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Abstract

The food enzyme considered in this opinion is aqualysin 1 (EC 3.4.21.111), produced from the genetically modified strain *Bacillus subtilis* LMGS 25520 by Puratos NV. The production strain was not detected in the food enzyme. Aqualysin 1 is intended to be used in baking processes. Based on the maximum use level recommended and individual consumption data from the EFSA Comprehensive European Food Consumption Database, dietary exposure to the food enzyme–total organic solids (TOS) was estimated to be up to 2.13 mg TOS/kg body weight per day in European populations. Genotoxicity tests indicated no genotoxic concerns. The allergenicity was evaluated by searching for similarity of the amino acid sequence to those of known allergens and 23 matches were found (20 respiratory and 3 dermal allergens). However, the Panel considered that there are no indications for food allergic reactions to the food enzyme. The genetic modifications performed, the manufacturing process, the compositional and biochemical data, the allergenicity and the genotoxicity assessment did not raise safety concerns. The Panel considered the margin of exposure (MOE) calculated from the no observed adverse effect level (NOAEL) determined from the repeated dose 90-day oral toxicity study and the estimated dietary exposure as insufficient to conclude that there is no safety concern for this food enzyme under the intended conditions of use. The Panel noted that recombinant DNA was present in all batches of the food enzyme tested.

Keywords: food enzyme, aqualysin 1, EC 3.4.21.111, *Bacillus subtilis*, genetically modified microorganism

Requestor: European Commission

Question number: EFSA-Q-2014-00920

Correspondence: fip@efsa.europa.eu

Panel members: Claudia Bolognesi, Laurence Castle, Kevin Chipman, Jean-Pierre Cravedi, Paul Fowler, Roland Franz, Konrad Grob, Rainer Gürtler, Trine Husøy, Sirpa Kärenlampi, Wim Mennes, Maria Rosaria Milana, Karla Pfaff, Gilles Riviere, Vittorio Silano, Jannavi Srinivasan, Maria de Fátima Tavares Poças, Christina Tlustos, Detlef Wölfle and Holger Zorn.

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1. Introduction

Article 3 of the Regulation (EC) No 1332/2008[1] provides definitions for 'food enzyme' and 'food enzyme preparation'.

> 'Food enzyme' means a product obtained from plants, animals or micro-organisms or products thereof obtained by a fermentation process using micro-organisms: (i) containing one or more enzymes capable of catalysing a specific biochemical reaction; and (ii) added to food for a technological purpose at any stage of the manufacturing, processing, preparation, treatment, packaging, transport or storage of foods.
>
> 'Food enzyme preparation' means a formulation consisting of one or more food enzymes in which substances such as food additives and/or other food ingredients are incorporated to maintain their properties and facilitate their stability storage, sale, standardisation, dilution or dissolution.

Before January 2009, food enzymes other than those used as food additives were not regulated or were only regulated as processing aids under the legislation of the Member States. On 20 January 2009, Regulation (EC) No 1332/2008 on food enzymes came into force. This Regulation applies to enzymes that are added to food to perform a technological function for the manufacture, processing, preparation, treatment, packaging, transport or storage of such food, including enzymes used as processing aids. Regulation (EC) No 1331/2008[2] established European Union (EU) procedures for the safety assessment and the authorisation procedure of food additives, food enzymes and food flavourings. The use of a food enzyme shall be authorised only if it is demonstrated that:

- it does not pose a safety concern to the health of the consumer at the level of use proposed,
- there is a reasonable technological need, and
- its use does not mislead the consumer.

All food enzymes currently on the EU market and intended to remain on that market, as well as all new food enzymes, shall be subjected to a safety evaluation by the European Food Safety Authority (EFSA) and approval via an EU Community list.

The 'Guidance on submission of a dossier on a food enzyme for evaluation' (EFSA, 2009a) lays down the administrative, technical and toxicological data required.

1.1. Background and Terms of Reference as provided by the requestor

1.1.1. Background as provided by the European Commission

Only food enzymes included in the Union list may be placed on the market as such and used in foods, in accordance with the specifications and conditions of use provided for in Article 7(2) of Regulation (EC) No 1332/2008 on food enzymes.

Three applications have been introduced by the company 'Novozymes A/S' for the authorisation of the food enzymes Lysophospholipase produced by a genetically modified strain of *Aspergillus niger* (strain NZYM-LP), Phospholipase from a genetically modified strain of *Aspergillus oryzae* (strain NZYM-PP) and Maltogenic amylase from a genetically modified strain of *Bacillus subtilis* (strain NZYM-OC), and one application by the company 'Puratos NV sa' for the authorisation of the food enzyme aqualysin 1 from a genetically modified strain of *Bacillus subtilis* (strain LMGS 25520).

Following the requirements of Article 12.1 of Commission Regulation (EU) No 234/2011[3] implementing Regulation (EC) No 1331/2008, the Commission has verified that the four applications fall within the scope of the food enzyme Regulation and contain all the elements required under Chapter II of that Regulation.

[1] Regulation (EC) No 1332/2008 of the European Parliament and of the Council of 16 December 2008 on Food Enzymes and Amending Council Directive 83/417/EEC, Council Regulation (EC) No 1493/1999, Directive 2000/13/EC, Council Directive 2001/112/EC and Regulation (EC) No 258/97. OJ L 354, 31.12.2008, p. 7–15.

[2] Regulation (EC) No 1331/2008 of the European Parliament and of the Council of 16 December 2008 establishing a common authorisation procedure for food additives, food enzymes and food flavourings. OJ L 354, 31.12.2008, p. 1–6.

[3] Commission Regulation (EU) No 234/2011 of 10 March 2011 implementing Regulation (EC) No 1331/2008 of the European Parliament and of the Council establishing a common authorisation procedure for food additives, food enzymes and food flavourings. OJ L 64, 11.3.2011, p. 15–24.

