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APPLICATION

APRIL 2016

TO:
FOOD STANDARDS AUSTRALIA NEW ZEALAND (FSANZ)

IN RELATION TO:
APPLICATION FOR APPROVAL OF PROTEASE AQUALYSIN 1 FROM A GENETICALLY MODIFIED STRAIN OF BACILLUS SUBTILIS AS A PROCESSING AID

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CONFIDENTIAL COMMERCIAL INFORMATION (CCI) INFORMATION

Additional material has been provided under CCI to support the Application for Sections 6.1.3; 6.1.4 and 6.4.1. This material has been provided separately to this Application document.

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ADMINISTATIVE INFORMATION

(As per section 3.1.1 B of the Application Handbook as at 1 March 2016)

Applicant: [redacted]

Organization: Puratos NV (Puratos)

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

Telephone: [redacted]

Email address: [redacted]

Primary contact: [redacted]

Nature of Business

(As per section 3.1.1 B of the Application Handbook as at 1 March 2016)

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 3.1.1 B of the Application Handbook as at 1 March 2016)

1. The following Scientific and Regulatory Consultants have been involved in the preparation, submission and stewardship of this application:
   - Dr Simon Brooke-Taylor, Brooke-Taylor & Co Pty Ltd
   - Fiona Fleming, 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

Status of Similar Applications

(As per Section 3.1.1 D of the Application Handbook as at 1 March 2016)

There are no similar current applications for approval of Aqualysin 1 as a processing aid.

Assessment Procedure

(As per section 3.1.1 F of the Application Handbook as at 1 March 2016)

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 3.1.1 G of the Application Handbook as at 1 March 2016)

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 3.1.1 I of the Application Handbook as at 1 March 2016)

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

2 PURPOSE OF THE APPLICATION

(As per section 3.1.1 D of the Application Handbook as at 1 March 2016)

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 Aqualysin 1 protease (EC 3.4.21.111) (hereafter Aqualysin 1) from *Bacillus subtilis* containing a protease gene from *Thermus aquaticus* in S18-4 Permitted Enzymes.

The food enzyme Aqualysin 1 is used as a processing aid in the manufacture of bakery products such as, but not limited to, bread, biscuits, steamed bread, cakes, pancakes, tortillas, wafers and waffles. Proteins provide functional properties during manufacture due to their ability to influence the dough's elasticity, plasticity and extensibility. Limited hydrolysis of the peptide bonds in gluten proteins with the help of proteases results in improved process ability which enhances the functional baking properties of these compounds.

3 JUSTIFICATION FOR THE APPLICATION

(As per section 3.1.1 D of the Application Handbook as at 1 March 2016)

3.1 NEED FOR THE PROPOSED CHANGE

(As per section 3.1.1 D of the Application Handbook as at 1 March 2016)

Schedule 18 - Processing Aids contains a list of permitted enzymes of microbial origin (S18-4 Permitted Enzymes). There is already approval for endo-protease (EC 3.4.21.26) from *Aspergillus niger.*

The source microorganism for the applicant's Aqualysin 1 is *Thermus aquaticus* 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 Aqualysin 1 enzyme and the genetically modified *Bacillus subtillis* as a host organism.

3.1.1 Purpose of using the processing aid

Proteases are interesting for their ability to digest/hydrolyse proteins (glutenin, gliadin) present in flour. Proteases hydrolyse large polypeptides into smaller peptides and amino acids decreasing the molecular weight of proteins. Endoproteinases are able to modify wheat gluten characteristics by proteolysis and, under specific controlled conditions, will not completely destroy the technical properties of gluten.

Proteases have a long history of use in the bakery sector. They are mostly used by bakers for reducing mechanical dough development requirements of unusually strong or tough gluten. They lower the viscosity and increase the extensibility of the dough.

The function of adding this specific protease is the prevention or retardation of staling during the baking process. Bread staling is a complex phenomenon. It is perceived as a softening of the crust, a hardening of the crumb and the disappearance of fresh bread flavour.

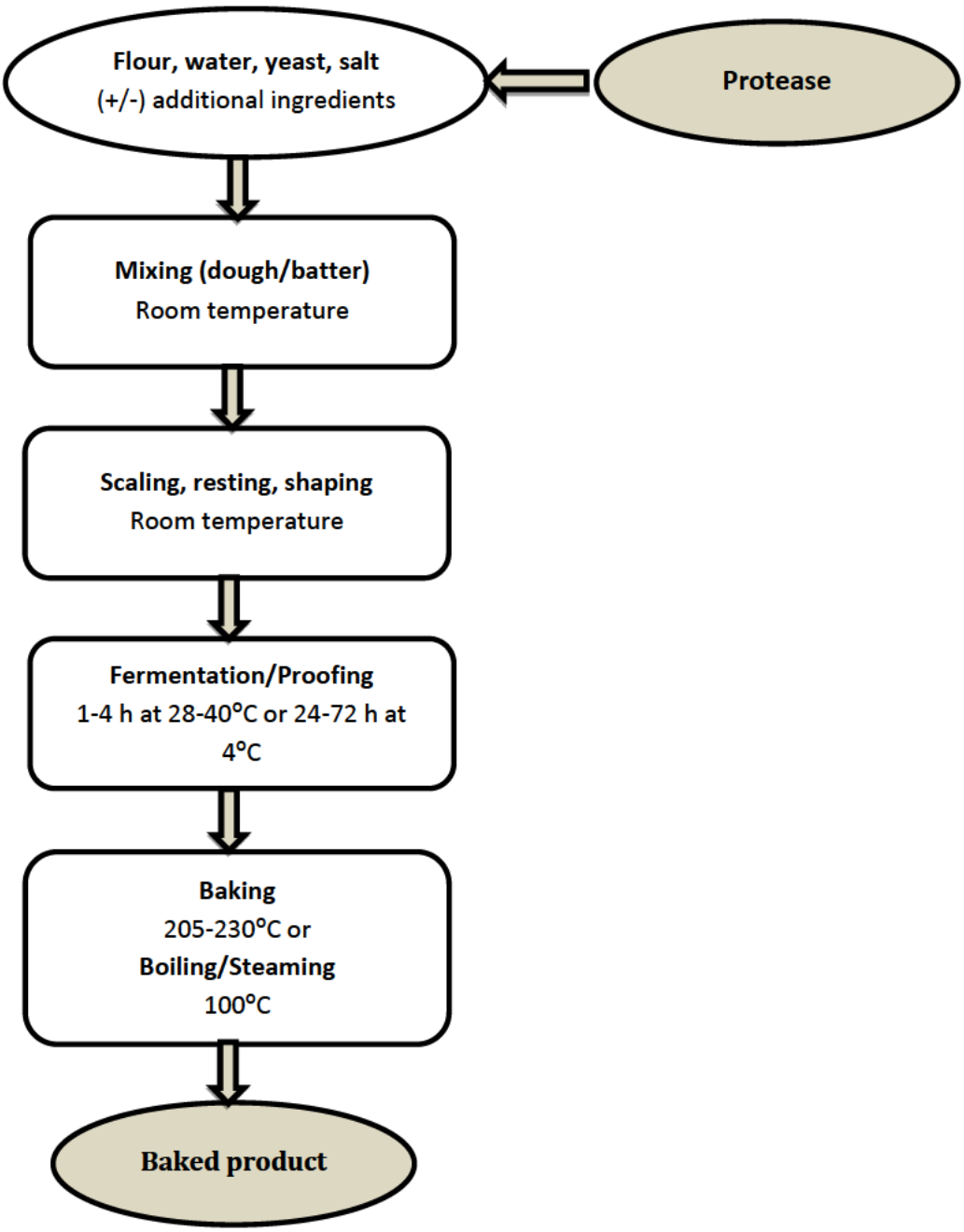
The protease preparation will be used as processing aid for the production of bakery products, fine bakery wares and similar products like bread, soft rolls, bagels, donuts, Danish pastry, hamburger rolls, pizza and pita bread, cake and other baked products where staling and inhibition thereof is a quality issue.

Proteases most used in baking are neutral proteases from fruits and from *Aspergillus oryzae* or *Bacillus subtilis*. These neutral proteases are far more active on gluten than the alkaline proteases, thus more difficult to use.

A thermophilic alkaline protease will be less active than other endoproteases already used during fermentation and kneading. The evolution of the technical properties of gluten, mostly strength and elasticity, will be better controlled during the process.

The food production process flow given below (Figure 1) illustrates the typical application of the food enzyme and shows the conditions under which the food enzyme may be used.

Figure 1: The Baking Process



3.2 ADVANTAGES OF THE PROPOSED CHANGE

(As per section 3.1.1 D of the Application Handbook as at 1 March 2016)

Proteases have a long history of use in the bakery sector. The benefits of the use of Aqualysin 1 in baking processes may include:

- Faster dough development upon mixing;
- Better dough machinability;
- Reduced dough rigidness which results in processing tolerance;
- Improved dough's structure and extensibility during the shaping or moulding step;
- Uniform shape of the bakery product, which might otherwise be impaired by processing of the dough;
- Regular batter viscosity, beneficial in the production process for e.g. waffles, pancakes and biscuits; and
- Improved short-bite of certain products like hamburger breads.

3.3 DISADVANTAGES OF THE PROPOSED CHANGE

(As per section 3.1.1 D of the Application Handbook as at 1 March 2016)

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

3.4 PUBLIC HEALTH AND SAFETY ISSUES

(As per section 3.1.1 D of the Application Handbook as at 1 March 2016)

The Applicant has not identified any public health and safety issues in relation to the approval of Aqualysin 1 for use in the Australia/New Zealand food supply.

Refer **Section 6** for information about the safety of the processing aid.

3.5 CONSUMER CHOICE

(As per section 3.1.1 D of the Application Handbook as at 1 March 2016)

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

Aqualysin 1 does not perform any technological function in the final foods containing ingredients prepared with this enzyme. Moreover, the food products prepared with this enzyme do not have characteristics or nutritional value other than what is expected by the consumer.

3.6 SUPPORT FOR THE PROPOSED CHANGE

(As per section 3.1.1 D of the Application Handbook as at 1 March 2016)

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.

