 2004; Veith *et al*., 2004) have been sequenced as well.

B. subtilis is a ubiquitous soil microorganism that contributes to nutrient cycling when biologically active due to the various enzymes produced by members of the species. *B. subtilis* is a gram-positive bacterium which multiplies and disseminates by asexual processes. Wild type strains produce endospores that allow it to endure extreme conditions of heat and desiccation in the environment. *B. subtilis* produces a variety of proteases and other enzymes that enable it to degrade a variety of natural substrates and contribute to nutrient cycling. However, under most conditions the organism is not biologically active but exists in the spore form (Alexander, 1977).

B. subtilis can grow at a pH above 5.5 and below 8.5, and shows optimal growth at pH 7. The species can grow between 25 and 52°C but grows optimally between 30 and 40°C. The species is an obligatory aerobe, except in the presence of glucose and nitrate, some anaerobic growth can occur (Claus and Berkeley, 1986).

Degree of Relatedness between Recipient and Donor(S)

The recipient and the donor do not belong to the same species. The donor is *Pseudoalteromonas haloplanktis* and the recipient is *Bacillus Subtilis*.

Description of Identification and Detection Techniques

The detection technique to demonstrate the absence of the GMM in the final product has been described by the European Food Safety Agency (EFSA, 2011)

Source and Natural Habitat of the Parental Microorganism

Pseudoalteromonas haloplanktis is an Antarctica psychrophilic bacterium. The strain was isolated from soil samples collected in the vicinity of the French Antartic station in Dumont D'Urville, Terre Adelie, Antartica (Collins & al. 2002).

Organism with Which Transfer of Genetic Material is known to occur under Natural Conditions and Presence of Indigenous Genetic Mobile Elements

According to Dubnau (1999), only for a rather limited number of bacterial species have the natural transformation systems been studied in great detail. *Bacillus Subtilis*, our parental strain, is one of them. However, the demonstration of absence of the production micro-organism in the final product shows that this characteristic is not a problem.

Regarding the transfer of DNA, there's no plasmid in the production strain. As the insert is chromosomally integrated, the frequency of transfer to other organisms is the same as for other genomic genes.

No gene that confers resistance/tolerance is present in the production strain.

Information on the Genetic Stability of the Recipient Microorganism

16S analysis were performed on the parental strain and the production strain (*Giz*α7101). The phylogenetic comparison showed that the production strain is indeed a *Bacillus subtilis*. The comparison with the recipient strain *Giz*α7101 showed 100% identity.

Further specific detail is provided in the CCI document.

Pathogenicity, Ecological and Physiological Traits

Pathogenicity: Pathogenic strains are not described in the Bergey's Manual[2] or in the ATCC[3] and other catalogues. The species *B. subtilis* does not appear on the list of pathogens in Annex III of

2 Bergey's Manual of Systematic Bacteriology
3 http://www.atcc.org/en.aspx, accessed 17.11.2015

Directive 2000/54/EC on the protection of workers from risks related to exposure to biological agent at work or on the list of pathogens from Belgium (Belgian Biosafety Server, 2010 – **Appendix 24**).

B. subtilis is exempted as a host of certified host-vector systems under the NIH Guidelines in the USA since 1994 (NIH, 1994 – **Appendix 25**). The US EPA has added *B. subtilis* to the list of exempted organisms in 1997 (US EPA, 1997). *B. subtilis* is a low-risk-class microorganisms, i.e., category 1 of the European Federation of Biotechnology (Frommer *et al*., 1989), and it can be used under the lowest containment level at large scale, GILSP, as defined by OECD (1992) – **Appendix 26**.

QPS status: The European Food Safety Agency (EFSA) maintains a list of the biological agents to which the Qualified Presumption of Safety (QPS) assessment can be applied. In 2007, the Scientific Committee set out the overall approach to be followed and established the first list of the biological agents. The QPS list is reviewed and updated annually by the Panel on Biological Hazards (BIOHAZ). If a defined taxonomic unit does not raise safety concerns or if any possible concerns can be excluded, the QPS approach can be applied and the taxonomic unit can be recommended to be included in the QPS list. The safety of *B. subtilis* as a production microorganism has been assessed by EFSA and been accorded QPS status provided the qualification requirements are met (EFSA, 2007).

Secondary metabolites: A review of the literature by the US EPA (1997) failed to reveal the production of metabolites of toxicological concern by *B. subtilis*. Although *B. subtilis* has been associated with outbreaks of food poisoning (Gilbert *et al*., 1981 and Kramer *et al*., 1982 as cited by Logan, 1988), the exact nature of its involvement has not been established. Unlike the case in these outbreaks of food poisoning, where apparently *B. subtilis* was isolated from a food source, the strains used for food enzyme production are not present in the processed food as only the enzyme preparation is used in the processing.

B. subtilis, like other closely related species in the genus, *B. licheniformis*, *B. pumulis* and *B. megaterium*, have been shown to be capable of producing lecithinase, an enzyme which disrupts membranes of mammalian cells. However, there has not been any correlation between lecithinase production and human disease in *B. subtilis*.

Concern on possible involvement of *B. cereus*-like enterotoxins in the rare cases where some Bacillus strains have been associated with food poisoning caused the Scientific Committee on Animal Nutrition (SCAN) to require specific testing of industrially used Bacillus strains (European Commission, 2000). Subsequent testing showed the absence of *B. cereus* – like enterotoxins (Pedersen *et al*., 2002).

In 2011 EFSA updated the guidance contained in the SCAN opinion stating that it now seems unlikely that *B. cereus*-like enterotoxins are produced in species other than the *B. cereus* group, and any toxigenic potential in other species appears far more likely to arise from the production of surfactins (EFSA, 2011). A PCR detection of non-ribosomal peptide synthase genes is suggested to be adequate to identify surfactin-positive strains. However, among the 22 publically available full genome sequences of Bacilli of the *B. subtilis* cluster the frequency of genes encoding for lipopeptide production is 100%, indicating that the presence of such genes are widespread, and this would not be a valid test.

EFSA launched a consultation (EFSA, 2013) on a revision of the guidance document in which the test for the presence of non-ribosomal peptide synthase genes is no longer a requirement, but detailed described new assays for haemolysis and cell toxicity will be requested instead.

This requirement is still based on the assumption that lipopeptides are the cause of the few incidents of food poisoning. However, the link between lipopeptide genes and food poisoning has not been documented in the scientific literature, and is not supported in the new studies with food products well known for their lipopeptide content, like the Japanese food Natto. The long history of safe use of Natto is also taken into account in the EFSA opinion on the safety of Vitamin K2 produced using a *B. subtilis* var *natto* strain (EFSA, 2008). In addition in a recent 28-day toxicity of Surfactin C produced by *B. subtilis* the no-observed-adverse-effect level (NOAEL) was 500 mg/kg following oral administration in rats, indicating the very low to almost non-toxicity of surfactin (Hwang *et al*., 2009).

In 2014, EFSA updated the guidance concerning the evaluation of Bacillus and lipopeptide's analysis is no longer required.

Metabolites of human toxicological concern are usually produced by microorganisms for their own protection. Microbes in natural environments are affected by several and highly variable abiotic (e.g. availability of nutrients, temperature and moisture) and biotic factors (e.g. competitors and predators). Their ever changing environments put a constant pressure on microbes as they are prompted by various environmental signals of different amplitude over time. In the wild this results in continuous adaptation of the microbes through inducing different biochemical systems; e.g. adjusting metabolic activity to current availability of nutrients and carbon source(s), or activation of stress or defense mechanisms to produce secondary metabolites as 'counter stimuli' to external signals (Klein and Paschke, 2004; Earl *et al*., 2008). On the contrary, 'environmental' conditions of microbial production strains during industrial scale fermentation have been optimized and 'customized' to the biological requirements of the strain in question (see review by Parekh *et al*., 2000). Thus, the metabolic activity and growth of a particular microbial production strain during the fermentation process (primarily the 'exponential growth phase') will focus on efficiently building cell biomass which in turn produces the molecule of interest. Industrial fermentations are run as monocultures (i.e. no external competitors or predators) with optimal abiotic conditions. Hence, there are no strong environment signals that would induce stress (e.g. starvation, competitive environment and low/high temperature) or defense mechanisms (e.g. production of antibiotic, antiviral or neurotoxic molecules). Biosynthesis of stress and/or defense secondary metabolites of toxicological relevance by industrial microbial production organisms during the fermentation process is thus highly unexpected (Sanchez and Demain, 2002) and is furthermore avoided from an economical perspective to optimize production.