1.1.2. Terms of Reference

The European Commission requests the European Food Safety Authority to carry out the safety assessments of the food enzymes Lysophospholipase produced by a genetically modified strain of *Aspergillus niger* (strain NZYM-LP), Phospholipase from a genetically modified strain of *Aspergillus oryzae* (strain NZYM-PP), Maltogenic amylase from a genetically modified strain of *Bacillus subtilis* (strain NZYM-OC) and aqualysin 1 from a genetically modified strain of *Bacillus subtilis* (strain LMGS 25520) in accordance with Article 17.3 of Regulation (EC) No 1332/2008 on food enzymes.

1.2. Interpretation of the Terms of Reference

The present scientific opinion addresses the European Commission's request to carry out the safety assessment of the food enzyme aqualysin 1 from the genetically modified *Bacillus subtilis* strain LMGS 25520.

1.3. Information on existing authorisations and evaluations

The French food authorities have evaluated and authorised the use of the food enzyme from the genetically modified *Bacillus subtilis* strain LMGS 25520 for food manufacturing processes.

2. Data and methodologies

2.1. Data

The applicant submitted a dossier in support of the application for authorisation of the food enzyme aqualysin 1 produced with genetically modified *Bacillus subtilis* strain LMGS 25520. The food enzyme is intended to be used in baking processes.

2.2. Methodologies

The assessment was conducted in line with the principles described in the EFSA 'Guidance on transparency in the scientific aspects of risk assessment' (EFSA, 2009b) and following the relevant existing guidances from the EFSA Scientific Committee.

The current 'Guidance on the submission of a dossier for safety evaluation of a food enzyme' (EFSA, 2009a) has been followed for the evaluation of this application with the exception of the exposure assessment, which was carried out in accordance with the methodology described in the 'CEF Panel statement on the exposure assessment of food enzymes' (EFSA CEF Panel, 2016).

3. Assessment

3.1. Technical data

3.1.1. Identity of the food enzyme

IUBMB nomenclature: Aqualysin 1
Systematic name: –
Synonyms: Caldolysin; AQN
IUBMB No: EC 3.4.21.111
CAS No: 88747-68-6
EINECS No: Not available

3.1.2. Chemical parameters

The aqualysin 1 peptidase produced with the genetically modified *B. subtilis* strain LMGS 25520 is initially synthesised as a single polypeptide of [redacted]. The enzyme is secreted and activated by cleavage of N- and C-terminal sequences, resulting in a functional enzyme of [redacted] (calculated molecular mass [redacted]). The sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) pattern showed one main protein band and confirmed the molecular mass.

The food enzyme was tested for α-amylase and xylanase activities, which were below the limits of detection of the applied assays. No other enzymatic activities have been reported by the applicant.

Data on the chemical parameters of the food enzyme were provided for three batches used for commercialisation and three batches used for the toxicological tests (Table 1). The average total organic solids (TOS) of the three food enzyme batches for commercialisation was 5.0%; the values ranged from 4.62% to 5.42%.

The average enzyme activity/mg TOS ratio of the three food enzyme batches for commercialisation was 0.063 aqualysin 1 Units/mg TOS; the values ranged from 0.046 to 0.083 U/mg TOS (Table 1).

Table 1: Compositional data of the food enzyme

Parameter	Units	Batches					
		1	2	3	4[(a)]	5[(b)]	6[(c)]
Aqualysin 1 activity	U/mL batch[(d)]	2.83	4.12	2.49	1.51	3.03	31.1
Protein	%	1.43	2.71	2.89	NA[(f)]	NA[(f)]	5.83
Ash	%	0.76	0.75	0.86	3.13	0.59	4.36
Water	%	94.62	94.29	93.72	94.79	96.57	71.78
Total organic solids (TOS)[(e)]	%	4.62	4.96	5.42	2.08	2.84	23.86
Aqualysin 1 activity/mg TOS	U/mg TOS	0.061	0.083	0.046	0.073	0.107	0.130

(a): Batch used for bacterial reverse mutation test.
(b): Batch used for chromosomal aberration test.
(c): Batch used for the repeated dose 14-day and 90-day oral toxicity study (batch after concentration).
(d): U/mL: Protease Unit/mL (see Section 3.3).
(e): TOS calculated as 100% - % water - % ash.
(f): NA: not analysed.

The food enzyme complies with the specification for lead (not more than 5 mg/kg) as laid down in the general specifications and considerations for enzymes used in food processing (FAO/WHO, 2006). In addition, the applicant provided data demonstrating that the levels of arsenic, cadmium and mercury were below the limits of detection of the employed methodologies.

No antimicrobial activity was detected in any of these batches (FAO/WHO, 2006).

The food enzyme complies with the microbiological criteria as laid down in the general specifications and considerations for enzymes used in food processing (FAO/WHO, 2006), which stipulate that *Escherichia coli* and *Salmonella* species are absent in 25 g of sample and total coliforms are not more than 30 colony forming units (CFU) per gram.

The applicant has provided information on the identity of the antifoam agent used. Taking into account the nature and properties of the antifoam agent, the manufacturing process and the quality assurance system implemented by the applicant, the Panel considers its use as of no safety concern.

The compositional data provided for the food enzyme batches are considered sufficient.

3.1.3. Properties of the food enzyme

The food enzyme aqualysin 1 is an alkaline serine peptidase, which catalyses the hydrolysis of peptide bonds in proteins resulting in the generation of polypeptides of different lengths.

The aqualysin 1 peptidase activity is determined based on the hydrolysis of the substrate *N*-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide and is expressed in Protease Units/mL (U/mL). The analytical principle is based on the hydrolysis of the substrate, resulting in the release of the yellow *p*-nitroaniline (reaction conditions: pH 7.5, temperature 70°C, reaction time 20 min). The concentration of released *p*-nitroaniline is determined spectrophotometrically at 415 nm. One unit of protease is defined as the amount of enzyme that liberates 1.9 μmole of 4-nitroaniline per minute from a 1.02 mM *N*-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide solution at pH 7.5 and 70°C. The assay includes a pre-incubation of the food enzyme for 90 min at 70°C in the reaction buffer.