3.7 REGULATORY IMPACT INFORMATION

(As per section 3.1.1 D.1 of the Application Handbook as at 1 March 2016)

3.7.1 Costs and Benefits of the application

(As per section 3.1.1 D.1.1 of the Application Handbook as at 1 March 2016)

Costs and Benefits – Consumer

The potential benefit to consumers include:

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

The proposed amendment places no additional economic cost on consumers.

Costs and Benefits - Industry and Business

The benefits for industry that may be obtained in the final foods when produced with the help of Aqualysin I protease may include:

- Faster dough development upon mixing
- Better dough machinability
- Reduced dough rigidness which results in processing tolerance
- Improved dough's structure and extensibility during the shaping or moulding step
- Uniform shape of the bakery product, which might otherwise be impaired by processing of the dough
- Regular batter viscosity, beneficial in the production process for e.g. waffles, pancakes and biscuits
- Improved short-bite of certain products like hamburger breads.

Use of Aqualysin 1 will be at the discretion of business, therefore there are no direct costs imposed on industry.

Costs and Benefits – Government

The proposed amendment places no additional regulatory costs on government beyond the initial regulatory cost of approving Aqualysin 1 as a processing aid.

3.7.2 Impact on International Trade

(As per section 3.1.1 D.1.2 of the Application Handbook as at 1 March 2016)

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 Aqualysin 1 is approved for use (outlined under Section 5).

4 INFORMATION TO SUPPORT THE APPLICATION

(As per section 3.1.1 E of the Application Handbook as at 1 March 2016)

4.1 Data Requirements

Refer to **Section 6** for information about the processing aid.

4.2 FSANZ Act Objectives

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

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

- 6.2 - Information on the Safety of an Enzyme Processing Aid;
- 6.3 - Additional Information Related to the Safety of an Enzyme Processing Aid Derived from a Microorganism;
- 6.4 - Additional Information Related to the Safety of an Enzyme Derived from a Genetically-Modified microorganism; and
- 6.5 – Information related to the Dietary Exposure to the processing aid.

(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.

Proteases have been used for the described technological function in food processes for many years and has been specifically approved in many countries including Australia and New Zealand.

There are no reasons to assume that the use of this food enzyme could lead to a food which would be misleading in terms of nature, freshness, the quality of the ingredients used or the nutritional quality of the final food. The application of food enzymes does not lead to the potential use of raw materials of inferior, unsafe quality. Instead, food enzymes are used to compensate natural variations of the agricultural raw material; liberate the full potential of the raw material and to support production processes which are more environmental friendly. Enzymatic processes in general occur in nature and they therefore do not influence the "naturalness" of the production process or the final food. Considering the above, there are no reasons to believe that the use of Aqualysin I protease in bakery products could be misleading for the consumer.

4.3 POLICY GUIDELINES

(As per section 3.3.2 of the Application Handbook as at 1 March 2016)

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 to achieve a solely technological function should be permitted where:

Specific Order Policy Principles – Technological Function	Section of Application
a) the purpose for adding the substance can be articulated clearly by the manufacturer (i.e. the 'stated purpose'); and	2 & 3.1
b) the addition of the substance to food is safe for human consumption; and	6
c) the amounts added are consistent with achieving the technological function; and	6
d) the substance is added in a quantity and a form which is consistent with delivering the stated purpose; and	6
e) no nutrition, health or related claims are to be made in regard to the substance.	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 04.03.2016.

5 INTERNATIONAL AND OTHER NATIONAL STANDARDS

(As per section 3.1.1 J of the Application Handbook as at 1 March 2016)

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

5.1 INTERNATIONAL STANDARDS

(As per section 3.1.1 J.1 of the Application Handbook as at 1 March 2016)

Aqualysin 1 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**).

5.2 OTHER NATIONAL STANDARDS OR REGULATIONS

(As per section 3.1.1 J.2 of the Application Handbook as at 1 March 2016)

5.2.1 Existing Authorisations and Evaluations

The food enzyme Aqualysin 1 has been evaluated and/or authorized in Canada, France and the USA as set out in Table 1.

Table 1: Non-exhaustive list of authorisations & evaluations of Aqualysin I Protease produced by *Bacillus subtilis*

Authority	Description	Reference
Canada	Protease Bacillus subtilis var.	Appendix 2 (B.16.100, Table V, P.6)
France	Serin-protease from Bacillus subtilis	Appendix 3
USA	GRAS self-affirmation	Appendix 4

5.2.2 Other Authorisations and Evaluations

Similar food enzymes have not as yet been evaluated by EFSA. However, within the EU and internationally, food enzymes similar to the one described in this application have already been evaluated.

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 summarizes not only authorized food enzymes with the same enzyme activity, but also authorized food enzymes from the same producing organism. As documented below in Tables 2 and 3, protease from various micro-organisms (including genetically modified ones) is 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 protease) produced by the same production organism, *Bacillus subtilis*

Authority	Food enzyme	Reference
JECFA	α-acetolactate α-amylase branching maltogenic xylanase	Appendix 5
Australia / New Zealand	α-acetolactate α-amylase β-amylase β-glucanase hemicellulase maltogenic α- pullulanase	ANZ Food Standards Code Schedule 18 – Permitted Enzymes – 18-4 (Copy not provided with Application)
Canada	α-acetolactate Amylase amylase Glucanase Pullulanase xylanase	Appendix 2
France	α-acetolactate α-amylase β-glucanase exo-α-amylase branching Pullulanase xylanase	Appendix 3
USA	asparaginase	Appendix 4 - GRN476
	1,4-α-glucan branching enzyme	Appendix 4 - GRN406
	branching glycosyltransfer	Appendix 4 - GRN274
	pectate lyase	Appendix 4 - GRN114

Table 2: Non-exhaustive list of authorized food enzymes (other than protease) produced by the same production organism, *Bacillus subtilis*		
Authority	**Food enzyme**	**Reference**
	Pullulanase	Appendix 4 - GRN205 and GRN20
	bacterially-derived carbohydrases	Appendix 4 - §184.1148

Table 3: Non-exhaustive list of authorized protease from production organisms including *Bacillus subtilis*		
Authority	**Production organism**	**Reference**
JECFA	*Bacillus cereus*	Appendix 5
	Aspergillus Niger	
	Eschetichia Coli K12	
	Kluyveromyces lactis	
	Bacillus subtilis	
	Aspergillus oryzae	
	Streptomyces fradiae	
	Bacillus Licheniformis	
	Fusarium Venenatum	
Australia / New Zealand	*Aspergillus niger*	ANZ Food Standards Code - Schedule 18 – Permitted Enzymes – 18-4
Canada	*Aspergillus oryzae*	Appendix 2
	Aspergillus niger	
	Bacillus subtilis	
	Micrococcus caseolyticus	
	Bacillus licheniformis	
France	*Fusarium venenatum*	Appendix 3
	Aspergillus oryzae	
	Aspergillus niger	
	Bacillus amyloliquefaciens	
	Bacillus licheniformis	
	Geobacillus caldoproteolyticus Rokko	
	Rhizomucor miehei	
	Micrococcus caseolyticus	
	Bacillus subtilis	
	Aspergillus wentii	
	Endothia parasitica	
	Mucor pusillus	
USA	*Trichoderma reesei*	Appendix 4 - GRN 333

Table 3: Non-exhaustive list of authorized protease from production organisms including *Bacillus subtilis*		
	Aspergillus oryzae	Appendix 4 - GRN 90
	Aspergillus niger	Appendix 4 - GRN 89

6 SUBSTANCES ADDED TO FOOD - PROCESSING AIDS

(As per section 3.3.2 of the Application Handbook as at 1 March 2016)

6.1 TECHNICAL INFORMATION ON THE PROCESSING AID

(As per section 3.3.2 A of the Application Handbook as at 1 March 2016)

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

6.1.1 Information on the type of processing aid

(As per section 3.3.2 A.1 of the Application Handbook as at 1 March 2016)

Endopeptidases are characterized by their preferential action on the peptide bonds in the inner regions of the polypeptide chain away from the N and C termini. The endopeptidases are divided into four subgroups based on their catalytic mechanism: (1) serine proteases, (2) aspartic proteases, (3) cysteine proteases and (4) metalloproteases.

Serine proteases are characterized by the presence of a serine group in their active site. Serine proteases are found in the exopeptidase, endopeptidase, oligopeptidase and omega peptidase groups.

Serine proteases have broad substrate specificities including esterolytic and amidase activity. Serine proteases are recognized by their irreversible inhibition by 3,4-dichloroisocoumarin (3,4-DCI), L-3-carboxytrans 2,3-epoxypropyl-leucylamido (4-guanidine) butane, diisopropylfluorophosphate (DFP), phenylmetylsulfonyl fluoride (PMSF) and tosyl-L-lysine chloromethyl ketone (TLCK).

Serine proteases are generally active at neutral and alkaline pH, with an optimum between pH 7 and 11. Their molecular weight ranges between 18 and 35 kDa. The isoelectric points of serine proteases are generally between pH 4 and 6. The optimal pH of alkaline proteases is around pH 10. Their isoelectric point is around pH 9 (Rao et al, 1998).

Serine alkaline proteases hydrolyze a peptide bond which has tyrosine, phenylalanine or leucine at the carboxyl side of the splitting bond. Serine proteases usually follow a two-step reaction for hydrolysis in which a covalently linked enzyme-peptide intermediate is formed with the loss of the amino acid or peptide fragment. This acylation step is followed by a deacylation process which occurs by a nucleophilic attack on the intermediate by water, resulting in hydrolysis of the peptide. (Rao et al, 1998).

Aqualysin I exhibits low specificity towards esters of amino acids with small hydrophobic or aromatic residues at the P1 position. This enzyme from the extreme thermophile, *Thermus aquaticus*, is an alkaline serine peptidase. It has three subsites, S1, S2 and S3, in the substrate binding site and a tripeptide length is enough for substrate binding and hydrolysis. The preferred amino acids at the S1 site are Ala and Phe, at the S2 site are Ala and Norleucine and at the S3 site are Phe and Ile. These specificities are similar to those of EC 3.4.21.64 (peptidase K) and EC 3.4.21.62 (subtilisin BPN'). The enzyme displays broad specificity for cleavage of insulin B-chain and hydrolyses elastin substrates such as succinyl-(Ala)n-p-nitroanilide and some peptide esters (Tanaka et al, 1998a, 1998b).