Most industrial *B. subtilis* strains are from safe lineages that have been repeatedly tested according to the criteria laid out in the Pariza & Johnson publication (Pariza and Johnson, 2001).

Concerning antimicrobial resistance, the guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance (EFSA, 2012 - FEEDAP) replacing the technical guidance updating the criteria used in the assessment of bacterial resistance to antibiotics of human or veterinary importance (EFSA 2008 - FEEDAP), recommend to identify the potential resistance to antibiotics of microorganisms used as additive in the feed sector. If the microorganisms are used as

such and are present in the final product and if they present an antibiotic resistance, this situation could be a problem due to the potential transmission of this character. Therefore, the FEEDAP recommend that any bacterial strain carrying an acquired resistance to antimicrobial that is shown to be due to the acquisition of genetic determinant presents the greatest potential for horizontal spread and should not be used as a feed additive.

In Section 7.4.2.2 a test can be found demonstrating the absence of the microorganism in the final product. As the microorganism is not present in the final product, the problem exposed by the FEEDAP panel in the mentioned documents is not an issue for this dossier.

In addition, in Section 7.4.1.3, tests are presented proving the absence of resistance genes to antibiotics.

Description of History of Use

Uses: *B. subtilis* is one of the most widely used bacteria for the production of enzymes and specialty chemicals. Industrial applications include production of amylase, protease, inosine, ribosides, and amino acids. Uses of proteases include use in detergent products and for dehairing and batting in the leather industry. Uses of amylases include desizing of textiles and starch modification for sizing of paper (Erikson, 1976; Ferrari *et al*., 1993).

Food use safety: *B. subtilis* is ubiquitous in the environment (soil, water, plants and animals) and as a result can be also found in food (de Boer and Diderichsen, 1991). The bacterium has already been used for decades for the production of food enzymes with no known reports of adverse effects to human health or the environment (de Boer and Diderichsen, 1991). Alpha-amylase enzyme preparation from *B. subtilis* has been used commercially since 1929, when it was used in the manufacture of chocolate syrup to reduce its viscosity (Reed, 1966).

Recently the US Food and Drug Administration reviewed the safe use of food-producing enzymes from recombinant microorganisms, including *B. subtilis* (Olempska-Beer *et al*., 2006). An extensive risk assessment of *B. subtilis*, including its history of commercial use has been published by the US EPA (1997). It was concluded that *B. subtilis* is neither a human pathogen nor is it toxigenic.

Food enzymes derived from *B. subtilis* strains (including recombinant *B. subtilis* strains) have been evaluated by JECFA and many countries which regulate the use of food enzymes. For an extensive overview of countries that accepted *B. subtilis* as a safe production organism for a broad range of food enzymes, please refer to Section 6.

History of Previous Genetic Modification

Obtention of the host strain

Starting strain

The starting strain was obtained from the "BGSC" (Bacillus Genetic Stock Center – Department of Biochemistry – The Ohio State University – Columbus – Ohio – USA) and is a derivative of the well-known and widely used *Bacillus subtilis* Marburg 168 which is considered as the type strain of this species.

The derivative of the *B. subtilis* Marburg 168 strain is the ***B. subtilis* DB105** and was obtained under BGSCID number 1E51. To allow genetic modifications of the starting strain, four auxotrophic mutations have been first introduced in this strain by the classical method of congression (Cutting, S. & Vander Horn P.B. 1990) using chromosomal DNA from the *Bacillus* 1A445 strain.

The *Bacillus* 1A445 strain was obtained from BGSC (Bacillus Genetic Stock Center) then a deletion in a xylanase has been transferred in this strain by congression.

The donor strain is *B. subtilis* WDEX2 which was obtained in the Applicant's research laboratory and directly derived from *B. subtilis* Marburg 168. A deletion of 2412 base pairs has been introduced into the genome of *B. subtilis* Marburg 168 leading to the deletion of the XynA gene. The genetic map showing the precise sequence deleted is presented in the CCI materials under Section 7.4.1.

Finally, an additional mutation in a xylanase gene (*xyn A*) found in the *Bacillus subtilis* R21 strain has been also added by congression.

The *Bacillus* R21 strain was obtained from BGSC (Bacillus Genetic Stock Center) and BGSCID for this strain is 1A651.

Further specific detail is provided in the CCI document.

7.4.1.2 Characteristics of the Origin of the Inserted Sequences (Donor Organism)

DNA from Defined Donor Organisms

The xylanase producing bacterial strain was isolated from soil samples collected in the vicinity of the French Artic station in Dumont D'Urville, Terre Adelie, Antartica (Collins et al 2002).

Pseudoalteromonas haloplanktis (previously classified as *Alteromonas haloplanktis*) is a bacteria initially incorporated in the risk 2 class of microorganisms. Indeed some strains of *Pseudoalteromonas haloplanktis* subsp *tetraodonis* (*Alteromonas tetraodonis*) have been described as potential producers of tetrodoxin (Simidu & al 1990). Since then, *Alteromonas tetraodonis* (*Pseudoalteromonas haloplanktis* subsp. *tetraodonis*) has been recognized as a separated species (Ivanova & al. 2001).

Pseudoalteromonas haloplanktis should therefore be considered as a true risk 1 class of microorganism. Furthermore, it is classified as such by the ATCC (American Type Culture Collection)

(Figure 5) and the DSMZ (DeutscheSammlung von Mikroorganismes und Zellkulturen GmbH) (Figure 6). More recent articles have also shown that *Pseudoalteromonas* has a probiotic effect on fishes (cited in the review of Verschuere & al 2000).

Figure 5: Description of *Pseudoalteromonas haloplanktis* as belonging to the risk 1 class of microorganism by the American Type Culture Collection

Organism:	*Pseudoalteromonas haloplanktis* (ZoBell and Upham) Gauthier et al. emend. Ivanova et al.; deposited as *Pseudomonas* sp.		
Designations:	B-16 [NCMB 19]	**Isolation:**	marine clam, Canada
Depositors:	RA MacLeod		
Biosafety Level:	1	**Shipped:**	freeze-dried
Growth Conditions:	ATCC medium 209: Marine Cytophaga medium Temperature: 26.0C		
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits.		

Figure 6: Description of *Pseudoalteromonas haloplanktis* as belonging to the risk 1 class of microorganism by the DSMZ.

Name: *Pseudoalteromonas haloplanktis* **(Type species)** Authors: (ZoBell and Upham 1944) Gauthier et al. 1995 emend. Ivanova et al. 2001 Status: New Combination Literature: Int. J. Syst. Bacteriol. 45:759; Risk group: 1 (German classification) Comment: elevation of *Pseudoalteromonas haloplanktis* subsp. *tetraodonis* to species rank reduces *Pseudoalteromonas haloplanktis* subsp. *haloplanktis* to *Pseudoalteromonas haloplanktis*; emended description: IJSEM 51:107Type strain: ATCC 14393, DSM 6060 Synonyms: *Alteromonas haloplanktis* (basonym)

Synthetic DNA

Not relevant

Nucleic Acids directly extracted from environmental sample

Not relevant

7.4.1.3 Description of the Genetic Modification

Characteristic of the Vector

Description of the expression cassette used for production of the enzyme in *B. subtilis*

The expression cassette comprises the following elements:

- part of a promoter of *Bacillus subtilis*
- the coding sequence of the *Pseudoalteromonas haloplanktis* gene
- a signal peptide sequence and a terminator sequence of *Bacillus subtilis*.

Construction of an integration plasmid

Schematic description of the procedure (see **Figure 7)**

The integration plasmid drawn on the figure comprises the following elements:

- An *Escherichia coli* plasmid vector (e.g. pSK+, pBR322,…) to allow propagation in *E. coli*
- A *Bacillus subtilis* marker (Cm^R). The marker used is the gene coding for the chloramphenicol acetyltransferase that gives to *Bacillus subtilis* the ability to grow in the presence of chloramphenicol in the medium. The use of this gene in *Bacillus subtilis* has been first described by Ehrlich, S.D. 1978. It can be recovered by a double restriction enzyme digestion from the plasmid pHV60 described by Gleave et al (1990). This plasmid is identical to the

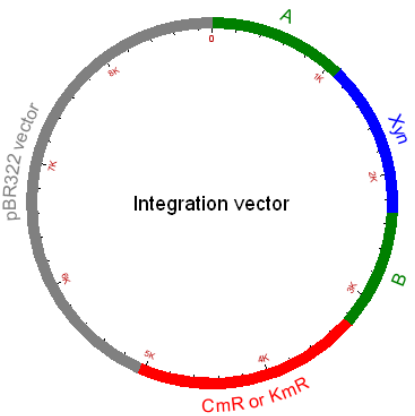
plasmid pHV32 described by Primrose and Ehrlich (1981). Another possibility is to use a gene coding for the resistance to Kanamycin. This is done by using the gene coding for kanamycin resistance recovered from plasmid pJH1 as described by Trieu-Cuot (1983).

