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Safety evaluation of the food enzyme xylanase from a genetically modified *Bacillus subtilis* strain TD160(229)

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Abstract

The food enzyme considered in this opinion is an endo-1,4-β-xylanase (EC 3.2.1.8) produced with a genetically modified *Bacillus subtilis* strain from Puratos N.V. (Belgium). The genetic modifications do not raise safety concerns. The food enzyme contains neither the production organism nor recombinant DNA. The endo-1,4-β-xylanase is intended to be used in baking processes. Based on the maximum use levels recommended for the baking processes, dietary exposure to the food enzyme–total organic solids (TOS) was estimated on the basis of individual data from the EFSA Comprehensive European Food Consumption Database. This exposure estimate is up to 0.008 mg TOS/kg body weight per day in European populations. The food enzyme did not induce gene mutations in bacteria nor clastogenic activity in human lymphocytes. Therefore, there is no concern with respect to genotoxicity. The subchronic toxicity was assessed by means of a repeated dose 90-day oral toxicity study in rodents. A no observed adverse effect level was derived, which, compared with the dietary exposure, results in a sufficiently high margin of exposure. The allergenicity was evaluated by searching for similarity of the amino acid sequence to those of known allergens; no matches were found. The Panel considered that there are no indications for food allergic reactions to this xylanase. Based on the microbial source, genetic modifications performed, the manufacturing process, the compositional and biochemical data provided, the findings in the toxicological studies and allergenicity assessment, this food enzyme does not give rise to safety concerns under the intended conditions of use.

Keywords: food enzyme, xylanase, endo-1, 4-β-xylanase, 4-β-D-xylan xylanohydrolase, EC 3.2.1.8, *Bacillus subtilis*, genetically modified microorganism

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Note: The full opinion will be published in accordance with Article 12 of Regulation (EC) No 1331/2008 once the decision on confidentiality will be received from European Commission. To avoid confusion, the original version of the scientific opinion has been removed from EFSA Journal, but is available on request, as is a version showing all the changes made.

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1. Introduction

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

> 'Food enzyme' means a product obtained from plants, animals or microorganisms or products thereof including a product obtained by a fermentation process using microorganisms: (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 facilitate their storage, sale, standardisation, dilution or dissolution.

Before January 2009, food enzymes other than those used as food additives were not regulated or were 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 in the manufacture, processing, preparation, treatment, packaging, transport or storage of such food, including enzymes used as processing aids. Regulation (EC) No 1331/2008[2] set up 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,
- 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.

Four applications have been introduced by the companies 'Advanced Enzyme Technologies Ltd', 'DuPont Nutrition Biosciences ApS.', 'Amano Enzyme Inc' and 'Puratos NV sa' for the authorisation of the food enzymes Amylase from *Bacillus amyloliquefaciens* (strain BANSC), Beta-amylase from barley (*Hordeum vulgare*), Triacylglycerol lipase from *Rhizopus niveus* (strain AE-N) and Xylanase from a genetically modified strain of *Bacillus subtilis* TD160(229).

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

1.1.2. Terms of Reference

The European Commission requests EFSA to carry out the safety assessments on the food enzymes Amylase from *Bacillus amyloliquefaciens* (strain BANSC), Beta-amylase from barley (*Hordeum vulgare*), Triacylglycerol lipase from *Rhizopus niveus* (strain AE-N) and Xylanase from a genetically modified

[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, pp. 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, pp. 1–6.

strain of *Bacillus subtilis* TD160(229) 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 request to carry out the safety assessment of the food enzyme Xylanase from a genetically modified *Bacillus subtilis* strain TD160 (229).

1.3. Information on existing authorisations and evaluations

The applicant reports that the French and Australian/New Zealand authorities have evaluated and authorised the use of xylanase from self-cloned *B. subtilis* in a number of food and beverage manufacturing processes. Conditions of use were not specified. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has evaluated xylanase from genetically modified self-cloned *B. subtilis* (FAO/WHO, 2004).