Aqualysin I is maximally active against various substrates at 70 – 80 °C and in the pH range around 10. Aqualysin I has four cysteine residues, forming two disulphide bonds, and, furthermore, in its C-terminal extended sequence there are two cysteine residues. The putative disulphide bonds seem to be the reason for the heat stability of the enzyme (Kwon and Matsuzawa, 1988).

Aqualysin I possesses at least two Ca2+ binding sites, one for tightly bound Ca2+ (stronger binding site) and the other for weakly bound Ca2+ (weaker binding site), and the weakly bound Ca2+ plays an absolutely essential role in the thermostability of the enzyme (Lin et al, 1999).

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

The benefits of the use of added protease in typical food (ingredient) processes are described in this application. The beneficial effects mentioned in the application are of value to the food chain because they lead to more consistent product quality. Moreover, the applications lead to improved effective production processes, resulting in better production economy and environmental benefits such as reduced losses of raw materials and therefore the production of less waste. A similar application has been specifically approved for a number of years in Denmark and France, which - together with the extensive use for decades in a number of EU countries - demonstrates the product performs the intended function in food processes.

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. A food producer who would add much higher doses than the needed ones would experience excessive costs as well as negative technological consequences, as described in Section 6.5.1.

The Applicant recommends a range from 2300-12000mU/kg of raw material, depending on the applications and processes. It is the responsibility of the user to define the level of use (functional vs financial).

6.1.2 Information on the identity of the processing aid

(As per section 3.3.2 A.2 of the Application Handbook as at 1 March 2016)

Enzyme

Name of the enzyme protein: aqualysin 1

Synonyms: caldolysin

Abbreviations: None

EC (IUBMB) number: EC 3.4.21.111

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

EC 3	Hydrolases
EC 3.4	Acting on peptide bonds (peptidases)
EC 3.4.21	Serine endopeptidases
EC 3.4.21.111	Aqualysin 1

CAS No: Not applicable

Enzyme Preparation

Commercial Name:

This enzyme is already sold under different commercial names:

- Premix X-220

A Technical Data Sheet for Premix X-220 is provided as **Appendix 6.**

Host Organism

Name: *Bacillus subtilis* Raα3114.

The organism is deposited under the number LMGS-25520 at the node of the Belgian Coordinated 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: *Thermus aquaticus*

The donor organism is the LMG8924 strain of *Thermus aquaticus*. The cloning and the characterization of the gene coding for the protease of this strain has been described by Kwon et al (1988).

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

(As per section 3.3.2 A.3 of the Application Handbook as at 1 March 2016)

Commercial enzymes, whether used in the production of food, feed or for technological purposes, are biological isolates of variable composition. Food enzymes are concentrates containing a specific enzyme protein, whose activity (also called main or principal activity) can be used for a specific, intended technological purpose in food processing. Apart from the enzyme protein in question, microbial food enzymes also contain some substances derived from the producing micro-organism and the fermentation medium. These constituents consist of organic material (proteins, peptides, amino acids, carbohydrates, lipids, secondary enzymes) and inorganic salts. As has been established by JECFA (FAO/WHO, 2006), the percentages of these organic materials are summarized and expressed as Total Organic Solids (TOS). The TOS value is an internationally accepted method to describe the chemical composition of commercial food enzymes. The ratio between the enzyme activity and TOS is an indication of the relative purity of the enzyme.

Protein content and relative purity of the food enzyme Aqualysin 1 from *Bacillus subtilis* was measured, and the TOS values were calculated, in 3 batches. The result is shown in the following Table.

Table 4: Composition and Specification of the Commercial food enzyme

Batch no	RA 1401	RA 1402	RA 1403	Mean
Ash (%)	0.76	0.75	0.86	0.79
Water (%)	94.62	94.29	93.72	94.21
TOS (%)	4.62	4.95	5.42	4.99
Activity (mU/ml)	2825	4115	2493	3144
mUnits/mg TOS	61	83	46	63.33
Protein (g/l)	14.26	27.08	28.88	23.4

Certificates of Analysis (COA) are provided in **Appendix 7.**

The methods by which the ash and dry matter content (to calculate the TOS) and protein values are measured are standardized and/or validated methods and given in **Appendix 7.**

The method, by which the enzyme activity is measured, including an explanation of the Units, is given in **Appendix CCI A1.**

Regarding the potential for specific chemicals which are used in the fermentation and downstream processing might end up in the food enzyme please refer to Section 6.1.4 - manufacturing process.

Information on the specific properties of the enzyme protein, such as Molecular Mass, amino acid sequence, post translational modification are provided in Section 6.1.3.1.

It is proposed that the food enzyme Aqualysin 1 should comply with the internationally accepted JECFA specifications for chemical and microbiological purity of food enzymes (FAO/WHO, 2006) and with the French purity criteria of enzymes (AR 19/10/2006):

Table 5: Specifications for chemical and microbiological purity of the food enzyme

Contaminant	Norm
Lead	Less than 5 mg/kg
Cadmium	Less than 0.5 mg/kg
Mercury	Less than 0.5 mg/kg
Arsenic	Less than 3 mg/kg
Aerobic microorganisms (30°C)	Less than 50,000 cfu per gram
Total coliforms	Not more than 30 cfu per gram
Escherichia coli	Absent in 25 grams
Salmonella sp.	Absent in 25 grams
Staphylococcus aureus	Absent in 1 gram
Clostridia sulphite reducing	Less than 30 cfu per gram
Antimicrobial activity	Not detected
Mycotoxins	Not applicable for bacterial enzymes

The proof that the food enzyme Aqualysin 1 complies with these specifications is shown by the analyses on various different batches provided in **Appendix 8.**

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

The Aqualysin 1 protein from *Bacillus subtilis* has been determined by Puratos NV from the DNA of the cloned gene and consists of a monomer with 528 amino acids with the signal peptide or 500 amino acids without the signal peptide.

AVLGGCQMASRSDPTPTLAEAFWPKEAPVYGLDDPEAIPGRYIVVFKKGKGQSLLQGGITTLQARL
APQGVVVTQAYTGALQGFAAEMAPQALEAFRQSPDVEFIEADKVVRAWATQSPAPWGLDRIDQR
DLPLSNSYTYTATGRGVNVYVIDTGIRTTHREFGGRARVGYDALGGNGQDCNGHGTHVAGTIGGV
TYGVAKAVNLYAVRVLDCNGSGSTSGVIAGVDWVTRNHRRPAVANMSLGGGVSTALDNAVKNSIA
AGVVYAVAAGNDNANACNYSPARVAEALTVGATTSSDARASFSNYGSCVDLFAPGASIPSAWYTS
DTATQTLNGTSMATPHVAGVAALYLEQNPSATPASVASAILNGATTGRLSGIGSGSPNRLLYSLLSS
GSGSTAPCTSCSYYTGSLSGPGDYNFQPNGTYYYSPAGTHRAWLRGPAGTDFDLYLWRWDGSR
WVTVASSTGPTSEESLSYSGTAGYYLWRIYAYSGSGMYEFWLQRP

A signal peptide sequence is used for the secretion of the enzyme. Its Molecular Mass is 28 Da.

Further detail is provided under **Confidential Commercial Information (6.1.3)**

6.1.3.2 Information on post translational modification of the enzyme protein

The N-terminal signal peptide, is removed *in vivo* during translocation through the bacterial outer membrane by a single peptidase. The N-terminal pro-sequence of aqualysin I is required for the stability of the aqualysin I precursor and for proper folding of the protease domain. The C-terminal pro-sequence of aqualysin I is required for the secretion of the enzyme in the form of the 38 kDa precursor. The C-terminal pro-sequence stabilizes the partially folded aqualysin I precursor against folding to the final conformation and keeps the precursor structure in a translocation-competent conformation, thereby facilitating the translocation of the precursor across membranes and supports protein secretion. Processing of the N-terminal and C-terminal pro-sequences is done by the proteolytic activity of aqualysin I itself at 65°C. (Kurosaka et al, 1996).

When the aqualysin I gene is expressed aqualysin I is not secreted into the extracellular medium but found in the outer membrane as a precursor with the C-terminal and the pro-domain (38 kDa protein). The precursor is autocatalytically processed to mature aqualysin I (28 kDa protein) by treatment at 65°C. The N-terminal pro-domain of aqualysin I precursor is essential for the correct folding of the active aqualysin I. The C-terminal pro-domain of aqualysin I precursor probably guides the precursor through the outer membrane, since the precursor is bound to the outer membrane (Kim et al, 2002). The C-terminal pro-domain also plays an important role in the extracellular secretion of the precursor in *Thermus thermophilus* (Kim et al, 1997).

6.1.3.3 Information and rationale on protein engineering of the enzyme protein

Not applicable. Protein engineering has not taken place.

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

The purified Aqualysin I 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.

This producing strain of *Bacillus subtilis* is known for its capacity of producing xylanase and alpha-amylase activities.

As these are relevant in bakery processes, the activities have been measured.

Table 6: Xylanase and alpha- Amylase Activities

Type	Activity			Method
	RA-1401	RA-1402	RA-1403	
Xylanase (BDXU/ml)	1*	0	0	Azo-xylan (Megazyme) using bacterial xylanase standard curve **Appendix CCI A1**
Alpha-amylase (skb/ml)	0	0	0	Synchron CX4 **Appendix CCI A1**

* Values under 10 are not significant.