- A DNA fragment recovered from the host strain chromosome that will serve as target sequence to integration (A & B). The sequences chosen for integration can be found in the scientific literature. Care should be taken not to damage essential genes. The DNA fragment representing the fusion between the promoter and the xylanase gene coding sequence (XYN).

A genetic map of the integration vector is presented below. This vector is used to integrate the promoter-xylanase fusion gene into the target sequences in the genome of the recipient strain.

All vectors used for the integration of the promoter-xylanase fusion gene in target genes have the same design.

Figure 7: A genetic map of the integration vector



Transformation of a host strain

The plasmid constructed above can be used to transform either a strain devoid of any copy of the fusion or a strain that contains already one or more copies of the xylanase gene.

The transformants are selected for their resistance to chloramphenicol.

Due to the inability of the plasmid to replicate in *Bacillus subtilis* cell, the only way to obtain transformants resistant to chloramphenicol is to have the plasmid integrated in the chromosome. Homologous recombination is very powerful in *Bacillus* (Dubnau, D. 1993. & Lacks, S.A. 1988.). This mechanism allows the integration of the plasmid at one of the two target sequences (A or B) that are present.

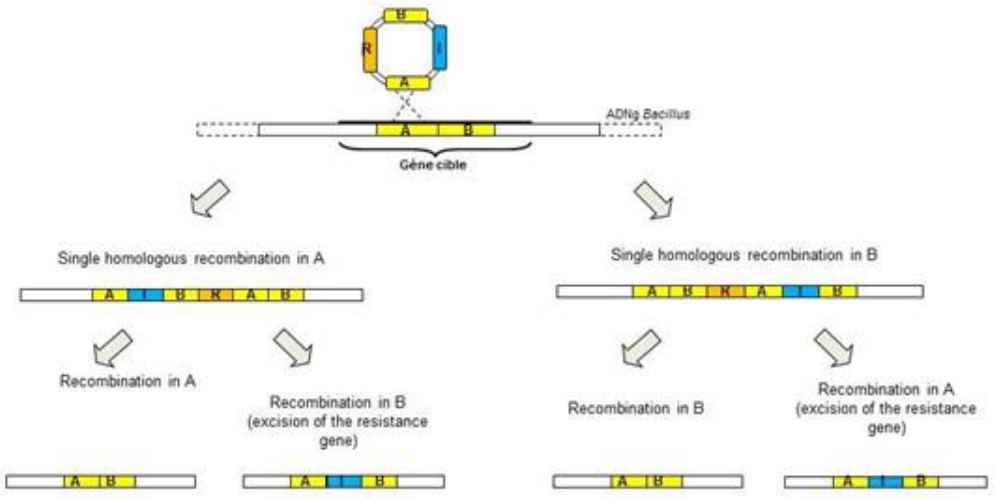
Obtention of exogenous sequence-free strain

The integration obtained in the previous section is stable as long as the selection pressure (eg. presence of chloramphenicol) is maintained. However, if chloramphenicol is omitted from the cultivation medium, loss of the integrated plasmid can occur by homologous recombination. In the example of **Figure 8**, if the homologous recombination occurs between two identical "A" sequences, all the integrated sequences of the plasmid will be lost. However, homologous recombination can also occur between two identical "B" sequences. In this case, the DNA fragment between A and B sequences in the original plasmid will be kept integrated into the host chromosome.

The distinction between the two events (recombination in "A" or in "B") is made by Southern blotting using the xylanase gene as a probe. As the copies integrated in the chromosome reside in different locations, they will all display a distinct pattern by hybridization.

This mechanism gives a transformed strain that has no exogenous DNA into its chromosome. No selection marker (eg. the chloramphenicol acetyltransferase gene) is necessary to keep the xylanase gene integrated since there are no more closely linked homologous sequences that could lead to the loss of the gene of interest.

Figure 8: DNA recombination after integration of the plasmid



Controls

Two methods can be followed to check for the absence of exogenous DNA:

- The loss for resistance to chloramphenicol or kanamycin
- Southern blotting using resistance gene as probe.

Successive integration of several copies

As mentioned above the integration plasmid can be used to transform a strain that has already one or more copies integrated into its chromosome. The search for strains containing only endogenous DNA integrated is performed in the same manner.

Another method used to obtain the same type of strain is to transform the host strain (which contains already one or more copies of the gene of interest) with the chromosomal DNA of a strain that contains the entire plasmid integrated and select for chloramphenicol resistance. After transformation, the search for strain with an extra copy of the xylanase gene and no exogenous DNA is made as described before.

The number of copies inserted in the genome of the final production strain is provided in the CCI section.

Information Related to the Genetic Modification

Stability of the modified production organism

There is no exogenous DNA in the production strain. The only way of having instability in this strain could arise from recombination between two integrated copies of the promoter xylanase fusion gene. This is very unlikely since these copies are separated by genes necessary for the survival of the strain. Homologous recombination between the copies would eliminate these essential genes.

Furthermore the production of enzyme by the production strain is quite constant showing that there is no loss of ”xylanase gene copies”.

Control of the absence of vector DNA and of antibiotic resistance marker genes

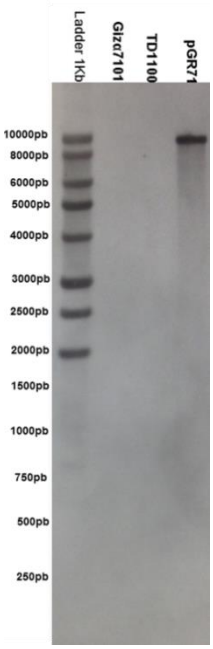
The antibiotic resistance genes used during the genetic modification process including also the construction of the recipient strain are:

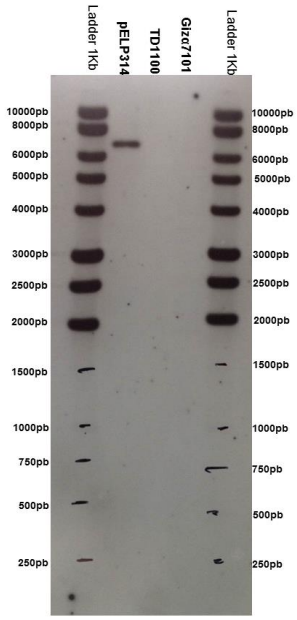
- kanamycin resistance gene: the absence of the kanamycine resistance gene in the recipient strain is demonstrated below by Southern analysis.

- kanamycine resistance gene: the vector used for introducing the xylanase gene copies, may contain a kanamycin resistance marker gene ; the absence of the kanamycine resistance gene in the final production strain, *Bacillus Giz*α7101, is demonstrated below by Southern analysis.

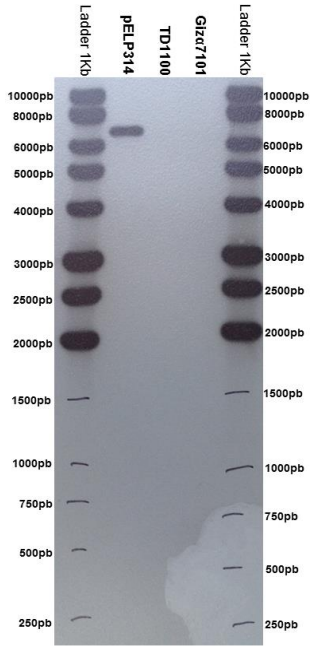
- ampicillin resistance gene: the vector used for introducing the xylanase gene copies, contains an ampicillin resistance marker gene ; the absence of the ampicillin resistance gene in the final production strain, *Bacillus Giz*α7101, is demonstrated below by Southern analysis.

- chloramphenicol resistance gene: the vector used for introducing the xylanase gene copies, may contain a chloramphenicol resistance marker gene ; the absence of the chloramphenicol resistance gene in the final production strain, *Bacillus Giz*α7101, is demonstrated below by Southern analysis.