2. Data and methodologies

2.1. Data

The applicant has submitted a dossier supporting the application for authorisation of the food enzyme xylanase produced with a genetically modified *Bacillus subtilis* strain TD160(229). 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 dossier with the exception of the exposure assessment, which was carried out in accordance to 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:	Endo-1,4-β-xylanase
Systematic name:	4-β-D-Xylan xylanohydrolase
Synonyms:	Xylanase; endo-1,4-D-β-xylanase
IUBMB No.:	EC 3.2.1.8
CAS No.:	9025-57-4
EINECS No.:	232-800-2.

3.1.2. Chemical parameters

The xylanase produced with the genetically modified *Bacillus subtilis* strain TD160(229) consists of a [redacted], including a signal sequence of [redacted], which is cleaved off during the secretion of the enzyme. The molecular mass of the mature protein, derived from the amino acid sequence, was calculated to be [redacted] The apparent molecular mass based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) pattern is about [redacted], equivalent to the calculated molecular mass of the food enzyme.

The food enzyme was tested for other enzyme activities, i.e. amylase and protease, which were below the limits of detection (LOD), except for one commercial batch with a very low amount of amylase and protease activities. No other enzymatic side activities have been reported by the applicant.

Data on the chemical parameters of the food enzyme have been provided for three batches used for commercialisation (1, 2 and 3) and additional three batches (4, 5 and 6) used for toxicological testing (Table 1). The average total organic solids (TOS) of the three commercial food enzyme batches was 1.9%; the values ranged from 1.4% to 3.0% (Table 1). The six food enzyme batches presented in Table 1 are liquid concentrates with no added diluents.

The average enzyme activity/TOS ratio of the three food enzyme batches for commercialisation was 41.92 Skalar xylanase units (SXU)/mg TOS; the values ranged from 35.97 to 45.57 SXU/mg TOS (Table 1).

Table 1: Compositional data of the food enzyme

Parameter	Units	Batches					
		1	2	3	4[(a)]	5[(b)]	6[(c)]
Xylanase activity	SXU/g batch[(d)]	638	619	1,079	297	283	1,001
Protein	%	1.2	1.1	2.2	NA[(e)]	NA	1.9
Ash	%	2.3	2.4	2.0	NA	1.21	2.7
Water	%	96.3	96.2	95.0	NA	97.4	95.1
Total organic solids (TOS)[(f)]	%	1.4	1.4	3.0	1.4	1.39	2.2
Xylanase activity/mg TOS	SXU/mg TOS	45.57	44.21	35.97	21.21	20.36	45.50

(a): Batch for bacterial reverse mutation test.
(b): Batch for *in vitro* mammalian chromosome aberration test and repeated dose 90-day oral toxicity study.
(c): Batch for *in vivo* mammalian erythrocyte micronucleus test.
(d): SXU/g: Skalar xylanase unit/g (see Section 3.1.3).
(e): NA: not analysed.
(f): TOS calculated as 100% − % water − % ash.

The food enzyme complies with the specification for lead (≤ 5 mg/kg) as laid down in the general specifications and considerations for enzymes used in food processing (FAO/WHO, 2006). The Panel considered that the concentrations of As, Cd and Hg are not of concern as they are well below the specification levels set for food additives (As: 3 mg/kg; Cd and Hg: 1 mg/kg) (EU Regulation 231/2012[3]). 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 Panel considered the compositional data provided for the food enzyme as sufficient.

3.1.3. Properties of the food enzyme

Xylanase catalyses the hydrolysis of 1,4-β-D-xylosidic linkages in xylan resulting in the generation of (1→4)-β-D-xylan oligosaccharides of different lengths.

The xylanase activity is measured based on the hydrolysis of xylan and is expressed in SXU/g. The analytical principle is based on hydrolysis of xylan to reducing carbohydrates (reaction conditions: pH 4.5, 30°C and 30 min). After 30 min, the enzymatic reaction is stopped by the addition of neocuproine at 95°C. It reacts with the reducing sugars producing a colour, which is measured spectrophotometrically at 460 nm. One SXU is defined as the amount of enzyme that liberates 1 micromole of reducing sugars (measured as xylose equivalents) from beech wood xylan in 1 min/mL under the standard assay conditions.