The aqualysin 1 has been characterised regarding its activity depending on temperature and pH. The pH profile has been measured within a pH range of 4.5–10.5 at 30–80°C; the enzyme is active up to pH 10.5 with an optimum of 9.5 at 70°C. The temperature profile has been measured from 30 up to 80°C at pH 4.5 to 10.5 and the aqualysin 1 is active at temperatures up to 80°C with an optimum of 70°C at pH 9.5. The aqualysin 1 shows approximately 97% residual activity after incubation for 250 min at 70°C. The stability of the enzyme decreases at temperatures above 70°C, showing only approximately 5.3% residual activity after 250 min of incubation at 80°C, and no residual activity after 25 min at 90°C.

3.1.4. Information on the source material

3.1.4.1. Information on the genetically modified microorganism

The aqualysin 1 production strain *B. subtilis* LMGS 25520 is deposited in the Belgian Co-ordinated Collection of Microorganisms (BCCM/LMG – Bacterial Culture Collection) located at the University of Gent, under deposit number LMGS 25520.

3.1.4.2. Characteristics of the parental and recipient microorganisms

The parental microorganism is the bacterium *B. subtilis,* strain [redacted]

3.1.4.3. Characteristics of the donor organisms

The donor for the aqualysin 1 peptidase gene is the thermophilic bacterium [redacted]

3.1.4.4. Description of the genetic modification process

The production strain *B. subtilis* LMGS 25520 was developed by [redacted]

3.1.4.5. Safety aspects of the genetic modification

[redacted]

[redacted]

3.1.5. Manufacturing process

The food enzyme is manufactured according to the Food Hygiene Regulation (EC) No 852/2004[4] and in accordance with current Good Manufacturing Practice (GMP).

The food enzyme is produced by a pure culture of *B. subtilis* strain LMGS 25520 in a contained, submerged, fed-batch fermentation system with conventional process controls in place.

After completion of the fermentation, the cells of the *B. subtilis* LMGS 25520 production strain are separated from the culture medium by microfiltration. The aqualysin 1 present in the filtrate is subsequently concentrated by ultrafiltration. Formulation into food enzyme preparation involves spray-drying after addition of [redacted]

The absence of the production microorganism in the food enzyme was demonstrated in [redacted] The agar medium used allowed screening for the presence of the production strain. All samples were tested in triplicate.

Recombinant DNA was detected in three food enzyme batches, tested in triplicate, by PCR-analysis amplifying a fragment of about [redacted] representing the complete aqualysin 1 coding sequence.

The Panel considered the information provided on the manufacturing process as sufficient.

3.1.6. Safety for the environment

The production strain could not be detected in the food enzyme. However, recombinant DNA was demonstrated to be present in all batches tested (see Section 3.1.5) (EFSA GMO Panel, 2011). The applicant provided laboratory transformation tests with samples from production batches were negative and argued that presence of recombinant DNA was therefore not a concern. However, the CEF Panel did not consider these laboratory-based data as relevant for the risk assessment.

On the other hand, no sequences that cause safety concern (such as antibiotic resistance genes or genes encoding known toxins) have been introduced in the production strain, therefore the Panel is of the opinion that the recombinant DNA present in the food enzyme does not pose a risk to the environment.

3.1.7. Case of need and intended conditions of use

The food enzyme is intended to be used in baking processes at a recommended use level of up to 190 mg TOS/kg flour.

The food enzyme aqualysin 1 is added to the raw materials during the preparation of dough. It is used to hydrolyse gluten proteins (glutenin, gliadin), thus contributing to reduce the viscosity, increase the extensibility and improve the structure of the dough. This results in better processability of the dough and in more uniform products.

[4] Regulation (EC) No 852/2004 of the European Parliament and of the Council of 29 April 2004 on the hygiene of food additives. OJ L 226, 25.6.2004, p. 3–21.

3.1.8. Reaction and fate in food

Aqualysin 1 catalyses the hydrolysis of peptide bonds in proteins. It is specific in its action, not known to catalyse other reactions than hydrolysis of peptide bonds in proteins resulting in the generation of polypeptides of different lengths. Such reaction products are naturally present in protein-containing foods.

Owing to the substrate specificity of aqualysin 1, no unintended reaction products in foods are to be expected under the proposed conditions of use. In addition, the information and data provided indicate that aqualysin 1 is inactivated during processing under the intended use conditions.

3.2. Dietary exposure

Exposure estimates were calculated using the methodology described in the CEF Panel statement on the exposure assessment of food enzymes (EFSA CEF Panel, 2016). The assessment of the food process covered in this opinion involved the selection of relevant food groups and the application of process and technical conversion factors (Appendix B). These input data were subject to a stakeholder consultation through open calls,[5] and adjusted in accordance with feedback received.

3.2.1. EFSA Comprehensive European Food Consumption Database

Since 2010, the EFSA Comprehensive European Food Consumption Database (hereafter the EFSA Comprehensive Database[6]) has been populated with detailed national data on food consumption. Competent authorities in European countries provide EFSA with data regarding the level of food consumption by individual consumers, as taken from the most recent national dietary survey in their country (EFSA, 2011a).

The food consumption data gathered by EFSA were collected using different methodologies and thus direct country-to-country comparisons should be made with caution. Depending on the food category and the level of detail used in exposure calculations, uncertainties might be introduced owing to subjects possibly underreporting and/or misreporting of consumption amounts. Nevertheless, the EFSA Comprehensive Database is the best available source of food consumption data across Europe.

Food consumption data from the population groups, infants, toddlers, children, adolescents, adults and the elderly, were used for the exposure assessment. For the present assessment, food consumption data were available from 33 different dietary surveys carried out in 19 European countries (Appendix A).