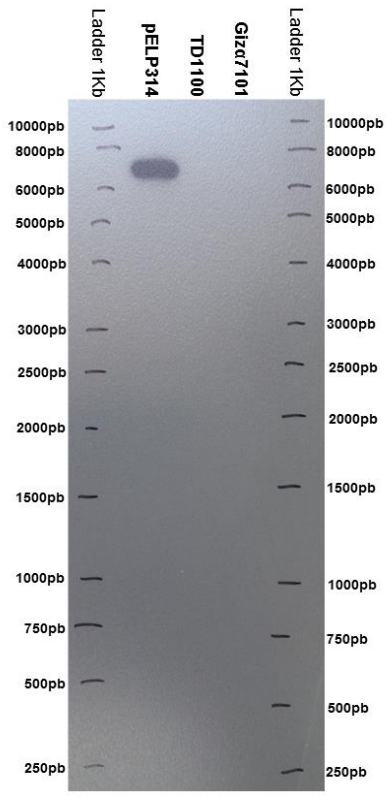




These results confirm the absence of the kanamycin resistance gene from plasmid pGR71 and pELP314 in the final production strain, Bacillus subtilis Gizα7101.



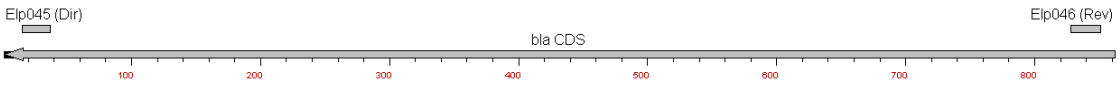
These results confirm the absence of the ampicillin resistance gene in the final production strain, *Bacillus subtilis* GIZα7101.



These results confirm the absence of the chloramphenicol resistance gene in the final production strain *Bacillus subtilis Giza*7101.

The ampicillin marker gene is the one present on pBR322 plasmid (Bolivar et al, 1977) from which are derived the different plasmids used during the construction of the expression vector.

Schematic view of the gene sequence is presented below with the region covered by the probe used in Southern Blot.

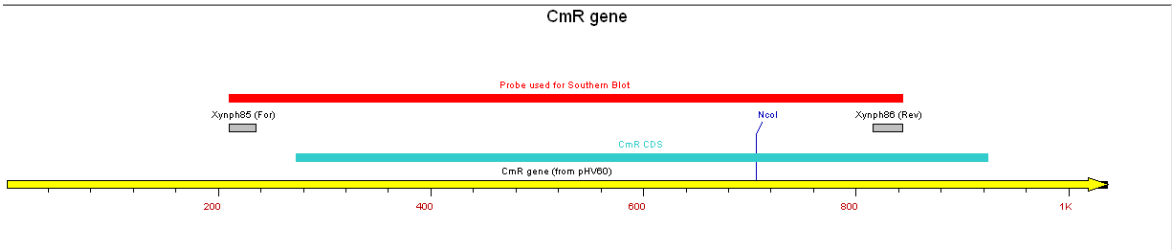


Coding sequence from ATG start codon to TAA stop codon (sequence is in reverse complement) is presented below with probe sequence underlined and primers used to generate the probe in bold:

TTACCAATGCTTAA**TCAGTGAGGCACCTATCTCAGC**GATCTGTCTATTTCGTTCATCCATAGTTGC
CTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAA
TGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGG
GCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGA
AGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTGCTGGCATCGT
GGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTA
CATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTA
AGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCAT
CCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGC
GACCGAGTTGCTCTTGCCCGGCGTCAACACGGGATAATACCGCGCCACATAGCAGAACTTTAAAA
GTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCC
AGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTG
GGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGG**GAATAAGGGCGACACGGAAATGT**TG
AATACTCAT

The chloramphenicol marker gene was recovered by restriction enzyme digestion from the plasmid pHV60 (Gleave et al, 1990).

Schematic view of the gene sequence is presented below with the region covered by the probe used in Southern Blot.



Gene sequence is presented below with probe sequence underlined and primers used to generate the probe in bold; the coding sequence from ATG start codon to TAA stop codon is highlighted in blue:

GATCCATCAAGATAAGAAAGAAAAGGATTTTTCACTACGCTCAAATCCTTTAAAAAAACACAAAAGA
CCACATTTTTTAATGTGGTCTTTTATTCTTCAACTAAAGCACCCATTAGTTCAACAAACGAAAATTGG
ATAAAGTGGGATATTTTTAAAATATATATTTATGTTACAGTAATATTGACTTTTAAAAAAGGATTGATT
CTAAT**GAAGAAAGCAGACAAGTAAGCCTCC**TAAATTCACTTTAGATAAAAATTTAGGAGGCATATC
AAATGAACTTTAATAAAATTGATTTAGACAATTGGAAGAGAAAAGAGATATTTAATCATTATTTGAAC
CAACAAACGACTTTTAGTATAACCACAGAAATTGATATTAGTGTTTTATACCGAAACATAAAACAAG
AAGGATATAAATTTTACCCTGCATTTATTTTCTTAGTGACAAGGGTGATAAACTCAAATACAGCTTTT
AGAACTGGTTACAATAGCGACGGAGAGTTAGGTTATTGGGATAAGTTAGAGCCACTTTATACAATT
TTTGATGGTGTATCTAAAACATTCTCTGGTATTTGGACTCCTGTAAAGAATGACTTCAAAGAGTTTT
ATGATTTATACCTTTCTGATGTAGAGAAATATAATGGTTCGGGGAAATTGTTTCCCAAAACACCTAT
ACCTGAAAATGCTTTTTCTCTTTCTATTATTCCATGGACTTCATTTACTGGGTTTAACTTAAATATCA
ATAATAATAGTAATTACCTTCTACCCATTATTACAGCAGGAAAATTCATTAATAAAGGTAATTCAATA
TATTTA**CCGCTATCTTTACAGGTACATCATTCTG**TTTGTGATGGTTATCATGCAGGATTGTTTATGA
ACTCTATTCAGGAATTGTCAGATAGGCCTAATGACTGGCTTTTATAATATGAGATAATGCCGACTGT
ACTTTTTACAGTCGGTTTTCTAATGTCACTAACCTGCCCCGTTAGTTGAAGAAGGTTTTTATATTAC
AGCTCCAGATCCATATCCTTCTTTTG

The kanamycine marker gene was recovered by restriction enzyme digestion from the plasmid pJH1 (Trieu-Cuot et al, 1985).

Schematic view of the gene sequence is presented below with the region covered by the probe used in Southern Blot.

Coding sequence from ATG start codon to TAA stop codon is presented below with probe sequence underlined and primers used to generate the probe in bold:

ATGGCTAAAATGAGAATATCACCGGAATTGAAAAAACTGATCGAAAAATACCGCTGCGTAAAAGAT
ACGGAAGGAATGTCTCCTGCTAAGGTATATAAGCTGGTGGGAGAAAATGAAAACCTATATTTAAAA
ATGACGGACAGCCGGTA**TAAAGGGACCACCTATGATG**TGGAACGGGAAAAGGACATGATGCTAT
GGCTGGAAGGAAAGCTGCCTGTTCCAAAGGTCCTGCACTTTGAACGGCATGATGGCTGGAGCAAT
CTGCTCATGAGTGAGGCCGATGGCGTCCTTTGCTCGGAAGAGTATGAAGATGAACAAAGCCCTGA
AAAGATTATCGAGCTGTATGCGGAGTGCATCAGGCTCTTTCACTCCATCGACATATCGGATTGTCC
CTATACGAATAGCTTAGACAGCCGCTTAGCCGAATTGGATTACTTACTGAATAACGATCTGGCCGA
TGTGGATTGCGAAAACTGGGAAGAAGACACTCCATTTAAAGATCCGCGCGAGCTGTATGATTTTTT
AAAGACGGAAAAGCCCGAAGAGGAACTTGTCTTTTCCCACGGCGACCTGGGAGACAGCAACATCT
TTGTGAAAGATGGCAAAGTAAGTGGCTTTATTGATCTTGGGAGAAGCGGCAGGGCGGACAA**GTGG**
TATGACATTGCCTTCTGCGTCCGGTCGATCAGGGAGGATATCGGGGAAGAACAGTATGTCGAGCT
ATTTTTTGACTTACTGGGGATCAAGCCTGATTGGGAGAAAATAAAATATTATATTTTACTGGATGAA
TTGTTTTAG

7.4.1.4 Information related to the GMM

Description of the genetic traits or phenotypic characteristics and in particular, any new traits and characteristics which may be expressed or no longer expressed

According to the genetic construction of the producing GMM strain *Bacillus Gizα7101*, the following phenotypic characteristics are no longer expressed:

- The strain is deficient in extracellular alkaline and neutral proteases;
- The strain is deficient in resident xylanase activities; and
- The strain is unable to sporulate.