The xylanase activity has been characterised under different temperature and pH conditions. The temperature profile has been measured from 30°C up to 70°C at pH 2.0–8.0. The optimum is at 50°C, pH 6.0. The xylanase is active at temperatures up to 70°C (approximately 40% relative activity at 70°C, pH 6.0). The pH profile showed 20% relative activity at pH 7.0, 50°C. The thermostability of the

[3] Regulation (EU) No 231/2012 of the European Parliament and of the Council of 9 March 2012 laying down specifications for food additives listed in Annexes II and III to Regulation (EC) No 1333/2008 of the European Parliament and of the Council. OJ L 83/1, 22.3.2012.

xylanase was tested at 50°C and 60°C after incubating up to 240 min at pH 4.5. At this pH, the xylanase stability decreases rapidly at 50°C and very rapidly at 60°C, showing 10% residual activity after 240 min incubation at 50°C, and no residual activity after 10 min at 60°C. The activity itself was measured under standard assay conditions.

3.1.4. Information on the source material

3.1.4.1. Information on the genetically modified microorganism

The xylanase production strain *Bacillus subtilis* strain TD160(229) is deposited in the Belgian Co-ordinated Collection of Microorganisms, University of Gent, with the deposit number LMG S-28355.

3.1.4.2. Characteristics of the recipient and parental microorganisms

The parental microorganism is *B. subtilis* [redacted], a derivative of *Bacillus subtilis* [redacted] The recipient strain is *B. subtilis* [redacted]

Bacillus subtilis has been recommended for Qualified Presumption of Safety (QPS) status, with the qualification that the absence of acquired antibiotic resistance genes and toxigenic activity are verified for the specific strain used (EFSA BIOHAZ Panel, 2017). The recipient strain was identified as *B. subtilis* by 16S rDNA analysis and showed no cytotoxic activity in Vero cells (Pedersen et al., 2002). [redacted] Consequently, the parental strain is presumed to be safe for production purposes.

The recipient strain, *B. subtilis* [redacted] has been developed from the parental strain *B. subtilis* [redacted]

3.1.4.3. Characteristics of the donor organisms

The donor for the xylanase gene was *B. subtilis* [redacted]

3.1.4.4. Description of the genetic modification process

The production strain *Bacillus subtilis* TD160(229) was developed from the recipient strain [redacted]

[redacted]

3.1.4.5. Safety aspects of the genetic modification

[redacted]

3.1.5. Manufacturing process

The food enzyme is manufactured according to the Food Hygiene Regulation (EC) No 852/2004,[4] with food safety procedures based on HACCP (Hazard Analysis and Critical Control Points), and in accordance with current Good Manufacturing Practice (GMP).

The food enzyme is produced by a pure culture in contained, submerged, fed-batch fermentation system with conventional process controls in place. The identity and purity of the culture are checked at each transfer step from frozen vials to the end of fermentation.

The downstream processing includes recovery, purification and concentration, formulation and packaging. The food enzyme produced is recovered from the fermentation broth after biomass separation via filtration. Further purification and concentration involve [redacted] a series of filtration steps, including ultrafiltration and final sterile filtration.

Subsequently, the food enzyme concentrate is formulated and commercialised as a liquid or a solid product. To this end, the concentrated food enzyme solution is standardised by addition of [redacted] used as a carrier for the manufacturing of dry enzyme preparations.

The absence of the production microorganism in the food enzyme was demonstrated [redacted]

No recombinant DNA was detected in four batches tested in triplicate. [redacted]

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

3.1.6. Safety for the environment

The production strain and its recombinant DNA were not detected in the final product. Therefore, no environmental risk assessment is required (EFSA GMO Panel, 2011).

3.1.7. Reaction and fate in food

The xylanase catalyses the hydrolysis of 1,4-β-D-xylosidic linkages in xylan, resulting in the generation of (1→4)-β-D-xylan oligosaccharides of different chain lengths. These reaction products are naturally present in xylan-containing foods.