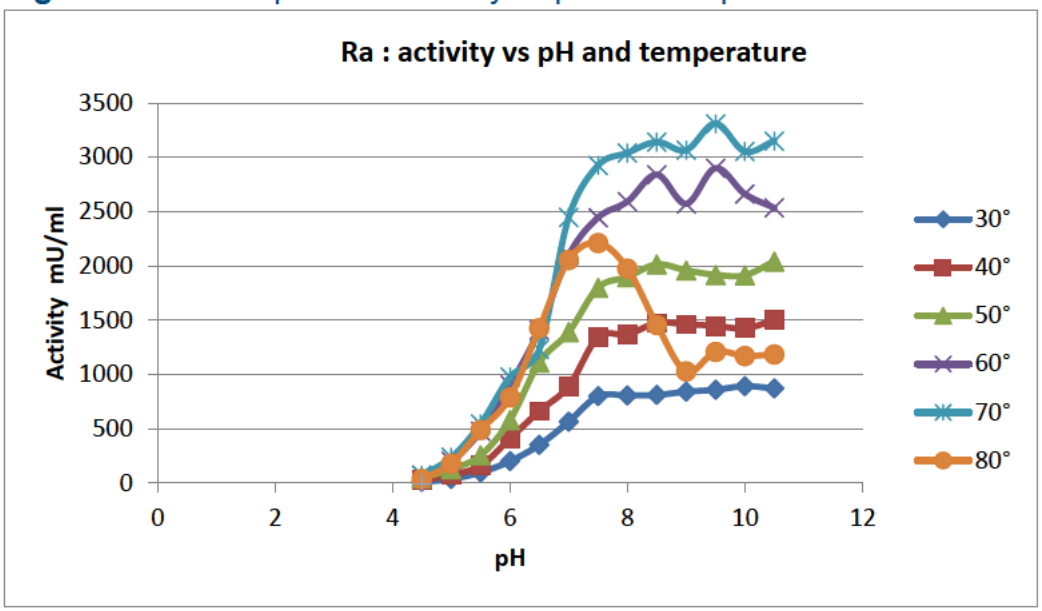
A Certificate of Analysis (CoA) is provided in **Appendix 9.**

Performances assays run with various enzymes batches in bread making never suggest that, when measurable, the residual enzymatic side activities have a technical impact in the application.

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

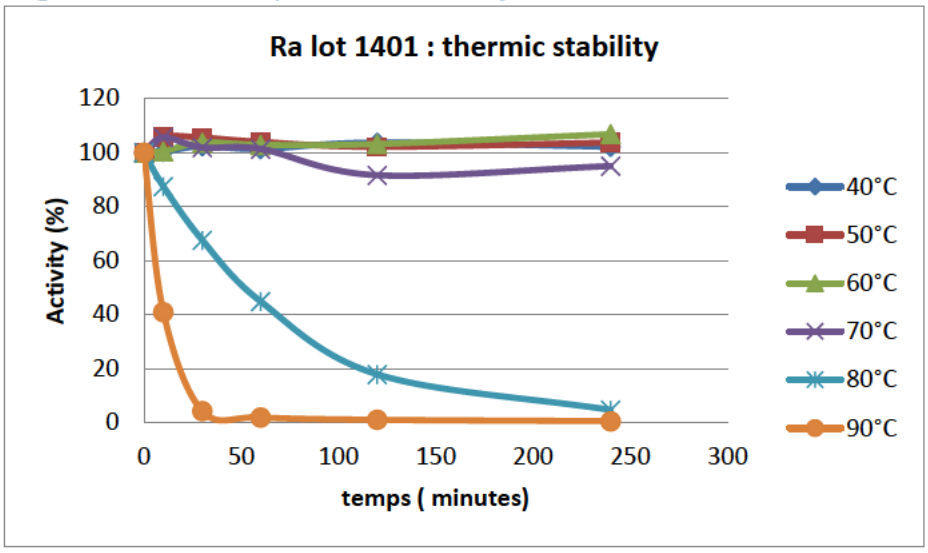
The activity of the food enzyme Aqualysin I from *Bacillus subtilis* was measured under various pH and temperature conditions using the analytical method provided in **Appendix CCI A1.** The results are presented in Figures 2 & 3 below.

Figure 2: Relative protease activity vs pH and temperature



It can be concluded from the given figures above, the food enzyme Aqualysin I from *Bacillus subtilis* exhibits activity from pH 7.0, and from 30°C to 80°C. The optimum pH is at 9.5, whereas the optimum temperature is around 70°C.

Figure 3: Relative protease stability



The Aqualysin I has a high thermostability until 90°c. At this temperature, the enzyme is rapidly denaturated.

6.1.3.6 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.

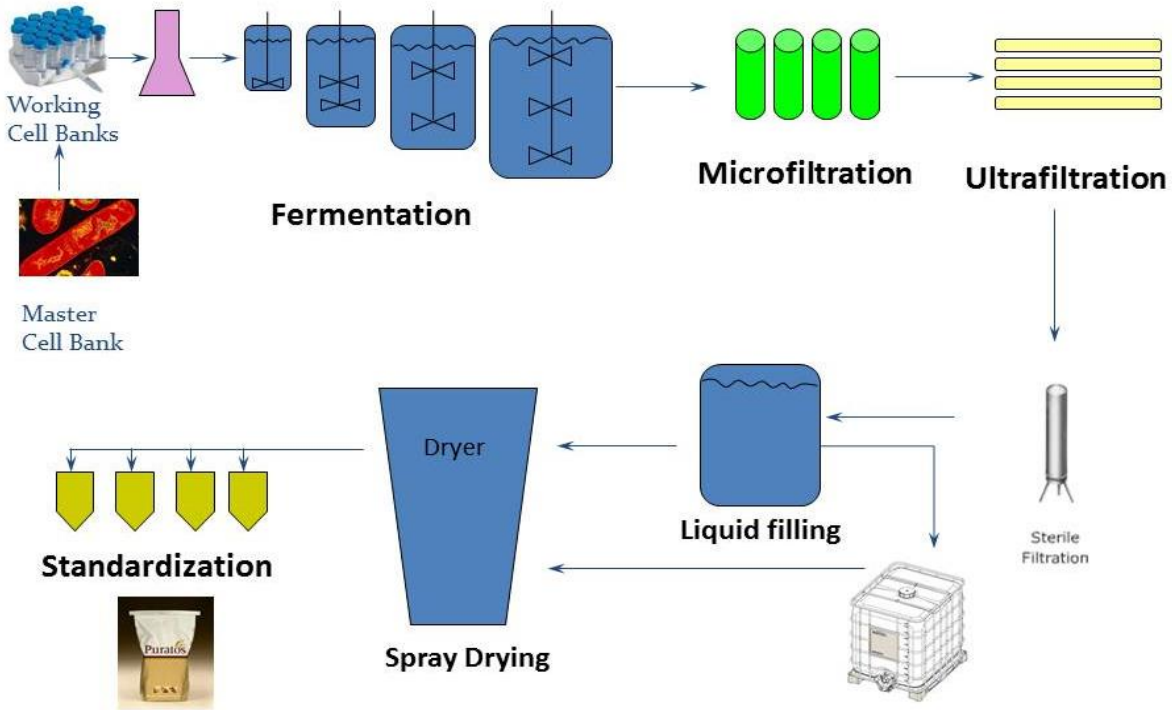
Tests of stability were performed on products at end of shelf-life and the results are provided in **Appendix 10**.

6.1.4 Manufacturing process

(As per section 3.3.2 A.4 of the Application Handbook as at 1 March 2016)

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



6.1.4.1 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 m^3. 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 fed-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 this food enzyme falls 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 protease 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. Control experiments have shown that no viable cells survived this treatment.

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 (food grade wheat maltodextrin). The enzyme preparation is thus granulated, reducing its dustiness.

The carrier of the commercial enzyme preparation is food grade wheat maltodextrin. 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 maltodextrin.

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).

Further detail is provided under **Confidential Commercial Information (6.1.4)**

6.1.4.2 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 -Food Hygiene Regulation.

A HACCP plan is applied to the production of Aqualysin 1 to manage all potential risk that may come from fermentation**.**

The HACCP plan is provided with the **CCI material (6.1.4 Manufacturing Process).**

A summary of the HACCP plan is provided as follows.

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 400-500 clones on milk LB with xylene agar to check the capacity of the clones to produce protease (100% is required to validate the WCB); and
- 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
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; and
- Dissolved oxygen content.

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.

6.1.5 Specification for identity and purity

(As per section 3.3.2 A.5 of the Application Handbook as at 1 March 2016)

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 3 – 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 complies with these specifications is shown by the analyses on various different batches **(Appendix 8).**

6.1.6 Analytical method for detection

(As per section 3.3.2 A.6 of the Application Handbook as at 1 March 2016)

The Aqualysin 1 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.

6.2 Information on the Safety of an enzyme processing aid

(As per section 3.3.2 C of the Application Handbook as at 1 March 2016)

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

(As per section 3.3.2 C.1 of the Application Handbook as at 1 March 2016)

Refer to overseas approvals in **Sections 5.1** and **5.2**

6.2.2 Information on the potential toxicity of the enzyme processing aid

(As per section 3.3.2 C.2 of the Application Handbook as at 1 March 2016)

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

Refer to overseas approvals in **Sections 5.1** and **5.2**

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

The Applicant considers that approval and use of this enzyme in other countries as outlined in Sections 5.1 and 5.2 demonstrates a history of safe human consumption.

6.2.2.3 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 (OECD, 1997a) of Commission Directive 2000/32/EC and the USA, EPA (TSCA) OPPTS harmonized guidelines.

The study report and results are presented in **Appendix 11.**

The assay uses tester strains TA97a, TA98, TA100, TA 102 and TA1535 selected to detect various types of mutagens. The test was performed with and without metabolic activation using an S9 activation system.

The sample was dissolved and diluted in sterile water. The sample was tested at the following concentration: 5, 1.6, 0.5, 0.16 and 0.05 mg/plate. An aliquot of the sterile water was used as negative control.

The test concentrations did not produce a two-fold increase of revertants or produce a clear dose related response in any of the tester strains.

The spot tests showed no zone of increased reversion or of toxicity.

The sample concentrations tested against the five strains did not meet the criteria for a potential mutagen.

The tested material was considered to be non-mutagenic.

Chromosomal aberration test

An *in vitro* chromosome aberration test in CHO cells was performed under GLP according to the OECD 473 guideline (**Appendix 12**). In this test, a 4 and 18-hour treatment period was evaluated for chromosomal aberrations in the absence of a metabolic activation system. In the presence of a metabolic activation system two 4-hour treatment periods were evaluated for chromosomal aberrations.