Consumption records were codified according to the FoodEx classification system (EFSA, 2011b).

3.2.2. Exposure assessment methodology

Chronic exposure was calculated based on individual consumption from the Comprehensive Database, averaged over the total survey period, excluding surveys with only one day per subject. High-level exposure/intake was calculated for only those population groups in which the sample size was sufficiently large to allow calculation of the 95th percentile (EFSA, 2011a).

The exposure per FoodEx category was subsequently added to derive an individual total exposure per day. Finally, these exposure estimates were averaged over the number of survey days and normalised for individual body weight (bw), resulting in an individual average exposure/day per kg bw for the survey period. This was done for all individuals in the survey and per age class, resulting in distributions of individual average exposure per survey and age class. Based on these distributions, the mean and 95th percentile exposures were calculated per survey for the total population and per age class.

3.2.3. Exposure to food enzyme–TOS according to the intended use proposed by the applicant

Exposure to the food enzyme–TOS was based on intended use and the recommended maximum use levels of the food enzyme–TOS provided by the applicant (190 mg TOS/kg flour). Food enzyme–TOS exposure was calculated from foods produced using a baking process.

Relevant food groups and/or individual foods were selected from the Comprehensive Database and were assumed to always contain the food enzyme–TOS at the maximum recommended use level. This will result in an overestimation of exposure to food enzyme–TOS.

[5] http://www.efsa.europa.eu/en/data/call/161110
[6] http://www.efsa.europa.eu/en/food-consumption/comprehensive-database

To facilitate matching of the reported use levels for baking processes with foods identified in the Comprehensive Database, the selected foods were disaggregated to ingredient level as appropriate, and converted into the corresponding raw material, i.e. flour, via the application of conversion factors (Appendix B). For example, consumption of 100 g of bread was converted into an intake of 70 g flour (recipe fraction of 0.7) and then multiplied by 190 mg TOS/kg flour, as provided by the applicant, to arrive at an exposure of 13.3 mg TOS/100 g bread.

Exposure to the food enzyme–TOS was calculated by multiplying values reported for each food category by their respective consumption amount per kilogram of body weight (kg bw) separately for each individual in the database. Table 2 provides an overview of the derived exposure estimates. Average and 95th percentile exposure to the food enzyme-TOS per age class, country and survey are reported in Appendix C – Table 1. The contribution of the food enzyme–TOS from each FoodEx category to the total dietary exposure is indicated in Appendix C – Table 2.

Table 2: Summary of estimated dietary exposure to food enzyme–TOS in six population groups

Population group	Estimated exposure (mg/kg bw per day)					
	Infants	**Toddlers**	**Children**	**Adolescents**	**Adults**	**The elderly**
Age range	3–11 months	12–35 months	3–9 years	10–17 years	18–64 years	≥ 65 years
Min–max of means (number of surveys)	0.133–0.580 (6)	0.497–1.200 (10)	0.521–1.134 (18)	0.311–0.754 (17)	0.224–0.458 (17)	0.216–0.399 (14)
Min–max of 95th percentiles (number of surveys)	0.770–1.633 (5)	1.118–2.024 (7)	0.975–2.132 (18)	0.557–1.493 (17)	0.433–0.901 (17)	0.409–0.718 (14)

bw: body weight.

3.2.4. Uncertainty analysis

In accordance with the guidance provided in the EFSA Opinion related to uncertainties in dietary exposure assessment (EFSA, 2007), the following sources of uncertainties have been considered and are summarised in Table 3.

Table 3: Qualitative evaluation of the influence of uncertainties on the dietary exposure estimate

Sources of uncertainties	Direction of impact
	Exposure to food enzyme–TOS
Model input data	
Consumption data: different methodologies/representativeness/ underreporting/misreporting/no portion size standard	+/–
Use of data from food consumption survey of a few days to estimate long-term (chronic) exposure for high percentiles (95th percentile)	+
Possible national differences in categorisation and classification of food	+/–
Model assumptions and factors	
FoodEx categories included in the exposure assessment were assumed to always contain the food enzyme–TOS	+
Exposure to food enzyme–TOS was always calculated based on the recommended maximum use level	+
Selection of broad FoodEx categories for the exposure assessment	+
Use of recipe fractions in disaggregation FoodEx categories likely to contain the food enzyme	+/–
Use of technical factors in the exposure model	+/–

TOS: total organic solids.
+: uncertainty with potential to cause overestimation of exposure; –: uncertainty with potential to cause underestimation of exposure.

The conservative approach applied to the exposure estimate to food enzyme–TOS, in particular, assumptions made regarding the occurrence and use levels of this specific food enzyme, is likely to have led to a considerable overestimation of the exposure.

3.3. Toxicological data

The Panel considered the test items used for the *in vitro* genotoxicity tests (Table 1, batches 4 and 5) as representative of the commercial food enzyme (Table 1, batches 1–3).

Batch 6 (Table 1) has been used for the repeated dose oral toxicity studies. It is a concentrate of the food enzyme prepared by the applicant to achieve a high dose within the volume limitations of a gavage study.

The aqualysin 1 activity (U/mL) of batch 6 is approximately 10 times higher than the average activity (U/mL) of the three commercial batches presented in Table 1. In contrast, the TOS content is increased upon concentration by only a factor of approximately 5, with the consequence that the activity/mg TOS in batch 6 is approximately twice as high as the average activity/mg TOS of the commercial batches.

This indicates that the employed concentration procedure did not only result in the loss of water but apparently also led to a loss of TOS constituent(s). Information on the part of the TOS that has been removed is not available.

On the other hand, there is also variability between the activities/mg TOS in the commercial batches. For example, the values for batches 2 and 3 differ by a factor of approximately two. This is comparable to the difference between the activity/mg TOS between batch 6 and the average of the commercial batches.

Therefore, the Panel considered batch 6 as acceptable to be used in the feeding study, however the Panel noted that uncertainties regarding the composition of TOS in this food enzyme concentrate remain.