[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, pp. 3–21.

The data and information provided indicate that the xylanase is inactivated during baking processes.

3.1.8. Case of need and intended conditions of use

This xylanase is intended to be used in baking processes at a recommended use level up to 0.752 mg TOS/kg flour.

In baking processes, the xylanase food enzyme is added to the raw materials during the preparation of the dough. It is used to hydrolyse (arabino)xylans, which interact with gluten and bind water, so contributing to the reduction of dough viscosity. The decrease in dough viscosity facilitates the handling of the dough, gives improved crumb structure and increases the volume.

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 processes covered in this opinion involved selection of relevant food groups and 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 on the level of food consumption by individual consumers, as taken from the most recent national dietary survey in their country (EFSA, 2011a). New consumption surveys recently added to the Comprehensive Database were also taken into account in this assessment.

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

Food consumption data from the following 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, 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 (Appendix B) 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 kilogram of body weight 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.

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

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 (Section 3.1.8). Food enzyme–TOS exposure was calculated from foods produced involving 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.

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 0.752 mg TOS/kg flour, as provided by the applicant, to arrive at an exposure of 0.05 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 separately for each individual in the database. Table 2 provides an overview of the derived exposure estimates. The 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	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
	Estimated exposure (mg/kg body weight per day)					
Min–max of means (number of surveys)	0.000–0.002 (6)	0.002–0.005 (10)	0.002–0.004 (18)	0.001–0.003 (17)	0.001–0.002 (17)	0.001–0.002 (14)
Min–max of 95th percentiles (number of surveys)	0.003–0.006 (5)	0.004–0.008 (7)	0.004–0.008 (18)	0.002–0.006 (17)	0.002–0.004 (17)	0.002–0.003 (14)

3.2.4. Uncertainty analysis

Uncertainties in the exposure assessment of the food enzyme have been discussed above. 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	+/–

Sources of uncertainties	Direction of impact
	Exposure to food enzyme–TOS
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 based on the description of the food process provided by the applicant (based on examples given by applicant)	+
Use of recipe fractions in disaggregation FoodEx categories likely to contain the food enzyme	+/–

+: uncertainty with potential to cause over-estimation 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 on the occurrence and use levels of this specific food enzyme, is likely to have led to a considerable over-estimation of the exposure.

3.3. Toxicological data

The batches used for the toxicological assays are described in Table 1. They differ in their enzyme activities. Batch number 6 showed a xylanase activity comparable to the commercial batches, while batches 4 and 5 have lower enzyme activities per g food enzyme and per mg TOS compared to the commercial batches.

3.3.1. Genotoxicity

3.3.1.1. Bacterial reverse mutation test

In order to investigate the potential to induce gene mutations in bacteria, the Ames test was performed according to Organisation for Economic Co-operation and Development (OECD) Test Guideline No. 471 of Chemicals. (OECD, 1983a), No. 472 OECD Test Guideline (OECD, 1983b) and the Proposal for Replacement of Guidelines 471 and 472, bacterial reverse mutation test (OECD, 1997a) and following Good Laboratory Practice (GLP), in *Salmonella* Typhimurium (strains TA1535, TA100, TA1537 and TA98) and in *Escherichia coli* WP2*uvrA* pKM *101*, in the presence or absence of metabolic activation by S9-mix. Two experiments in triplicate were carried out using five different concentrations of the food enzyme (50, 150, 500, 1,500 and 5,000 μg TOS/plate), appropriate positive control chemicals, and sodium acetate buffer as a negative control. The first test was a standard plate incorporation assay, and the second test was performed as pre-incubation assay. All positive control chemicals showed a distinct increase of induced revertant colonies, confirming the sensitivity of the tests and the efficacy of the S9-mix. Upon treatment with the food enzyme, there was no increase in revertant colony numbers or cytotoxicity. Therefore, the Panel concluded that the food enzyme has no mutagenic activity under the conditions employed in this study.