Structure and amount of any vector and/or donor nucleic acid remaining in the GMM

The detailed genetic construction of the producing strain is fully described under 7.4.1.1 and 7.4.1.3

The structure of the expression cassette is presented under 7.4.1.3.

The structure and amount of any vector and/or donor nucleic acid remaining in the GMM are presented under 7.4.1.3. The number of copies inserted in the genome of the final production strain is 7.

The proof of the numbers of copies in provided in the Southern Blot on paragraph 7.4.1.4.

Stability of the Genetic Traits in the GMM

The control for long-term stability of the production strain is made as follow: a single colony is picked from an agar slant and inoculated at 35°C in 50 ml of medium. When the culture reaches the stationary phase, 50µl are transferred to a new medium and incubated again at the same temperature. This procedure is repeated for a total of at least 10 successive subcultures. This process corresponds thus to more than 100 generations (compared to the production process where there are about 25 generations). Total chromosomal DNA is extracted from colonies obtained after plating the last culture and subjected to Southern analysis. The pattern of hybridization is the same for all colonies tested and corresponds to the pattern of the original strain.

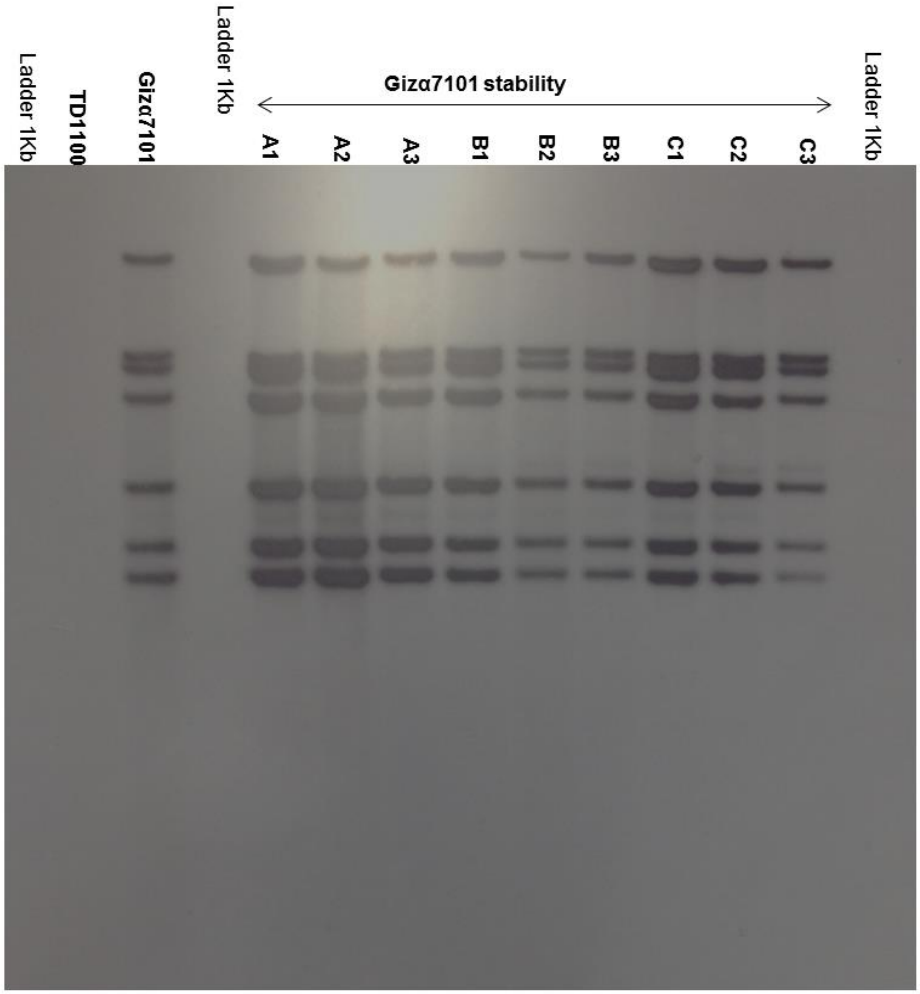
A Southern blot analysis was performed with 10 different strains samples:

- The recipient strain derived from *B. subtilis* 168 and into which the copies of the xylanase gene were subsequently introduced to obtain the final production strain *Bacillus Gizα7101*, serving as negative control.
- *Bacillus Gizα7101*, the final production strain, serving as positive control.
- *Bacillus Gizα7101* clones, 3 clones obtained after plating the last culture of 10 successive cultures of the final production strain in 3 replicates.

Total chromosomal DNA were extracted using the kit Wizard® Genomic DNA Purification (Promega), were digested by restriction enzymes and run on a 1% agarose gel. The DNA was then blotted on a nylon membrane and hybridized with a DNA fragment corresponding to the xylanase gene.

The Southern blot hybridisation results are shown below:

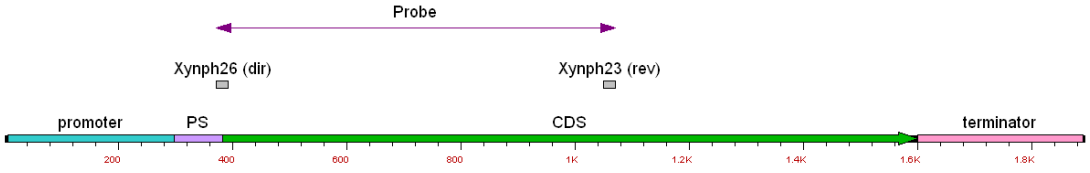
Figure 9: Southern blot hybridization results



The pattern of hybridization is the same for all colonies tested and corresponds to the pattern of the original strain.

The genetic map of the expression vector and the probe sequence is shown on the map below.

Figure 10: Genetic map of the expression vector and probe sequence



Rate and Level of Expression of the New Genetic Material and Activity of the Expressed Proteins

The best way to quantify the expression level of the inserted genetic material is to quantity the activity of the enzyme produced. The level of activity, the method of analysis and the mode of action of the enzyme are described in 7.1.3 and 7.1.6.

Description of Identification and Detection Techniques

The techniques used for identification and detection of the inserted sequences are presented under 7.4.1.3 and corresponding Southern blot hybridization results are presented under 7.4.1.4. The techniques for detection and verification of absence of the vector in the GMM are presented under 7.4.1.3.

Information on the Ability to Transfer Genetic Material to Other Organisms

The living production strain is maintained in contained conditions until it has been destroyed after the fermentation.

In addition this strain did not have been described as a strain capable of DNA transfer by natural system such as conjugation, transduction, etc. Furthermore there is no mobile element described for the production strain.

Some rDNA was detected in the final product. However the probability of transfer of this rDNA is low.

Transformation of competent *Bacillus subtilis* strain with various amount of DNA extracted from the enzyme preparation. No transformant could be isolated.

As demonstrated in Section 7.4.2.2 the GMM is not present in the final product. Therefore, no active transfer could occur.

The inserted sequence is located in the chromosomes. It's not link to mobile genetic elements and no sequence could enhance gene transfer or integration into the genome of other microorganisms.
The sequence is coding for a xylanase active at lower temperature than those useful for a microorganism in Europe. The donor micro-organism lives in Antartica. Those very specific ecological factors are not met in Europe and therefore no selective advantage could occur due to an unforeseen gene transfer.

History of Previous Uses or Environmental Release of the GMM

The GMM has never been released in the environment. The absence of the GMM in the final product is demonstrated in Section 7.4.2.2.

Safety for Humans and Animals

The host strain is derived from *Bacillus subtilis* 168, the type strain of this species.

Bacillus subtilis has a long history of safe use (de Boer, A.S. and Diderichsen, B. 1991). Furthermore, the FDA has given to enzyme preparations obtained from *Bacillus subtilis* **GRAS** status. The production strain should therefore be regarded as safe.

Bacillus subtilis is classified as QPS (qualified presumption of safety) by EFSA (2007).

The process described in the present Application has been evaluated by the Scientific Institute of Public Health – Division of Biosafety and Biotechnology (SBB). The strains concerned by this process belong to **class 1** level of containment according to the European legislation (directive 98/81/EC as amended).

Regarding the eventual production of toxins by *Bacillus subtilis* as expressed, the scheme recommended for the testing the *Bacillus subtilis* strain was followed - Guidance on the assessment of the toxigenic potential of Bacillus species used in animal nutrition (EFSA 2014 - FEEDAP).