3.3.1. Genotoxicity

3.3.1.1. Bacterial reverse mutation test

To investigate the potential of aqualysin 1 to induce gene mutations in bacteria, a bacterial reverse mutation assay (Ames test) was performed according to OECD Test Guideline 471 (OECD, 1997a) and following Good Laboratory Practice (GLP). Five strains of *Salmonella* Typhimurium (TA 97a, TA 98, TA 100, TA 102 and TA 1535) were used in the presence or absence of metabolic activation (S9-mix), applying plate incorporation method. The highest test sample concentration was also tested using the spot procedure. Two experiments were carried out using five different concentrations: 50, 160, 500, 1,600 and 5,000 μg food enzyme/plate, corresponding to 1.04, 3.33, 10.4, 33.3 and 104 μg TOS/plate, appropriate positive controls and sterile water as a negative control. All positive controls induced significant increases in revertant colony numbers, confirming the sensitivity of the tests and the efficacy of the S9-mix. The spot tests showed no zone of increased reversion or of toxicity. Due to cytotoxicity to strain TA 100, no analysis could be performed in the first experiment with metabolic activation (at concentrations of 160, 500, 1,600 and 5,000 μg food enzyme/plate). Therefore, in the second experiment six additional concentrations (0.5, 1.6, 5, 16, 50 and 160 μg food enzyme/plate, corresponding to 0.01, 0.03, 0.10, 0.33, 1.04 and 3.33 μg TOS/plate) were tested with S9-mix in strain TA 100. Upon treatment with the food enzyme, there was no increase in revertant colony numbers above control values in any of the strains, with or without metabolic activation.

The Panel concluded that the food enzyme aqualysin 1 did not induce gene mutations in the bacterial reverse mutation assay under the test conditions employed for this study.

3.3.1.2. *In vitro* mammalian chromosomal aberration test

The *in vitro* mammalian chromosomal aberration test was carried out in Chinese Hamster Ovary (CHO) cells according to OECD Test Guideline 473 (OECD, 1997b) and following GLP. Two experiments were performed. In the first experiment, applying a short treatment, duplicate cultures were exposed to the food enzyme for 4 h in the presence and absence of S9-mix, followed by 18 h recovery period. The following concentrations were tested: 500, 1,000 and 1,500 μg food enzyme/mL in the presence of S9-mix (corresponding to 14.2, 28.4 and 42.6 μg TOS/mL) and 200, 300 and 500 μg food enzyme/mL in the absence of the S9-mix (corresponding to 5.7, 8.5 and 14.2 μg TOS/mL). In the second experiment, two treatment conditions were applied: (i) continuous treatment (18 + 0 h), where cultures were exposed to

the food enzyme for 18 h without S9-mix at 750, 1,000 and 1,500 μg food enzyme/mL (corresponding to 21.3, 28.2 and 42.6 μg TOS/mL) and (ii) short treatment in the presence of S9-mix at 1,000, 1,300 and 2,000 μg food enzyme/mL (corresponding to 28.2, 36.2 and 56.8 μg TOS/mL). Two hundred metaphases were scored per experimental point. The reductions in mitotic index did not exceed 41% of negative control values at any concentration of food enzyme tested. The frequency of chromosomal aberrations in treated cultures was comparable to the values detected in negative controls and within the range of the laboratory historical solvent control data. A statistically significant increase in endoreduplicated cells was observed after short treatment in the presence of S9-mix together with a dose-related decrease of mitotic index up to 50% of negative control values; based on the OECD TG 473 (2014) the increase in the endoreduplicated cells is possibly due to cell cycle perturbation or cytotoxicity. The positive controls induced a significant increase in the frequency of cells with structural chromosomal aberrations. The Panel concluded that the food enzyme aqualysin 1 did not induce chromosomal damage in CHO cells under the test conditions employed for this study.

3.3.2. Subchronic toxicity

3.3.2.1. Repeated dose 14-day oral toxicity study

A repeated dose 14-day oral toxicity study in rodents was performed as a dose range finding study to evaluate the potential toxicity of the food enzyme following daily oral gavage for a further repeated dose 90-day oral toxicity study to be performed in the same species. GLP was not claimed.

Three groups of three male and three female Sprague–Dawley rats (Crl CD®(SD)IGS BR) received the food enzyme (batch 6) at the dose levels of 36.3, 145.1 or 290.1 U/kg bw per day, under a dosage-volume of 1.17, 4.67 or 9.33 mL/kg bw per day (referred to as low, mid and high dose) by oral gavage for 2 weeks. These doses correspond to 279, 1,116 and 2,232 mg TOS/kg bw per day. The control group received water by gavage at a constant dosage-volume of 9.33 mL/kg bw per day. The test item was provided as 'ready-to-use' dosage form with an enzymatic activity of 31.1 U/mL.

One high-dose female was sacrificed on day 10 for ethical reasons, showing the following clinical signs: hunched posture, piloerection, thin appearance, coldness to the touch, hypoactivity, ventral recumbency, tremors, abdominal breathing, half-closed eyes and soiled areas. A slight body weight loss, associated with a low food intake was recorded between days 4 and 8. At necropsy, black discoloration of the thymus and red discoloration in the lungs were observed in this animal.

No statistically significant differences in body weight and food intake were observed between control and all other exposed animals. A lower body weight gain (−48%) and a slightly lower food consumption (−14%) were recorded in high-dose males between days 1 and 4 but these findings were without effect on final mean body weight.

In high-dose animals hypersalivation (in all animals) was observed from the first week of treatment period. Piloerection (3/3 males, 2/3 females), hypoactivity (2/3 males), dyspnoea (3/3 males), soiled areas (2/3 males) and hunched posture (1/3 males and 1/3 females) appeared during the second week of the treatment period.