In the presence of a metabolic activation system, a relative mitotic index of up to 51 and 45% was reported for the main and repeat test, respectively. In the absence of a metabolic activation system, the main test with a 4-hour pulse treatment showed no reduced relative mitotic index. However, in the repeat test, covering an 18-hour continuous treatment, a relative mitotic index of 50% was reported. Although in the 4-hour pulse treatment no profound cytotoxicity is reported, the 18-hour continuous treatment showed an adequate cytotoxicity without an induction of chromosome aberrations. Based on the results of this test, it was concluded that the test substance did not induce clastogenicity.

In the presence of a metabolic activation system, an induction of endoreduplicated cells was reported in the treatment groups compared to the concurrent negative control in both the main and repeat test (see Table 7). In the treatment groups in the absence of a metabolic activation system no increase of endoreduplicated cells could be found.

Table 7: Results of endoreduplicated cell scoring in the presence of metabolic activation system (S9) in the chromosome aberration test

Concentration	Percentage of endoreduplicated cells (%)	
	Main test	Repeat test
(negative control -S9) 0 µg/ml	0	0
(negative control +S9) 0 µg/ml	3.5	2
500 µg/ml	8	.
1000 µg/ml	12	12
1300 µg/ml	-	8
1500 µg/ml	12.5	.
2000 µg/ml	-	4.5

Looking at these data it is observed that in the negative control group in the absence of a metabolic activation system, no endoreduplication was observed in contrast to the negative control in the presence of a metabolic activation system. The significant differences in endoreduplicated cells present in both negative control groups may point at a relation between the presence of a significant amount of endoreduplicated cells and the metabolic activation system used. The significant induction of endoreduplication observed in combination with the test substance is nevertheless remarkable, although a dose response relation is not very evident. Taken into account that the molecular weight of the test substance is 18-35 kDa, passive uptake by the cells is assumed to be negligible (Kamei, 2009). Furthermore, the protease activity of the test substance combined with the presence of a metabolic activation system may also be a confounding factor.

It is demonstrated by the results of the mutagenicity tests that Aqualysin 1 protease does not induce clastogenic effects. A possible interaction with topoisomerase II**,** the main mechanism via which endoreduplication is regulated, can however not be excluded.

Nevertheless, it should be noted that:

- endoreduplication was already increased due to the presence of the metabolic activation system used;
- enzymes are not assumed to directly interact with DNA, whereas endoreduplication might be related with an interaction with topoisomerase II activity;
- indirect mechanisms inducing endoreduplication are considered threshold related. As such, a certain exposure threshold level is assumed, below which adverse effects are not to be considered;
- the molecular weight of the substance (18-35 kDa) is such that absorption as a whole molecule is not assumed upon oral uptake;
- at the intended use for baking, fine bakery products and similar applications, the enzyme activity will be lost due to processing (heating) steps before consumption; and
- in the sub-chronic (90-day) repeated dose toxicity study in rats, as well as in the other toxicity studies performed, no treatment related systemic effects relevant for human health, were reported.

In conclusion, Aqualysin 1 protease is not considered to have mutagenic properties. Furthermore, it can be concluded that a possible relation with endoreduplication is not considered of health relevance, taken into account a threshold mechanism, its molecular weight preventing uptake via the oral route, inactivation during processing and lacking toxicity in a 90 day repeated dose toxicity test, performed with dose levels reportedly 200 times higher than the maximal daily intake calculated for human.

6.2.2.4 Sub-chronic oral toxicity test

The objective of this study was to evaluate the potential toxicity of the protease Aqualysin 1 from *Thermus aquaticus* expressed in *Bacillus subtilis* following daily oral administration to rats for 13 weeks.

The study design was based on the following guidelines:

- OECD Guideline No. 408 (OECD, 1998)
- Commission Regulation (EC) No.440/2008

Three groups of 10 male and 10 female Sprague-Dawley rats received the test item at a dose level of 12,800, 25,600 or 38,400 mU/kg bw/ day. The gavage was performed during 13 weeks under a constant dosage-volume of 0.41, 0.82 and 1.23 ml/kg bw/day, respectively. In addition, one group of 10 males and 10 females received 1.23 ml/kg bw/day of drinking water treated by reverse osmosis as control.

The protease was provided as “ready-to-use” dosage form at an enzyme activity of 31,095 mU/ml.

The dose-levels were selected taking into account the maximum concentration recommended for bread production, namely 1,200,000 mU/100 kg flour, the maximum daily intake of bread based on EFSA Comprehensive European Food Consumption Database of March 2011 (400 g/day for children weighting 30kg and 500 g/day for adults of 60 kg) and the results of the 2-week toxicity study by oral route for which the NOAEL has been established at 36,266.67 mU/kg bw/day.

The dose-levels that were chosen, corresponds to safety factors of 100, 200 and 300.

The animals were checked daily for mortality and clinical signs. Detailed clinical examinations were performed before the beginning of the treatment and then once a week. Body weight and food consumption were recorded once a week during the study. A Functional Observation Battery (FOB) was performed for all animals once in week 11. Body weight end food consumption was recorded once a week during the study.

Ophthalmological examinations were performed on all animals before the treatment and on control and high-dose groups on completion of the treatment period. Haematology and blood chemistry were performed on all animals at the end of the treatment period.

On completion of the treatment period, the animals were sacrificed and a full macroscopic post-mortem examination was performed. Designated organs were weighed and selected tissue specimens were preserved.

A microscopic examination was performed on selected tissues from all animals from control and high-dose groups and on macroscopic lesions from all the low- and intermediate-dose animals.

There was no premature death or unscheduled sacrifice during the study, except a single female of the low-dose group that was prematurely sacrificed in week 13 after blood sampling.

Prior to death ptyalism was observed as well as soilure on the neck an ears. Lesions were observed at the right eye. No macroscopic or microscopic findings were observed.

During the 13-week treatment period, mainly dose related ptyalism was observed in treated groups in both sexes. These observations were considered treatment related but were considered as non-adverse as it is commonly observed in studies by gavage.

Increased incidence of soiled body parts was observed in all treated females. As it was also observed in the controls, this was considered to be of minor toxicological importance.

The other clinical signs observed during the study were not dose related or are signs commonly observed in laboratory rats treated by gavage under similar conditions (e.g. alopecia, thinning of hair, scabs, etc.). These signs were consequently considered to be unrelated to the treatment.

No change was observed at the FOB.

No abnormal response to handling during the study or abnormal pupil size, grooming, gait, behaviour, defecation or urination.

No salivation, lacrimation, exophthalmos, tremors, twitches, clonic or tonic convulsions, hypo- or hyperactivity, ataxia, hypotonia and stereotypy were observed in animals from both sexes.

A few females of all groups had abnormal fur appearance. As these signs are often seen in laboratory animals and since there was no relation to dose-level, this was considered as without any toxicological significance.

All animals showed normal responses to various kinds of stimuli. There was no difference between tested animals and control for landing foot splay or rectal temperature.

There was no effect on the number of movements and rearing movements.

The mean body weight was unaffected by the treatment.

Moderate increases in the mean body weights and body weight changes of treated females were observed from week 2 to week 11. No clear relationship was observed and these changes did not correlate with changes in food intake. A relationship to the treatment cannot be excluded. These findings were considered of low toxicological significance.

Food intakes were similar in treated animals and in the control groups.

Food consumption in male of the high-dose group was minimally increased in comparison with the controls. As this was not clearly dose-related, it was considered without toxicological significance.

No relevant ophthalmological findings were observed at the end of the treatment period except one case of chorioretinopathy in one male of the high-dose group. This is commonly observed in untreated laboratory rats of this strain and age. It was thus considered to be unrelated with the treatment.

No relevant changes were observed in the haematology parameters at the end of the treatment period.

Concerning blood biochemistry there were minimal and either of no biological or toxicological significance or unrelated to the treatment.

Minimally lower inorganic phosphorus levels were observed in females from the high-dose group as well as minor creatinine level increases in female from the intermediate- and high-dose groups. These changes of very slight amplitude and without any microscopic correlates were attributed to the enzyme but were considered as non-adverse.

A trend towards an increase in aspartate aminotransferase activity was noticed in males from the intermediate- and high-dose groups. This was not observed in females. As these changes were of minimal amplitude, there were considered to be without any toxicological significance.

Other statistically significant changes observed in treated animals (chloride, glucose and protein levels) were not dose related and consequently considered unrelated to the treatment.

Except the one female from the intermediate-dose group that has been sacrificed prematurely in week 13, no mortality has been recorded.

No test item-related organ weight differences were noted. The observed differences were not dose-related, minor and reflected the usual ranges of individual variations.

No macroscopic post-mortem lesion was noted.

All microscopic changes noted in treated rats were considered incidental changes as they were also occurring in the control animals. These findings were of low incidence and/or are common background findings for this strain of rats.

The test item was clinically well tolerated at all the dose-levels. No organ weight, macroscopic or microscopic changes related to the treatment were observed.

In conclusion, under the experimental conditions of this study, the No Observable Adverse Effect Level (NOAEL) was established at 38,400 mU/kg bw/day, in absence of clinical signs or death.

The report of the study and results are presented under **Appendix 13.**

6.2.2.5 Respiratory Toxicity

An acute inhalation study has been performed (**Appendix 14**) in compliance with the UK GLP standards following 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 Aqualysin I. 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 Aqualysin I is in excess of 5.07 mg/l of air.

6.2.2.6 Dermal irritation

An acute dermal irritation test has been performed (**Appendix 15**) in compliance with UK GLP standards following 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 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 assessed after 1, 24, 48, 72 hours and reported. No dermal reaction at the test site of any animal was detected.

Aqualysin I from Bacillus subtilis can be classified as a mid-irritant to rabbit skin according to the Draize classification scheme.

No corrosive effects were noticed.