Cytotoxicity test on Vero cells:

As requested by EFSA, cytotoxicity test with Vero cells was realized on the production strain *Bacillus subtilis Giz*α7101 (**Appendix 27**). As a conclusion of this test, *B. subtilis Giz*α7101 has no cytotoxic potential on Vero cells.

7.4.2 Information related to the product

7.4.2.1 Information Related to the Production Process

See Section 7.1.4.

7.4.2.2 Information Related to the Product Preparation Process

Demonstration of the Absence of the GMM in the Product

In order to demonstrate the presence/absence of the production strain, the following protocol was applied:

a) The cultivation medium is ‘Schaeffer’ medium which consists of sugar, proteins and salts and was specifically developed for the cultivation of *Bacillus* strains (Schaeffer et al, 1965). The growth conditions used are: 35°C, 200 rpm during 24 hours.

b) The detection sensitivity has been determined by addition of the production strain in known concentrations (in CFU/ml) in the enzyme preparation and then grown for 24 hours. The design of experiment and the detection sensitivity are presented in details in **Appendix 28 (CCI).**

c) The production strain could be differentiated from possible contaminating microorganisms by plating the cultures on LB agar plates containing 20 g/L beechwood xylan. The production strain will produce a clear halo due to xylan hydrolysis and the contaminating microorganisms will not.

d) Three different batches of the enzyme preparation were tested in triplicate for the presence/absence of the production strain. The results are presented in **Appendix 28 (CCI).**

The results obtained demonstrate the absence of the production strain in the three different batches tested. The sampling method is detailed in **Appendix 29.** In short, about 50 ml are sampled with a dedicated sampling flask from a batch of concentrate before formulation in a Multibox (~1000 L) after homogenization for 10 minutes.

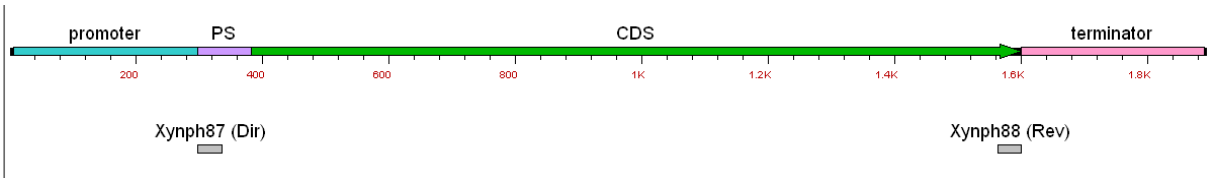
Information on the Inactivation of the GMM Cells and Evaluation of the Presence of Remaining Physically Intact Cells

As explained under 7.4.1, a sterile filtration step at the end of the purification process excludes the presence of remaining physically intact cells of the production microorganisms. This is validated by the demonstration of the absence of the GMM in the product (see under Section 7.4.2.2).

Information on the Possible Presence of Recombinant DNA

In order to demonstrate the presence/absence of recombinant DNA of the production strain, three batches where tested by PCR in triplicate.

a) The xylanase coding sequence was targeted in the genome of the final production strain using primers:



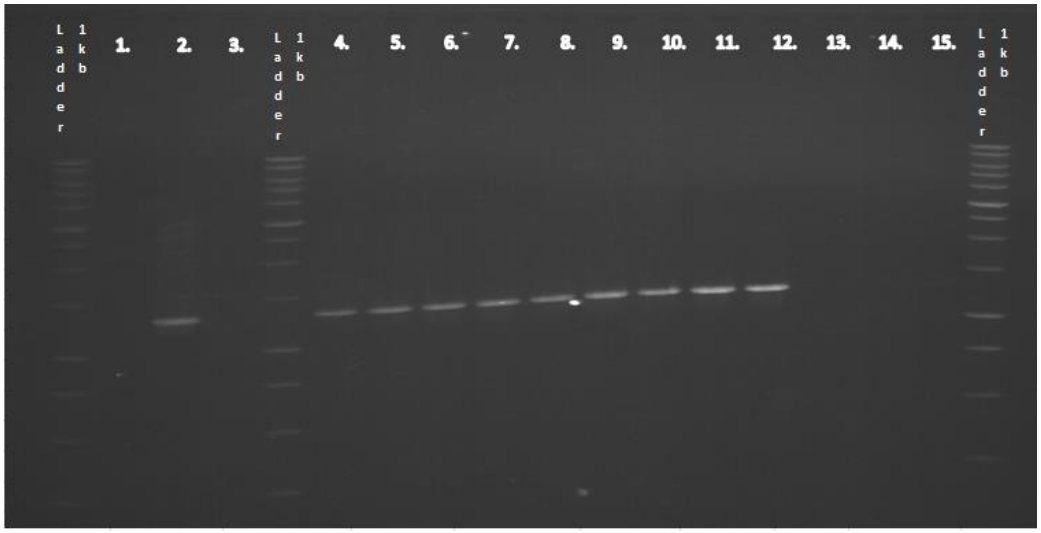
b) DNA extraction from the enzyme preparation was performed using the kit Wizard® Magnetic DNA Purification System for Food (Promega). Details of the procedure with the different steps followed (of which lysis step) and volumes can be found in the instructions for use from the supplier in **Appendix 31.** DNA sample preparation was done using 100 µl of starting material (see c) below for sampling details).

c) The sampling method is detailed in **Appendix 29**. In short, about 50 ml are sampled with a dedicated sampling flask from a batch of concentrate before formulation in a Multibox (~1000 L) after an homogenization of 10 minutes.

d) Three different batches of enzyme preparation were tested in triplicate for the presence/absence of recombinant DNA of the production strain. The results are presented below.

Recombinant DNA of the production strain is found in the three batches.

Well	Samples	Volume (µl)	H2O ajoutée (µl)
1	Blanco : H2O = template	0	5
2	Ctl+ : genomic DNA Gizα7101 10 ng/µl	5	0
3	Ctl- : genomic DNA TD1100 10 ng/µl	5	0
4	Extraction DNA Giz lot 1401 A	5	0
5	Extraction DNA Giz lot 1401 B	5	0
6	Extraction DNA Giz lot 1401 C	5	0
7	Extraction DNA Giz lot 1402 A	5	0
8	Extraction DNA Giz lot 1402 B	5	0
9	Extraction DNA Giz lot 1402 C	5	0
10	Extraction DNA Giz lot 1403 A	5	0
11	Extraction DNA Giz lot 1403 B	5	0
12	Extraction DNA Giz lot 1403 C	5	0
13	Extraction DNA Water A	5	0
14	Extraction DNA Water B	5	0
15	Extraction DNA Water C	5	0



Further information is provided under CCI (7.4.1 & 7.4.2)

7.5 INFORMATION RELATED TO THE DIETARY EXPOSURE TO THE PROCESSING AID

(As per section 3.3.2F of the Application Handbook 1 September 2013 - amended 1 June 2015)

7.5.1 Dietary Exposure

Commercial food enzyme preparations are generally used following the GMP/*Quantum Satis* (QS) principle, i.e. at a level not higher than the necessary dosage to achieve the desired final product – according to Good Manufacturing Practice. The amount of enzyme activity added to the raw material by the individual food manufacturer has to be determined case by case, based on the desired effect and process conditions and the flour quality. Therefore, the enzyme manufacturer can only issue a recommended enzyme dosage range. Such a dosage range is the starting point for the individual food producer to fine-tune their process and determine the optimal amount of enzyme that will provide the desired effect and nothing more. Consequently, from a technological point of view, there are no 'regular or maximal use levels' and endo β(1-4) xylanase is used according to the QS principle. A food producer who would add much higher doses than the needed ones would experience excessive costs as well as potentially negative technological consequences mainly with respect to quality.

Microbial food enzymes contain – apart from the enzyme protein in question – some substances derived from the producing micro-organism and the fermentation medium. The presence of all organic materials is expressed as Total Organic Solids (TOS). Whereas the dosage of a food enzyme depends on the enzyme activity present in the final food enzyme preparation, the dosage on basis of TOS is more relevant from a safety point of view. Therefore, the use levels are expressed in TOS.

Table 8 below shows the range of recommended use levels for each application where the food enzyme may be used.