In the mid-dose group, hypersalivation (in all animals) occurred from the first week of treatment period and piloerection (in almost all animals) from day 10, dyspnoea (2/3 males on days 10 or 11, 1/3 females on days 10 and 11) and hunched posture (1/3 females from day 13).

In the low-dose group, only piloerection (1/3 males) from day 10 and hypersalivation (1/3 males) from day 14 were recorded. No clinical signs were observed in females.

In the control group no clinical signs and pathological–anatomical changes were observed.

Based on the above results the applicant selected the low dose of this range finding study as high dose for the repeated dose 90-day oral toxicity study.

3.3.2.2. Repeated dose 90-day oral toxicity study in rodents

A repeated dose 90-day oral toxicity study in rodents was performed in accordance with OECD Test Guideline 408 (OECD, 1998) and following GLP. Three groups of 10 male and 10 female Sprague–Dawley rats received the food enzyme (batch 6) at the dose levels of 12.8, 25.6 and 38.4 U/kg bw per day (referred to as low, mid and high dose) by oral gavage. These doses correspond to 98.5, 197 and 295 mg TOS/kg bw per day. The control group received water by gavage under a constant dosage-volume of 1.23 mL/kg bw per day.

There were no premature deaths or unscheduled sacrifices during the study, except for a single low dose female, sacrificed prematurely on week 13 after blood sampling.

Moderate increases in the mean body weights and relative body weight gains of food enzyme-treated females were observed from week 2 up to week 11. No clear dose relationship was observed and these changes did not correlate with changes in food intake.

During the 13-week treatment period, mainly dose-related hypersalivation was observed in food enzyme-treated groups in both sexes. Increased incidence of fur and skin staining was observed in all food enzyme-treated female groups. These observations appeared to be associated with the delivery of the food enzyme but were considered as non-adverse.

Blood biochemistry investigations at the end of the treatment period showed minimally lower inorganic phosphorus levels in high-dose females (13%), and minimally elevated levels of creatinine (9%) in mid- and high-dose females. A trend towards an increase was observed in aspartate aminotransferase activity in mid- and high-dose (22%) males. The values are within the historical control range and not considered adverse.

No effects on food intake, ophthalmological findings, haematology, functional observation battery tests, organ weight, macroscopic or microscopic evaluations were observed. It was concluded that under the conditions of this repeated dose 90-day oral toxicity study the NOAEL is 38.4 U/kg bw per day, corresponding to 295 mg TOS/kg bw per day.

A comparison of the NOAEL (295 mg TOS/kg bw per day) from the 90-day study with the derived dietary exposure estimates of 0.521–1.134 mg/kg bw per day at the mean and 0.975–2.132 mg TOS/kg bw per day at the 95th percentile, resulted in a margin of exposure (MOE) of 138.

3.4. Allergenicity

The allergenicity assessment considers only the food enzyme and not any carrier or other excipient which may be used in the final formulation.

The allergenicity of aqualysin 1 produced with the genetically modified *B. subtilis* strain LMGS 25520 has been assessed by comparison of its amino acid sequence with those of known allergens, according to the EFSA Scientific opinion on the assessment of allergenicity of GM plants and microorganisms and derived food and feed of the Scientific Panel on Genetically Modified Organisms (EFSA GMO Panel, 2010). Using higher than 35% identity in a sliding window of 80 amino acids as criterion 23 matches were found, of which 20 matched with respiratory allergens (mainly fungal allergens) and 3 matched with dermal allergens.

Several cases of respiratory allergy following occupational inhalation of aerosols containing proteases or other enzymes have been reported (Martel et al., 2010). However, aqualysin 1 from *B. subtilis* strain LMGS 25520 is not described as an allergen and no food allergic reactions to this food enzyme have been reported. Although 20 of the matches found were with mainly occupational respiratory fungal allergens, several studies have shown that patients with occupational asthma to a food enzyme (e.g. α-amylase) can commonly ingest the corresponding enzyme without acquiring clinical symptoms of food allergy (Cullinan et al., 1997; Brisman, 2002; Poulsen, 2004; Armentia et al., 2009). The match with the three dermal allergens is not relevant in respect to the ingestion of the food enzyme as protein based dermal allergies (atopic dermatitis) are mainly triggered by respiratory exposure. Therefore there is no indication for potential allergenicity of the food enzyme aqualysin 1.

The cross-reactivity of food enzymes was studied by Bindslev-Jensen et al. (2006). There were no indications of cross-reactivity upon oral exposure to 19 tested food enzymes and the main allergens represented by the 400 patients (allergic to inhalation allergens, food allergens, allergens of bee or wasp) included in this study.

Taken together, the CEF Panel considers that there are no indications for food allergic reactions by dietary exposure to aqualysin 1 produced with *B. subtilis* strain LMGS 25520.

Conclusions

The genetic modifications performed, the manufacturing process, the compositional and biochemical data, the dietary exposure, as well as the allergenicity and the genotoxicity assessment did not raise safety concerns.

The Panel considered the MOE calculated from the NOAEL determined from the repeated dose 90-day oral toxicity study and the estimated dietary exposure as insufficient to conclude that there is no safety concern for this food enzyme under the intended conditions of use.

The Panel noted that recombinant DNA was demonstrated to be present in all batches of the food enzyme tested.

Documentation provided to EFSA

1) 'Application for authorisation of Aqualysin 1 with a genetically modified strain of *Bacillus subtilis* in accordance with Regulation (EC) No 1331/2008'. November 2014. Submitted by Puratos NV.
2) Summary reports on technical, toxicological and genetic modifications data were delivered by Hylobates Consulting/BiCT (Rome, Italy) on 22 February 2016, FoBiG GmbH (Freiburg, Germany) on 22 February 2015 and by the Technical University of Denmark (Søborg, Denmark) on 9 September 2015, respectively.
3) Additional information received from Puratos NV in March 2015.
4) Additional information received from Puratos NV in August 2017.