3.3.1.2. *In vitro* mammalian chromosome aberration test

The *in vitro* mammalian chromosome aberration test was carried out according to the OECD Test Guideline 473 (OECD, 1983c) and following GLP. Cultured human peripheral blood lymphocytes from a single donor, the proliferation of which was stimulated with phytohaemagglutinin (PHA), were treated with the food enzyme, culture medium (vehicle control) or appropriate positive controls. Two experiments were performed in duplicate. In the first experiment the cultures were exposed to the food enzyme (1,250, 2,500 and 5,000 μg dry matter/mL, corresponding to 527, 1,053 and 2,106 μg TOS/mL, based on dry matter of 3.3% according to study protocol), in the absence of S9 mix continuously for 19 and 43 h, while in presence of S9 mix, cells were treated for 3 h and harvested after 16 h or 40 h. Cultures treated with 5,000 μg dry matter/mL in both main tests showed reduction in mean mitotic index in the range 54–67% in the absence of S9-mix (short term and long term) and 7–17% in the presence of S9-mix (short term), compared with the solvent control value. Per culture, 200 lymphocytes were analysed for the presence of chromosomal aberrations, aneuploidy and

endoreduplication. Only cells with 44–46 chromosomes were considered. The positive controls caused statistically significant increases in the proportion of aberrant cells in each test, demonstrating the sensitivity of the test system and the efficacy of the S9-mix. The negative controls fell within the range of historical negative controls. Statistically significant increase in the number of chromosomal aberrations excluding gaps was observed after 43 h of continuous treatment with the highest concentration evaluated (5,000 μg dry matter/mL) in the absence of S9-mix (6.0% aberrant cells; historical range 0–4%). Nevertheless, the increase is considered biologically irrelevant since the increase was not reproducible in the replicate culture or in parallel test. In the presence of 5,000 μg dry matter/mL the mitotic index was reduced by 54%. For all other food enzyme concentrations used, the frequency of cells with chromosomal aberrations was similar to that of negative controls. The Panel concluded that the food enzyme did not induce chromosomal aberration in cultured human peripheral blood lymphocytes when tested up to 5,000 μg dry matter/mL (corresponding to ca. 2,106 μg TOS/mL) under the experimental conditions employed.

3.3.1.3. *In vivo* mammalian erythrocyte micronucleus test

The *in vivo* micronucleus test was performed according to OECD Guideline 474 (OECD, 1997b) and following GLP. Male and female mice Swiss Ico: OFI (IOPS Caw) received two treatments of the food enzyme by gavage at dose levels of 500, 1,000 and 2,000 mg food enzyme/kg bw per day (corresponding to 0, 11, 22 and 44 mg TOS/kg bw per day) at a 24-h interval. A preliminary toxicity test had shown that a dose of 2,000 mg food enzyme/kg per day, the limit dose for the micronucleus test, was tolerated; this level was, therefore, selected as an appropriate maximum. The negative control group received the vehicle, drinking water. A positive control group received a single treatment of cyclophosphamide at a dose of 50 mg/kg bw per day. Bone marrow smears were prepared 24 h after the last treatment (n = 10, five male and five female mice per group). For each animal, the number of micronucleated polychromatic erythrocytes (MNPE) was counted in 2,000 polychromatic erythrocytes. The polychromatic erythrocytes (PE) and normochromatic erythrocyte (NE) ratio was decided on by scoring a total of 1,000 erythrocytes. A record of the incidence of micronucleated mature erythrocytes was also kept. No statistically significant increases in the frequency of MNPE and no substantial decrease in the proportion of immature erythrocytes were observed in mice treated with the food enzyme, compared with vehicle control values. The positive control produced significant increases in the frequency of micronucleated immature erythrocytes. The Panel considered this study of limited validity because no data on bone marrow exposure were provided.

The Panel concluded on the basis of the *in vitro* studies that there is no concern for genotoxicity for the TOS enzyme tested.