Table 8: Recommended Enzyme Use Levels

Application	Raw material (RM)	Recommended use levels (GDXU/kg RM)	Maximal recommended use levels (GDXU/kg RM)
Baking	Flour	10-150	150

Endo β(1-4) xylanase from *Bacillus Subtilis* may be used in the manufacture of a wide variety of baked foods. Due to this wide variety of applications, the most appropriate way to estimate the human consumption in the case of food enzymes is using a Budget Method which calculates a Theoretical Maximum Daily Intake (TMDI) based on conservative assumptions regarding physiological requirements for energy from food and the energy density of food rather than on food consumption survey data.

The Budget Method was originally developed for determining food additive use limits, was used extensively by the Codex Committee on Food Additives and Contaminants during the Codex General

Standard for Food Additives and is known to result in highly conservative estimations of the daily intake. Potential dietary exposure is estimated using a standard adult of 60 kg and in children of 31 kg.

Bread consumption in Western Europe is stable, although it varies greatly between States. The Germans and Dutch eat the most bread on average at just under 60 kg per person per year while the UK, Denmark and Portugal are at the bottom of the list with an annual consumption of less than 37 kg bread.

Average bread consumption in Australia is 32.3kg per person per year (Euromonitor, 2013, **Appendix 30**). The Australian Health Survey (2011-12) **(Appendix 30)** reported that:

"*among the consumers of regular bread, the median amount consumed on a day was 72 grams (around 2 average slices), with males consuming more than females."*

The median for males was 82.0 grams and females were 66.0 grams. If this was scaled up to consumption on 365 days per year, the total consumption for males and females would be 29.9 kg and 24 kg respectively per year which is below the 32.3kg average reported by Euromonitor.

Bread consumption is not likely to notably grow since bread is eaten in nearly every household and it is unrealistic to expect bread to make any major inroads into other sectors.

Assuming a reference body weight of 60 kg for adults and 31 kg for children with an average consumption as reported in the INCA 2 study (afssa, 2006-2007) and at a maximal dose of 15000 IU/100 kg flour equivalent to 15000 IU/125 kg bread, the maximum daily intake of enzyme per consumer will be:

- For adults: 42 kg bread[4] * 15000 IU / (60 kg b.w. * 125 kg bread * 365 days) = 0.230 IU enzyme/kg b.w./day

- For children: 20 kg bread[5] * 15000 IU / (31 kg b.w. * 125 kg bread * 365 days) = 0.212 IU enzyme/kg b.w./day

According to the Euromonitor study, the consumption of bread by European extreme consumers is 90 kg bread per year and the average in all 28 MS is 50 kg (Euromonitor, 2013, Appendix 30).

The maximal intake of enzyme will be 0.493 IU/kg b.w/day**.** This is equivalent to 3.5 µg TOS/kg b.w/day.

The risk for consumers in Australia is even lower as the average bread consumption is over 10kg per person per year less per year.

In **Section 7.5.1** the recommended use levels of the enzyme endo β(1-4) xylanase are given, based on the raw materials used in the various food processes. For the calculation of the TMDI, the maximum use levels are chosen. Furthermore, the calculation takes into account how much bread is obtained per

4 afssa, Table 1, p 32 – 115g/day
5 afssa, Table 2, p 34 – 55.8g/day

kg flour (on average 125 kg bread is produced from 100 kg flour) and it is assumed that all the TOS will end up in the final product.

Table 9: TMDI calculation

Application	Raw material (RM)	Maximal recommended use level (UI/kg RM)	Maximal recommended use level (mg TOS/kg RM)	Final food	Ratio RM/final food	Maximal level in final food (UI/kg food)	Maximal level in final food (mg TOS/kg food)
Baking	Flour	150	1.44	Bread	0.8	120	1.15

The Total TMDI can be calculated on basis of the **maximal** values found in food, multiplied by the average consumption of food /kg body weight/day. Consequently, the Total TMDI will be:

TMDI in food (mg TOS/kg body weight/day)	Total TMDI (mg TOS/kg body weight/day)
1.15*90/60*365	**0.0047**

It should be stressed that this Total TMDI is based on conservative assumptions and represents a highly exaggerated value because of the following reasons:

- It is assumed that ALL producers of the above mentioned foodstuffs use the specific enzyme endoβ(1-4) xylanase from *Bacillus Subtilis*;
- It is assumed that ALL producers apply the HIGHEST use level per application;
- For the calculation of the TMDI's in food, only highest theoretical amount of TOS were selected;
- It is assumed that the amount of TOS does not decrease as a result of the food production process;
- It is assumed that the final food containing the calculated theoretical amount of TOS is consumed DAILY over the course of a lifetime;
- Assumptions regarding food and beverage intake of the general population are overestimates of the actual average levels (Douglass et al., 1997).

7.5.2 A list of foods or food groups likely to contain the processing aid or its metabolites

Table 10: Foods Groups and Foods likely to contain the Enzyme

Food Group	Food	Standard 1.3.1
Flour	Baker's flour	6.2 Flours, meals and starches
Baked cereal goods	Breads, Biscuits, Steamed bread, Cakes, Pancakes, Tortillas, Wafers, Waffles	7 Breads and Bakery Products 7.1 Breads and related products 7.2 Biscuits, cakes and pastries
Unbaked cereal goods	Pasta, Noodles and Snack goods	6.4 Flour products (including noodles and pasta)

7.5.3 The levels of residues of the processing aid or its metabolites for each food or food group

Maximal recommended use levels (GDXU/kg RM) 150.

7.5.4 For foods or food groups not currently listed in the most recent Australian or New Zealand National Nutrition Surveys (NNSs), information on the likely level of consumption

Not applicable

7.5.5 The percentage of the food group in which the processing aid is likely to be found or the percentage of the market likely to use the processing aid

Based on the information from other markets where this enzyme is already used, we will estimate that up to 20% of bread and other bakery products market could be produced with this specific endoxylanase.

7.5.6 Information relating to the levels of residues in foods in other countries

The enzyme is exclusively used for the production of bakery products and consequently not eliminated from the final product. The added enzyme is therefore remaining in the final food, but as protein as we have shown that there is no active enzyme present after the baking process. The amount of recommended xylanase addition in all countries where the xylanase is sold is the same.

7.5.7 For foods where consumption has changed in recent years, information on likely current food consumption

Not applicable.

REFERENCES

Alexander, M. (1977). Introduction to Soil Microbiology. John Wiley and Sons, Inc., New York

Beg, Q.K, Kapoor, M, Mahajan, L, Hoondal, G.S. (2001). Microbial xylanases and their industrial applications: a review. Appl Microbiol Biotechnol 56:326-338.

Bolivar, F., Rodriguez, R.L., Greene, P.J., Betlach, M.C., Heyneker, H.L., and Boyer, H.W. (1977). Construction and Characterization of New Cloning Vehicles. Gene, 2(1997) 95-113. Elsevier/north Holland Biomedical Press, Amsterdam.

Chen, X.H., et al. (2007). Comparative analysis of the complete genome sequence of the plant growth-promoting bacterium *Bacillus amyloliquefaciens* FZB42. Nat. Biotechnol. 25:1007-1014

Claus, D. and Berkeley R.C.W. (1986). Genus Bacillus Cohn 1872, pp. 1105-1139. In: P.H.N. Sneath, *et al*. (eds.), Bergey's Manuel of Systematic Bacteriology, Vol. 2. Williams and Wilkins Co., Baltimore, MD

Collins, T., Meuwis, M.A., Stals, I., Claeyssens, M., Feller, G. & Gerday, C. (2002). A novel family 8 Xylanase: Functional and physico-chemical characterization. J. Biol. Chem. 277, 35133-35139

Collins, T., Hoyoux, A., Gerday, C., Georis, J., Dauvrin T, and Feller G. (2002).). A novel family 8 Xylanase: Fundamentals and Applications. Poster.

Coorevits, A., De Jonghe, V., Vandroemme, J., Reekmans, R., Heyman, J., Messens, W., De Vos, P., Heyndrickx, M. (2008). Comparative analysis of the diversity of aerobic spore-forming bacteria in raw milk from organic and conventional dairy farms. Systematic and Applied Microbiology, 31(2):126-140

Cutting, S. & Vander Horn P.B. 1990. Genetic Analysis in Molecular Biology Methods for Bacillus. Edited by C.R. Harwood and S.M. Craig. John Wiley and Sons Ltd. 1990

De Boer, A.S. and Diderichsen, B. (1991). On the safety of *Bacillus subtilis* and *B. amyloliquefaciens*: a review. Appl. Microbiol. Biotechnol. 36(1):1-4

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

Dubnau, D. (1999). DNA Uptake in Bacteria. Annu Rev. Microbiol. 1999. 53:217-44

Dubnau, D. (1993). 39. Genetic Exchange and Homologous Recombination. p555-584. *In* Sonenshein A, Hoch J, Losick R (ed), *Bacillus subtilis and Other Gram-Positive Bacteria*. ASM Press, Washington DC.