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Abbreviations

bw	body weight
BCCM	Belgian Co-ordinated Collection of Microorganisms
CAS	Chemical Abstracts Service
CFU	colony forming units
CHO	Chinese Hamster Ovary
EC	Enzyme Commission
EINECS	European Inventory of Existing Commercial Chemical Substances
FAO	Food and Agricultural Organization
GLP	Good Laboratory Practice
GMO	genetically modified organisms
GMP	Good Manufacturing Practice
IUBMB	International Union of Biochemistry and Molecular Biology
JECFA	Joint FAO/WHO Expert Committee on Food Additives
MIC	minimum inhibitory concentration
MOE	margin of exposure
NOAEL	no observed adverse effect level
OECD	Organisation for Economic Cooperation and Development
PCR	polymerase chain reaction
RNA	ribonucleic acid
SDS–PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
QPS	Qualified Presumption of Safety
TOS	total organic solids
WHO	World Health Organization

Appendix A – Population groups considered for the exposure assessment

Population	Age range	Countries with food consumption surveys covering more than one day
Infants	From 12 weeks on up to and including 11 months of age	Bulgaria, Denmark, Finland, Germany, Italy, United Kingdom
Toddlers	From 12 months up to and including 35 months of age	Belgium, Bulgaria, Denmark, Finland, Germany, Italy, Netherlands, Spain, United Kingdom
Children[(a)]	From 36 months up to and including 9 years of age	Austria, Belgium, Bulgaria, Czech Republic, Denmark, Finland, France, Germany, Greece, Italy, Latvia, Netherlands, Spain, Sweden, United Kingdom
Adolescents	From 10 years up to and including 17 years of age	Austria, Belgium, Cyprus, Czech Republic, Denmark, Finland, France, Germany, Italy, Latvia, Spain, Sweden, United Kingdom
Adults	From 18 years up to and including 64 years of age	Austria, Belgium, Czech Republic, Denmark, Finland, France, Germany, Hungary, Ireland, Italy, Latvia, Netherlands, Romania, Spain, Sweden, United Kingdom
The elderly[(a)]	From 65 years of age and older	Austria, Belgium, Denmark, Finland, France, Germany, Hungary, Ireland, Italy, Romania, Sweden, United Kingdom

(a): The terms 'children' and 'the elderly' correspond, respectively, to 'other children' and the merge of 'elderly' and 'very elderly' in the Guidance of EFSA on the 'Use of the EFSA Comprehensive European Food Consumption Database in Exposure Assessment' (EFSA, 2011a).

Appendix B – FoodEx categories used to derive exposure estimates for the food enzyme–TOS and the respective conversion factors

FoodEx code	FoodEx category	Conversion factor from FoodEx food group to raw material[(a)]	Recipe fraction	mg TOS/kg flour
A.01	Grains and grain-based products (unspecified)	0.8	1	190
A.01.03	Grain milling products (unspecified)	1	1	190
A.01.03.001	Wheat milling products (unspecified)	1	1	190
A.01.03.001.001	Wheat flour, brown	1	1	190
A.01.03.001.002	Wheat flour, Durum	1	1	190
A.01.03.001.003	Wheat flour, white	1	1	190
A.01.03.001.004	Wheat flour, wholemeal	1	1	190
A.01.03.001.005	Graham flour	1	1	190
A.01.03.001.006	Wheat flour, gluten free	1	1	190
A.01.03.001.014	Wheat starch	1.2	1	190
A.01.03.002	Rye milling products (unspecified)	1	1	190
A.01.03.002.001	Rye flour, gluten free	1	1	190
A.01.03.002.002	Rye flour, light	1	1	190
A.01.03.002.003	Rye flour, medium	1	1	190
A.01.03.002.004	Rye flour, wholemeal	1	1	190
A.01.03.003	Buckwheat milling products (unspecified)	1	1	190
A.01.03.003.001	Buckwheat flour	1	1	190
A.01.03.004	Corn milling products (unspecified)	1	1	190
A.01.03.004.001	Corn flour	1	1	190
A.01.03.004.003	Corn starch	1.3	1	190
A.01.03.005	Oat milling products (unspecified)	1	1	190
A.01.03.005.002	Oat flour	1	1	190
A.01.03.005.004	Oat starch	1.2	1	190
A.01.03.006	Rice milling products (unspecified)	1	1	190
A.01.03.006.001	Rice flour	1	1	190
A.01.03.006.002	Rice flour white	1	1	190
A.01.03.006.003	Rice flour, instant	1	1	190
A.01.03.006.004	Rice starch	1.2	1	190
A.01.03.007	Spelt milling products	1	1	190
A.01.03.008	Other milling products (unspecified)	1	1	190
A.01.03.008.001	Amaranth flour	1	1	190
A.01.03.008.002	Barley flour	1	1	190
A.01.03.008.003	Chapatti flour	1	1	190
A.01.03.008.004	Flour mix, wheat/rye/barley/oats	1	1	190
A.01.03.008.005	Millet flour	1	1	190
A.01.03.008.007	Sorghum flour	1	1	190
A.01.04	Bread and rolls (unspecified)	1	0.7	190
A.01.04.001	Wheat bread and rolls	1	0.7	190
A.01.04.002	Rye bread and rolls	1	0.7	190
A.01.04.003	Mixed wheat and rye bread and rolls	1	0.7	190
A.01.04.004	Multigrain bread and rolls	1	0.7	190
A.01.04.005	Unleavened bread, crisp bread and rusk (unspecified)	1	0.8	190
A.01.04.005.001	Crisp bread, rye wholemeal	1	0.9	190