6.2.2.7 Eye irritation

An acute eye irritation test has been performed (**Appendix 16**) in compliance with UK GLP standards following 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 on three animals. The consequences on the eye were assessed at 1, 24, 48 and 72 hours after treatment. A very slight conjunctivitis was observed after 1 and/or 24 hour observations. The eyes were overtly normal at the 48-hour observation.

Aqualysin I from Bacillus subtilis can be classified as a minimal irritant (class 3 on 1 to 8 scale) to the rabbit eye according to a modified Kay and Calandra classification system.

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

Enzyme preparations used in the various toxicological tests are representative of the food enzyme to be used for the production of bread and other bakery products.

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

Table 8: Composition and Specification of the test material used for toxicological studies

Batch no	RA 07023 (commercial preparation)	RA 1101 (after concentration)	RA 206411125 (commercial preparation)
Ash (%)		4.36	
Water (%)		71.78	
TOS (%)		23.86	
Activity (mU/ml)	4000 mu/g	31095	4000 mu/g

Certificates of Analysis are provided as follows:

- **Appendix 14** - RA 07023: Acute Inhalation Toxicity Study (Appendix 12 of the study document)
- **Appendix 17** - RA 1101: after concentration
- **Appendix 12** - RA 206411125: Chromosomal Aberration Test

Summary of the toxicological data

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

- No mutagenic or clastogenic activity under the given test conditions was observed; and
- The sub-chronic oral toxicity study showed a No Observed Adverse Effect Level (NOAEL) of at least 38,400 mU/kg body weight/day. This is equivalent to 606 mgTOS/kg bw/day.

The Margin of Safety (MoS) for human consumption can be calculated by dividing the NOAEL by the Total Theoretical Maximal Daily Intake (TMDI). As was shown in Section 6.5 (Dietary Exposure) the Total TMDI of the food enzyme is 0.6229 mg TOS/kg body weight/day. Consequently, the MoS is:

MoS = 606/0.6229 = 972.8

Applying the common safety factor of 100, the MoS would be still of a factor 10.

The Margin of Safety in Australia and New Zealand will be much higher, as the TMDI is much lower, as can be seen in Section 6.5.

For technical reasons, overdoses could not occur. Indeed, an eventual overdose would provide bread with a shape that will not be accepted anymore by consumers (see Figure 11).

As explained in Section 6.5 (Dietary Exposure) the Total TMDI is highly exaggerated. Moreover, the NOAEL was based on the highest dose administered, and is therefore to be considered as a minimum value. Therefore, the actual MoS in practice will be some magnitudes higher than the level of use. Consequently, there is no safety reason for determining a maximum level of use.

The overall conclusion is that the use of the food enzyme Aqualysin I produced in *Bacillus subtilis* in bakery products is safe. Considering the safety factor – even when calculated by means of an overestimation of the intake via the Budget method – there is no need to restrict the use of the enzyme in bakery products.

Consequently, it is concluded that Aqualysin I from *Bacillus subtilis* can be used *Quantum Satis* in bakery products.

6.2.3 Information on the potential allergenicity of the enzyme processing aid

(As per section 3.3.2 C.3 of the Application Handbook as at 1 March 2016)

Intake Allergies

The amino-acid sequence for Aqualysin I enzyme protein from *Bacillus subtilis* has been determined, as described in Section 6.1.3 (CCI). Therefore, the homology searches as described in Section 4.2 of the EFSA CEF Guidance document on food enzymes (EFSA, 2009b) have been performed. At present, validated testing methods to predict the allergenicity of the 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 (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 18**).

Based on Full Fasta search method, sliding 80mer Window search method or 8mer Extract Match search method, 23 homologies where found. 20 homologies are linked to respiratory allergies and 3 homologies (line 9, 12 and 13) are linked to dermatology allergies. Tests (presented below) were performed concerning respiratory and dermal allergies.

The result presented exclude the respiratory and dermal allergenic potential of Aqualysin I.

Because they are proteins, enzymes could theoretically have the potential to cause allergic responses. However, in order to address allergenicity by ingestion, it may be taken into account that:

- The allergenic potential of enzymes was studied by Bindslev-Jensen *et al.* (2006) and reported in the publication: "*Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry*". The investigation comprised enzymes produced by wild-type and genetically modified strains as well as wild-type enzymes and Protein Engineered variants and comprised 400 patients with a diagnosed allergy to inhalation allergens, food allergens, bee or wasp. It was concluded from this study that ingestion of food enzymes in general is not likely to be a concern with regard to food allergy.
- Previously, the AMFEP Working Group on Consumer Allergy Risk from Enzyme Residues in Food performed an in-depth analysis of the allergenicity of enzyme products (Dauvrin et al., 1998). The overall conclusion was that – as opposed to exposure by inhalation – there are no scientific indications that the small amounts of enzymes in food can sensitize or induce allergy reactions in consumers after consumption.
- Enzymes when used as digestive aids are ingested daily, over many years, at much higher amounts when compared to enzymes present in food (up to 1 million times more). Wüthrich (1996) published a list of enzymes used as digestive aids and concluded that they are not potent allergens by ingestion.

Thus, there are no scientific indications that small amounts of enzymes in food can sensitize or induce allergic reactions in consumers through oral intake.

Additional considerations supporting the assumption that the ingestion of an enzyme protein is not a concern for food allergy should also be taken into account:

- The majority of proteins are not food allergens and based on previous experience, the enzyme industry is not aware of any enzyme proteins used in food that are homologous to known food allergens that have induced allergenic reaction.
- The food enzyme is used in very small amounts during food processing, resulting in extremely small amounts of the enzyme protein in the final food. A high concentration generally equals a higher risk of sensitization, whereas a low level in the final food equals a lower risk (Goodman et al., 2008).
- In the case where proteins are denatured, the tertiary conformation of the enzyme molecule is destroyed. In general, these alterations in conformation are associated with decrease in the antigenic reactivity in humans: in the vast majority of investigated cases, denatured proteins are much less immunogenic than the corresponding native proteins (Valenta and Kraft, 2002; Valenta, 2002; Takai *et al.*, 1997; Takai *et al.*, 2000; Nakazawa *et al.*, 2005; Kikuchi *et al.*, 2006).
- In addition, residual enzyme still present in the final food will be subjected to digestion in the gastro-intestinal system, which reduces further the risk of a potential enzyme allergenicity. While stability to digestion is considered as a potential risk factor for allergenicity, it is

believed that small protein fragments resulting from digestion are less likely to be allergenic (FAO/WHO, 2001; Goodman et al., 2008).

- Finally, enzymes have a long history of safe use in food processing, with no indication of any adverse effects or reactions. Moreover, a wide variety of enzyme classes (and structures) are naturally present in food. This is in contrast with most known food allergens, which are naturally present in a narrow range of foods.

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 6.1.4). 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 it can be considered safe under its normal conditions of use, i.e. no aerosol formation.

Normal dust prevention is always recommended and required to exclude general health concern in bakeries.

The only allergen present in the commercial enzyme preparation is the food grade wheat maltodextrin 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.

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

(As per section 3.3.2 C.4 of the Application Handbook as at 1 March 2016)

The food enzyme Aqualysin 1 has been evaluated and/or authorized in the following countries: Canada, France and the USA as set out in Section 5.1.

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

(As per section 3.3.2 D of the Application Handbook as at 1 March 2016)

6.3.1 Information on the source microorganism

(As per section 3.3.2 D.1 of the Application Handbook as at 1 March 2016)

The microorganism that is used for the production of Aqualysin I is the bacterium *Bacillus subtilis Raα3114.* 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:	subs. Subtilis
Synonyms:	*Vibrio subtilis, Bacillus uniflagellatus, Bacillus natto, Bacillus globigii*

The source material for the food enzyme is *Bacillus subtilis* LMGS-25520. The organism has been genetically modified to produce the enzyme. Information regarding the construction is provided in **Section 6.1.3.** Absence of antibiotic resistance genes and absence of genetically modified material (GMM) in the final product are demonstrated 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.

6.3.2 Information on the pathogenicity and toxicity of the source microorganism

(As per section 3.3.2 D.2 of the Application Handbook as at 1 March 2016)

The production organism for this enzyme, *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 status (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 (Processing Aids) of the Code permits the use of the following enzymes derived from B. Subtilis:

- α –acetolactate decarboxylase (EC 4.1.1.5);
- α- and β–amylase (EC 3.2.1.1 & EC 3.2.1.2);
- β-glucanase (EC 3.2.1.6);
- endo-1,4-beta-xylanase (EC 3.2.1.8);
- hemicellulose multicomponent enzyme (EC 3.2.1.78);
- maltogenic α–amylase (EC 3.2.1.133);
- metalloproteinase;
- Pullulanase (EC 3.2.1.41); and
- serine proteinase (EC 3.4.21.14).

In the US, several enzyme preparations from *B. Subtilis* have Generally Recognised as Safe (GRAS) status as covered in Section 5.

Therefore, *Bacillus subtilis* can be considered generally safe not only as a production organism of its natural enzymes, but also as a safe host for other safe gene products.

6.3.3 Information on the genetic stability of the source organism

(As per section 3.3.2 D.3 of the Application Handbook as at 1 March 2016)

This information is provided in Sections 6.4.1.3 and 6.4.1.4.

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

(As per section 3.3.2 E of the Application Handbook as at 1 March 2016)

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

(As per section 3.3.2 E.1 of the Application Handbook as at 1 March 2016)

6.4.1.1 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 a *Thermus aquaticus* and the recipient strain is a *Bacillus subtilis*.

Description of Identification and Detection Techniques

In order to demonstrate the presence/absence of the production strain in the enzyme preparation, 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, P., Millet, J. & Aubert, J.P. 1965.). The growth conditions used are: 37°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 20.**

c) The production strain could be differentiated from possible contaminating microorganisms by plating the cultures on LB agar plates containing 14 g/L milk powder. The production strain will produce a clear halo due to milk proteins hydrolysis and the contaminating microorganisms will not.

d) Three different batches of enzyme preparation were tested in triplicate for the presence/absence of the production strain. The results are presented in **Appendix 19**.

The results obtained demonstrate the absence of the production strain in the three different batches tested. The sampling method is detailed in **Appendix 20**. 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.