Earl, A.M., Losick, R. and Kolter, R. (2008). Ecology and genomics of *Bacillus subtilis*. Trends in Microbiology 16:269-275

EFSA (2007). Opinion of the Scientific Committee on a request from EFSA on the Introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA. The EFSA Journal 587, 1-16.

EFSA (2008). Technical Guidance. Update of the criteria used in the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. The EFSA Journal 2008;732;1-15

EFSA (2008). Scientific Opinion of the Panel on Dietetic Products, Nutrition and Allergies on Vitamin K2 added for nutritional purposes in foods for particular nutritional uses, food supplements and food intended for the general population and Vitamin K2 as a source of vitamin K added for nutritional purposes in foodstuffs, in the context of Regulation (EC) N°258/971. The EFSA Journal 822, 1-31.

EFSA (2009a). Opinion of the Scientific Committee on the Potential Risks Arising from Nanoscience and Nanotechnologies on Food and Feed Safety. The EFSA Journal, 958, 1-39.

EFSA (2011). Technical Guidance on the assessment of the toxigenic potential of Bacillus species used in animal nutrition. The EFSA Journal 2011; 9(11):2445.

EFSA (2011). Scientific Opinion. Guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use. EFSA Journal 2011;9(6):2193

EFSA (2012). Scientific Opinion. Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. The EFSA Journal 2012;10(6):2740

EFSA (2013). Public consultation on draft Guidance on the assessment of the toxigenic potential of Bacillus species used in animal nutrition, EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP).

EFSA (2014). Scientific Opinion. Guidance on the assessment of the toxigenic potential of *Bacillus species* used in animal nutrition. The EFSA Journal 2014;(12(5):3665.

Erikson, R.J. (1976). Industrial applications of the bacilli: A review and prospectus, pp. 406-419. In: D. Schlesinger (ed.), Microbiology. American Society for Microbiology, Washington, DC

European Commission (2000). Opinion of the Scientific Committee on Animal Nutrition on the safety use of Bacillus species in animal nutrition.

European Parliament, Regulation (EC) No 852/2004 of the European Parliament and of the Council of 29 April 2004 on the hygiene of foodstuffs (Food Hygiene Regulation (852/2004)).

FAO/WHO (2006). General Specifications and Considerations for Enzyme Preparations Used in Food Processing. Compendium of food additive specifications, sixty-seventh meeting. FAO JECFA Monographs 3, 2006 (ISBN 92-5-105559-9), 63-67.

Ferrari, E., Jamagin, A.S. and Schmidt, B.F. (1993). Commercial production of extracellular enzymes. In: Sonenshein, Hoch, Losick (eds.), *Bacillus subtilis* and other Gram-positive bacteria. American Society for Microbiology; Washington, DC, pp. 917-937

Frommer, W.B., Ager, B., Archer, L., Brunius, B., Collins, C.H., Donikian, R., Frontali, C.F., Hamp, S., Houwink, E.H., Kuenzi, M.T., Kramer, P., Lagast, H., Lund, S., Mhler, J.L., Normand-Plessier, F., Sargeant, K., Tuijnenburg Muijs, G., Vranch, S.P., Werner, R.G. (1989). Safe biotechnology III. Safety precautions for handling microorganisms of different classes. Appl. Microbiol. Biotechnol. 30:541-552

Gilbert, R.J and Kramer, J.M. (1981). *Bacillus cereus* and Other *Bacillus Species* in Foodborne Bacterial Pathogens, edited by Doye, M.P. Marcel Dekker Inc

Gleave, A.P., Mountain, A., and Thomas, C.M. (1990). Use of a novel cassette to label phenotypically a cryptic plasmid of *Bacillus subtilis* and map loci involved in it stable maintenance. Journal of General Microbiology (1990), 136, 905-912.

Hwang, Y.H., Kim, M.S., Song, I.B., Park, B.K., Lim, J.H., Park, S.C. and Yun, H.I. (2009). Subacute (28 day) toxicity of surfactin C, a lipopeptide produced by Bacillus subtilis, in rats. Journal of Health Science, 55(3):351-355

Ivanova, E.P., Romanenko, L.A., Matte, M.H., Glavur, R.M., Lysenko, A.M., Simidu, U., Kita-Tsukamoto., Sawabe, T., Vysotskii, M.V., Frolova, G.M., Mikhailov, V., Christen, R., and Colwemm, R.R. (2001). Retrieval of the species *Alteromonas tetradonis* Simidu et al 1991 as Pseudoalteromonas tetraodonis comb. Nov. and emendation of description. International Journal of Systematic and Evolutionary Microbiology (2001), 51, 1071-1078

Klein, D.A. and Paschke, M.W. (2004). Filamentous fungi: The indeterminate lifestyle and microbial ecology. Microbial Ecology 47:224-235

Kunst, F. *et al*. (1997). The complete genme sequence of the Gram-positive bacterium *Bacillus subtilis*. Nature 390:249-256

Lacks, S.A. (1988). Mechanisms of Genetic Recombination in Gram-Positive Bacteria. In Genetic Recombination edited by Kucherlapati, R., and Smith, G.R. (American Society for Microbiology,Washington, D.C), pp 43–86.

Logan, N.A. (1988). Bacillus species of medical and veterinary importance. J. Med. Microbiol. 25:157-165

Logan, N.A. and Berkeley, R.C.W. (1984). Identification of Bacillus strains using the API system. Journal of General Microbiology 130(7):1871-1882

Olempska-Beer, Z.S., Merker, R.I., Ditto, M.D., DiNivo, M.J. (2006). Food-processing enzymes from recombinant microorganisms – a review. Regul. Toxicol. Pharmacol. 2006 Jul; 45(2):144-158

Parekh, S., Vinci, V.A. and Strobel, R.J. (2000). Improvement of microbial strains and fermentation processes. Applied Microbiology and Biotechnology 54:287-301

Pariza, M.W. and Johnson, E.A. (2001). Evaluating the safety of microbial enzyme preparations used in food processing: update for a new century. Regulatory Toxicology and Pharmacology 33:173-186

Pedersen, P.B., Bjørnvad, M.E., Rasmusse, M.D., Petersen, J.N. (2002). Cytotoxic potential of industrial strains of Bacillus sp. Regulatory Toxicology and Pharmacology 36:155-161

Primrose, S.B. & Ehrlich, S.D. (1981). Isolation of Plasmid Deletion Mutants and Study of their Instability. Plasmid 6, 193-201.

Reed, T. (1966). “Enzymes in Food Processing”. Academic Press, New York, p.406

Rey, M.W., Ramaiya, P., Nelson, B.A., Brody-Karpin, S.D., Zaretsky, E.J., Tang, M., Lopez de Leon, A., Xiang , H., Gusti, V., Clausen, I.G., Olsen, P.B., Rasmussen, M.D., Andersen, J.T., Jørgensen, P.L., Larsen, T.S., Sorokin, A., Bolotin, A., Lapidus, A., Galleron, N., Ehrlich, S.D., Berka, R.M. (2004). Complete genome sequence of the industrial bacterium *Bacillus licheniformis* and comparisons with closely related Bacillus species. Genome Biol. 2004; 5(10):R77. Epub 2004 Sep 13

Sanchez, S. and Demain, A.L. (2002). Metabolic regulation of fermentation processes. Enzyme and Microbial Technology 31:895-906

Schaeffer, P., Millet, J. & Aubert, J.P. (1965). Catabolic Repression of Bacterial Sporulation. Microbiology: Vol 54.

Trieu-Cuot, P., and Courvalin, P. (1983). Nucleotide sequence of the *Streptococcus faecalis* plasmid gene encoding the 3’5”-aminoglycoside phosphotransferase type III. Gene, 23(1983) 331-341.

US EPA 1997, Federal Register, Volume 77, Issue 7 (Wednesday, January 11, 2012)

Veith, B., Herzberg, C., Steckel, S., Feesche, J., Maurer, K.H., Ehrenreich, P., Baumer, S., Henne, A., Liesegang, H., Merkl, R., Ehrenreich, A., Gottschalk, G. (2004). The complete genome sequence of *Bacillus licheniformis* DSM13, an organism with great industrial potential. J. Mol. Microbiol. Biotechnol. 2004; 7(4):204-211