FoodEx code	FoodEx category	Conversion factor from FoodEx food group to raw material[a]	Recipe fraction	mg TOS/kg flour
A.01.04.005.002	Crisp bread, rye, light	1	0.9	190
A.01.04.005.003	Crisp bread, wheat, wholemeal	1	0.9	190
A.01.04.005.004	Crisp bread, wheat, light	1	0.9	190
A.01.04.005.005	Rusk, light	1	0.9	190
A.01.04.005.006	Rusk, wholemeal	1	0.9	190
A.01.04.005.007	Pita bread	1	0.7	190
A.01.04.005.008	Matzo	1	0.9	190
A.01.04.005.009	Tortilla	1	0.7	190
A.01.04.006	Other bread	1	0.7	190
A.01.04.007	Bread products	1	0.7	190
A.01.07	Fine bakery wares (unspecified)	1	0.5	190
A.01.07.001	Pastries and cakes (unspecified)	1	0.5	190
A.01.07.001.001	Beignets	1	0.15	190
A.01.07.001.002	Buns	1	0.7	190
A.01.07.001.003	Cake from batter	1	0.25	190
A.01.07.001.004	Cheese cream cake	1	0.24	190
A.01.07.001.005	Cheese cream sponge cake	1	0.24	190
A.01.07.001.006	Chocolate cake	1	0.24	190
A.01.07.001.007	Chocolate cake with fruits	1	0.24	190
A.01.07.001.008	Cream cake	1	0.24	190
A.01.07.001.009	Cream cheese cake	1	0.24	190
A.01.07.001.010	Cream custard cake	1	0.24	190
A.01.07.001.011	Cream custard sponge cake	1	0.24	190
A.01.07.001.012	Croissant	1	0.5	190
A.01.07.001.013	Croissant, filled with chocolate	1	0.5	190
A.01.07.001.014	Croissant, filled with cream	1	0.5	190
A.01.07.001.015	Croissant, filled with jam	1	0.5	190
A.01.07.001.016	Croquembouche	1	0.15	190
A.01.07.001.017	Doughnuts	1	0.24	190
A.01.07.001.018	Clair	1	0.15	190
A.01.07.001.019	Flan	1	0.5	190
A.01.07.001.020	Fruit cake	1	0.6	190
A.01.07.001.021	Fruit pie	1	0.15	190
A.01.07.001.022	Cheese pie	1	0.15	190
A.01.07.001.023	Fruit tart	1	0.15	190
A.01.07.001.024	Gingerbread	1	0.6	190
A.01.07.001.025	Gougere	1	0.15	190
A.01.07.001.026	Kringles	1	0.25	190
A.01.07.001.027	Nut cream cake	1	0.24	190
A.01.07.001.028	Pancakes	1	0.25	190
A.01.07.001.029	Profiterole	1	0.15	190
A.01.07.001.030	Pyramid cake	1	0.25	190
A.01.07.001.031	Rhubarb flan	1	0.15	190
A.01.07.001.032	Scone	1	0.5	190
A.01.07.001.033	Sponge dough	1	0.25	190
A.01.07.001.034	Sponge cake	1	0.25	190

FoodEx code	FoodEx category	Conversion factor from FoodEx food group to raw material[(a)]	Recipe fraction	mg TOS/kg flour
A.01.07.001.035	Sponge cake roll	1	0.25	190
A.01.07.001.036	Muffins	1	0.25	190
A.01.07.001.037	Waffles	1	0.25	190
A.01.07.001.038	Apple strudel	1	0.15	190
A.01.07.001.039	Cream-cheese strudel	1	0.24	190
A.01.07.001.040	Cheese pastry goods from puff pastry	1	0.15	190
A.01.07.001.041	Croissant from puff pastry	1	0.6	190
A.01.07.001.042	Brioche	1	0.5	190
A.01.07.001.044	Lebkuchen	1	0.6	190
A.01.07.001.045	Dumpling	1	0.5	190
A.01.07.001.046	Cake marbled, with chocolate	1	0.5	190
A.01.07.001.047	Marzipan pie	1	0.25	190
A.01.07.001.048	Baklava	1	0.15	190
A.01.07.002	Biscuits (cookies)	1	0.9	190
A.01.07.002.001	Biscuits, sweet, plain	1	0.9	190
A.01.07.002.002	Biscuits, chocolate filling	1	0.81	190
A.01.07.002.003	Biscuits, cream filling	1	0.81	190
A.01.07.002.004	Biscuits, fruit filling	1	0.81	190
A.01.07.002.005	Biscuits, vanilla filling	1	0.81	190
A.01.07.002.006	Butter biscuits	1	0.81	190
A.01.07.002.007	Biscuit, iced	1	0.81	190
A.01.07.002.008	Speculaas	1	0.9	190
A.01.07.002.009	Biscuits, sweet, wheat wholemeal	1	0.9	190
A.01.07.002.010	Biscuits, oat meal	1	0.9	190
A.01.07.002.011	Biscuits, spelt meal	1	0.9	190
A.01.07.002.012	Biscuits, salty	1	0.9	190
A.01.07.002.013	Biscuits, salty, with cheese	1	0.81	190
A.01.07.002.014	Sticks, salty	1	0.81	190
A.17.03.003	Biscuits, rusks and cookies for children	1	0.9	190
A.18.04.001	Find bakery products for diabetics	1	0.5	190
A.19.01.001	Sandwich and sandwich-like meal	1	0.32	190
A.19.01.002	Pizza and pizza-like pies	1	0.3	190

TOS: total organic solids.

(a): Food and Agriculture Organization of the United Nations. Technical Conversion Factors for Agricultural Commodities. Available from: http://www.fao.org/economic/the-statistics-division-ess/methodology/methodology-systems/technical-conversion-factors-for-agricultural-commodities/en/

Appendix C – Dietary exposure estimates to the food enzyme-TOS in details

Information provided in this appendix is shown in an excel file (http://onlinelibrary.wiley.com/wol1/doi/10.2903/j.efsa.2018.5170/suppinfo).

The file contains two sheets, corresponding to two tables.

Table 1: Average and 95th percentile exposure to the food enzyme-TOS per age class, country and survey

Table 2: The contribution of the food enzyme–TOS from each FoodEx category to the total dietary exposure