Source and Natural Habitat of the Parental Microorganism

The donor organism is the LMG8924 strain of *Thermus aquaticus*. The cloning and the characterization of the gene coding for the protease of this strain has been described by Kwon et al. 1988

Strains of *Thermus aquaticus* have been isolated from a variety of thermal springs in Yellowstone National Park and from a thermal spring in California. The organism is an obligate aerobe and has a pH optimum of 7.5 to 7.8. The optimum temperature for growth is 70°C, the maximum 79°C and the minimum about 40°C. This micro-organism is worldly known for its DNA polymerase largely used for the PCR analysis.

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 to antibiotics is present in the production strain.

Information on the Genetic Stability of the Recipient Microorganism

16S analysis were performed on the parental strain (TD1100) and the recipient strain (Raα3114). The phylogenetic comparison showed that parental strain is indeed a *Bacillus subtilis*. The comparison with the recipient strain Raα3114 showed 100% identity.

Further specific detail is provided in the CCI document – 6.4.1.

Pathogenicity, Ecological and Physiological Traits

Pathogenicity: Pathogenic *B. subtilis* strains are not described in the Bergey's Manual or in the ATCC and other catalogues. The species *B. subtilis* does not appear on the list of pathogens in Annex III of 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 (**Appendix 21**). *B. subtilis* is listed as Risk Group 1 in the microorganism classification lists of the German Federal Institute for Occupational Safety and Health (BAuA) (**Appendix 22,** p36) and the Federal Office of Consumer Protection and Food Safety (BVL) (**Appendix 23,** p 6).

B. subtilis is exempted as a host of certified host-vector systems under the NIH Guidelines in the USA since 1994 (**Appendix 24**). The US EPA have added *B. subtilis* to the list of exempted organisms in 1997 (**Appendix 25**). *B. subtilis* is a low-risk-class microorganism, 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 in 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 recently 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. Lipopeptide's analysis are 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. This results in continuous adaptation of the microbes by 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 e.g. 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) replacing the technical guidance updating the criteria used in the assessment of bacterial resistance to antibiotics of human or veterinary importance (EFSA 2008), 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 6.4.2.2, 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 the final product there are no antimicrobial resistance genes which is covered in Section 6.1.3.

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

Obtaining 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 a xylanase gene. The genetic map showing the precise sequence deleted is presented in the CCI materials under Section 6.4.1.

Finally, an additional mutation in a xylanase gene 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.

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

DNA from Defined Donor Organisms

The donor organism is the LMG8924 strain of *Thermus aquaticus*. The cloning and the characterization of the gene coding for the protease of this strain has been described by Kwon et al (1988).

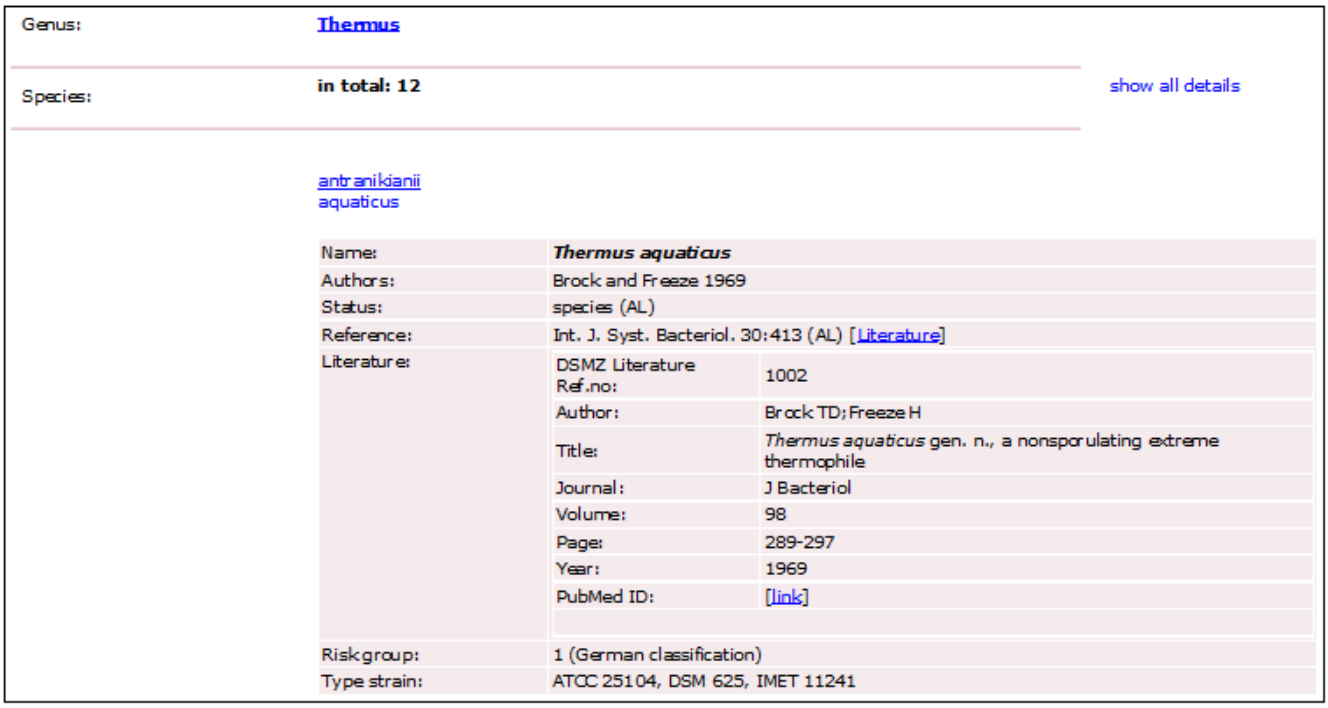
The DNA sequence is available in the GENBANK database (accession number D90108) and is shown in **Appendix 27**. Thermus aquaticus has been classified as a class 1 microorganism by the ATCC (American Type Culture Collection) (Figure 5) and the DSMZ (DeutscheSammlung von Mikroorganismes und Zellkulturen GmbH) (Figure 6).

Further specific detail is provided in the CCI document.

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

Organism:	Thermus aquaticus Brock and Freeze		
Designations:	YT-1 [DSM 625, JCM 10724, LMG 8924, NCAIM B.01703, NCIB 11243]	**Isolation:**	Hot spring, Yellowstone National Park
Depositors:	TD Brock		
Biosafety Level:	1	**Shipped:**	freeze-dried
Growth Conditions:	ATCC medium 461: Castenholz TYE medium		
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 *Thermus aquaticus* as belonging to the risk 1 class of microorganism by the DSMZ



Genus:	Thermus	
Species:	in total: 12	show all details
	antranikianii aquaticus	

Name:	***Thermus aquaticus***
Authors:	Brock and Freeze 1969
Status:	species (AL)
Reference:	Int. J. Syst. Bacteriol. 30:413 (AL) [Literature]
Literature:	

DSMZ Literature Ref.no:	1002
Author:	Brock TD; Freeze H
Title:	*Thermus aquaticus* gen. n., a nonsporulating extreme thermophile
Journal:	J Bacteriol
Volume:	98
Page:	289-297
Year:	1969
PubMed ID:	[link]

Risk group:	1 (German classification)
Type strain:	ATCC 25104, DSM 625, IMET 11241

Synthetic DNA

Not relevant

Nucleic Acids directly extracted from environmental sample

Not relevant

6.4.1.3 Description of the Genetic Modification

Characteristic of the Vector

The expression cassette comprises of the coding sequence of the *Thermus aquaticus* protease gene, combined with an appropriate signal peptide. These coding sequences are flanked by a promotor and terminator sequence.

The vector of expression was synthesized by Polymerase Chain Reaction (PCR). For the initial transformation, a DNA fragment recovered from the host strain chromosome that will serve as target sequence for integration and a *Bacillus subtilis* transformation marker are used. In a next step, the transformation marker is removed from the original transformant. The same mechanisms can be used to insert several copies of the gene of interest.

This mechanism gives **a transformed strain that has no exogenous DNA into its chromosome** besides the gene of interest

Description of the integration sites

The location of insertion of the gene of interest has been chosen depending on the functions of genes one wants to eliminate.

Further specific detail is provided in the CCI document.

6.4.1.4 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 Raα3114, 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 6.4.1.1 and 6.4.1.3

The structure of the expression cassette is presented under 6.4.1.3.

The structure and amount of any vector and/or donor nucleic acid remaining in the GMM are presented under 6.4.1.3. The number of copies inserted in the genome of the final production strain is 7.

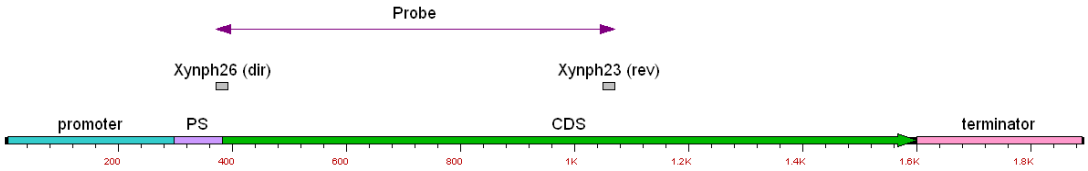
The proof of the numbers of copies in provided in the Southern Blot in 6.4.1.4.

Stability of the Genetic Traits in the GMM

Specific detail is provided in the CCI document.

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

Figure 7: 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 6.1.3 and 6.1.6**.**

Description of Identification and Detection Techniques

The techniques used for identification and detection of the inserted sequences are presented under 6.4.1.3 and corresponding Southern blot hybridization results are presented under 6.4.1.4. The techniques for detection and verification of absence of the vector in the GMM are presented under 6.4.1.3.

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.

Some DNA was detected in the final product. However, the probability of transfer of this DNA is extremely low. The transformability of the enzyme liquid concentrate was tested and shown not to be able to transform competent cells. It can be concluded that there will be no DNA dissemination.

As demonstrated in Section 6.4.2.2 the GMM is not present in the final product. Therefore, no active transfer could occur with environmental strains.

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 protease active at higher temperature than those useful for a microorganism in Europe. The donor micro-organism lives in hot springs in the Yellow stone

national park in USA. 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 6.4.2.2.

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 and *Bacillus subtilis* is classified as QPS (qualified presumption of safety) by EFSA (2007). The production strain should therefore be regarded as safe.

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* Raα3114 (**Appendix 28**). As a conclusion of this test, *B. subtilis* Raα3114 has no cytotoxic potential on Vero cells.

6.4.2 Information related to the product

6.4.2.1 Information Related to the Production Process

See Section 6.1.4.

6.4.2.2 Information Related to the Product Preparation Process

Demonstration of the Absence of the GMM in the Product

This is discussed under Description of Identification and Detection Techniques (6.4.1.1).

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

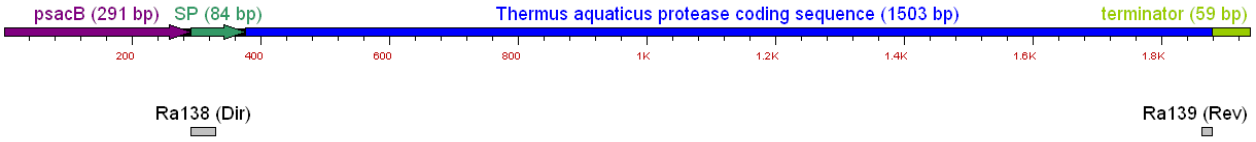
As explained under 6.4.1, a sterile filtration step at the end of the purification process excludes the presence of remaining physically intact cells of the production microorganisms. This is validated by the demonstration of the absence of the GMM in the product (see under Section 6.4.2.2).

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 protease coding sequence was targeted in the genome of the final production strain using primers:

Figure 8: Genetic map of the expression vector and probe sequence



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 29**. 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 20.** 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.

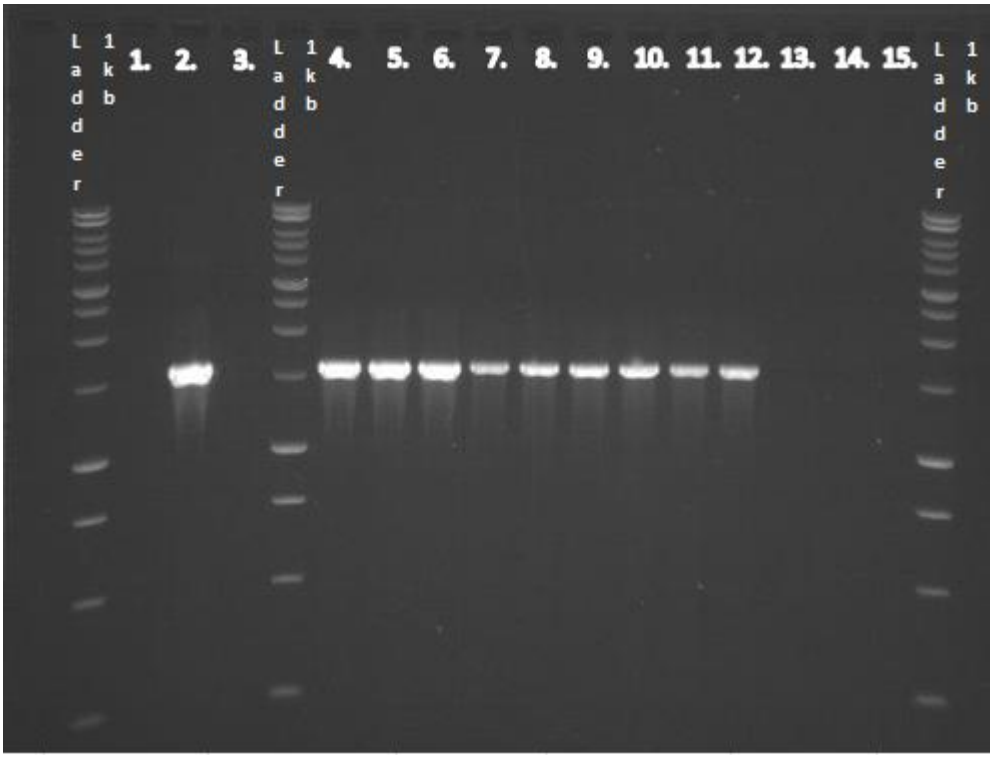
d) Three different batches of enzyme preparation were tested in triplicate for the presence/absence of recombinant DNA of the production strain. The results are presented below.

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

Table 9: Results - presence/absence of recombinant DNA of the production strain

Well	Samples	Volume (µl)	H2O ajoutée (µl)
1	Blanco : H2O = template	0	5
2	Ctl+: genomic DNA Raα3114 10 ng/µl	5	0
3	Ctl-: genomic DNA TD1100 10 ng/µl	5	0
4	Extraction DNA Ra lot 1401 A	5	0
5	Extraction DNA Ra lot 1401 B	5	0
6	Extraction DNA Ra lot 1401 C	5	0
7	Extraction DNA Ra lot 1402 A	5	0
8	Extraction DNA Ra lot 1402 B	5	0
9	Extraction DNA Ra lot 1402 C	5	0
10	Extraction DNA Ra lot 1403 A	5	0
11	Extraction DNA Ra lot 1403 B	5	0
12	Extraction DNA Ra 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

Figure 9: Results - presence/absence of recombinant DNA of the production strain



Further information is provided under CCI (6.4.2)

6.5 INFORMATION RELATED TO THE DIETARY EXPOSURE TO THE PROCESSING AID

(As per section 3.3.2 F of the Application Handbook as at 1 March 2016)

6.5.1 Dietary Exposure

Commercial food enzyme preparations are generally used following the *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. Therefore, the enzyme manufacturer can only issue a recommended enzyme dosage range. Such a dosage range is the starting point for the individual producer to fine-tune his process and determine the right 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 Aqualysin I 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 negative technological consequences.

The test showed below demonstrates that a high dose of the enzyme gives rapidly a bad structure to the bread which will be rejected by the consumer.

Figure 10: Impact of high usage dose of Aqualysin 1

Sample	Quantity Aqualysin 1 (mU/100 kg)
1	230.000
2	3.450.000
3	11.500.000
4	23.000.000
5	34.500.000

The table below shows the recommended use level for the food enzyme.

Table 10: Recommended Enzyme Use Levels – Aqualysin 1

Application	Raw material (RM)	Recommended use levels (mU/kg RM)	Maximal recommended use levels (mU/kg RM)
Baking	Flour	2300 – 12000	12000

Aqualysin 1 from *Bacillus Subtilis* may be used in the manufacture of a wide variety of bakery products. Due to this variety of applications, the most appropriate way to estimate the human consumption in the case of food enzymes is using the so-called Budget Method (Hansen, 1966; Douglass et al., 1997). This method enables to calculate 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 and is known to result in highly conservative estimations of the daily intake.

The conservative "budget method" has been used to assess potential dietary exposure in standard adult of 60kg 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 1,200,000 mU/125 kg of bread, the maximal daily intake of enzyme per consumer will be:

- For adults: 42 kg bread[2] * 1,200,000mU / (60 kg bw * 125 kg bread * 365 days) = 18.397mU enzyme/kg bw/day
- For children: 20 kg bread[3] * 1,200,000mU / (31 kg bw * 125 kg bread * 365 days) = 17.271mU enzyme/kg bw/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 member states is 50 kg (Euromonitor, 2013, **Appendix 30**).

The intake of enzyme by European extreme consumers (90 kg bread per year vs 60 kg on average in all MS) will be 39.45 mU/kg bw/day (Euromonitor 2013 – **Appendix 30**).

This is equivalent to 0. 6229 mg TOS/kg bw/day

The risk for consumers in Australia is lower as the average bread consumption per person per year is lower.

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.

The recommended use levels of the enzyme Aqualysin I are given, based on the raw materials used in the various baked food processes. For the calculation of the theoretical maximum daily intake (TMDI), the maximum use levels are chosen. Furthermore, the calculation takes into account how much bread is obtained per 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.

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

Table 11: Maximum level of the food enzyme in final baked food

Application	Raw material (RM)	Maximal recommended use level (mU/kg RM)	Maximal recommended use level (mg TOS/kg RM)	Final food	Ratio RM/final food	Maximal level in final food (mg TOS/kg food)
Baking	Flour	12000	189.48	Bread	0.8	151.58

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

Table 12: Total TMDI of the food enzyme

TMDI in food, calculation (mg TOS/kg body weight/day)	Total TMDI (mg TOS/kg body weight/day)
151.58*90/60*365	**0.6229**

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 Aqualysin I from *Bacillus subtilis*;
- For the calculation of the TMDI's in baked foods, only the highest theoretical amount of TOS was 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 enzyme is consumed DAILY over the course of a lifetime; and
- Assumptions regarding food intake of the general population are overestimates of the actual average levels (Douglass et al., 1997).

The risk for consumers in Australia is lower as the average bread consumption per person per year is lower.

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

(As per section 3.3.2 F.1 of the Application Handbook as at 1 March 2016)

Table 13: Foods Groups and Foods likely to contain the Enzyme

Food Group	Food	Schedule 15 (S15-5)
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)

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

(As per section 3.3.2 F.2 of the Application Handbook as at 1 March 2016)

Maximal recommended use levels are set out in Table 10:

Recommended Enzyme Use Levels – Aqualysin 1 [Table 10]

Application	Raw material (RM)	Recommended use levels (mU/kg RM)	Maximal recommended use levels (mU/kg RM)
Baking	Flour	2300 – 12000	12000

6.5.4 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

(As per section 3.3.2 F.3 of the Application Handbook as at 1 March 2016)

Not applicable

6.5.5 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

(As per section 3.3.2 F.4 of the Application Handbook as at 1 March 2016)

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 Aqualysin 1.

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

(As per section 3.3.2 F.5 of the Application Handbook as at 1 March 2016)

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 we have shown there is no active enzyme present after the baking process. The amount of recommended Aqualysin 1 addition in all countries where the enzyme is sold is the same.

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

(As per section 3.3.2 F.6 of the Application Handbook as at 1 March 2016)

Not applicable.

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