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

A repeated dose 90-day oral toxicity study was performed according to OECD Test Guideline 408 (OECD, 1981), and following GLP. Groups of 10 male and 10 female CD rats received the food enzyme orally via gavage for 13 weeks, at dose volumes of 10 mL/kg bw with dose levels of 0.1, 1 and 10 mL food enzyme/kg bw per day (referred to as low, mid and high dose groups). The highest dose corresponds to 147.3 mg TOS/kg bw per day. A similarly constituted control group received the vehicle (water).

No treatment-related deaths or effects on clinical signs, body weight, food and water consumption, food conversion efficiency, ophthalmoscopy, gross pathology and histopathological changes of organs and tissues were observed.

In haematology evaluation, both white blood cell (WBC) counts and lymphocyte counts were dose dependently decreased in males and both reached statistical significance at the high-dose group. The WBC and lymphocyte counts were also slightly decreased in females at the high dose.

In clinical chemistry evaluation, the prothrombin time was increased significantly in the low-dose groups of both sexes. This effect lacked a dose relationship and was not ascribed to treatment. Several intergroup differences in clinical chemistry parameters reached statistical significance, when compared with the controls, but these were minor, lacked a dose relationship or lacked consistency between the sexes and were, therefore, attributed to normal biological variation (bilirubin, total protein and sodium in males, creatinine, potassium and chloride in females).

The Panel derived a no observed adverse effect level (NOAEL) based on the haematological changes the mid dose level of this repeated dose 90-day oral toxicity study of 14.7 mg TOS/kg bw per day.

A comparison of the NOAEL (14.7 mg TOS/kg bw per day) from the 90-day study with the derived exposure estimates of 0.001–0.005 mg TOS/kg bw per day at the mean and from 0.002 to 0.008 mg

TOS/kg bw per day at 95th percentile, resulted in the margin of exposures (MOE) above 1,800, indicating that there is no safety concern.

3.4. Allergenicity

The potential allergenicity of this xylanase produced with the genetically modified *Bacillus subtilis* strain TD160(229) was assessed by comparing its amino acid sequence with those of known allergens according to the 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 window of 80 amino acids as the criterion, no matches were found.

Several cases of occupational allergy upon inhalation of aerosols containing xylanase or other enzymes have been reported (Martel et al., 2010). However, several studies have shown that adults with occupational asthma can ingest respiratory allergens without acquiring clinical symptoms of food allergy (Brisman, 2002; Poulsen, 2004; Armentia et al., 2009). In addition, no food allergic reactions to xylanase have been reported in the literature.

Bindslev-Jensen et al. (2006) investigated the possible cross-reactivity of 19 different commercial enzymes used in the food industry in allergic patients (400 patients allergic to inhalation allergens, food allergens, bee or wasp). In a few patients, a xylanase from a genetically modified *Aspergillus oryzae* gave positive results in a skin prick test and a histamine release test; however, these positive reactions are without clinical relevance as oral exposure to even high doses of this xylanase did not result in allergic reactions.

Taken together, the CEF Panel considers that there are no indications for food allergic reactions to this xylanase produced with *Bacillus subtilis* strain TD160(229).

The Panel notes that [redacted] is used as diluent and carrier of the food enzyme preparation. [redacted] contains substances and products causing allergies (respiratory and food allergies) and intolerances (gluten intolerance) (Regulation (EU) No 1169/2011)[7]. The food enzyme preparation might contain traces of [redacted] allergens and gluten, which may give rise to safety concerns in [redacted]-allergic and gluten-intolerant consumers.

Conclusions

Based on the genetic modifications performed, the manufacturing process, the compositional and biochemical data provided, the exposure assessment, the findings in the toxicological studies and allergenicity assessment, the Panel concluded that the food enzyme xylanase from *Bacillus subtilis* strain TD160(229) does not give rise to safety concerns under the intended conditions of use.

Documentation provided to EFSA

1) Dossier 'Xylanase produced by a genetically modified strain of *Bacillus subtilis* strain TD160 (229)'. Month 2014. Submitted by Puratos.
2) Preparatory work reports on technical data, toxicological data and on the genetic modifications were delivered by Hylobates Consulting/BiCT (Rome, Italy) on 29 April 2016, FoBiG GmbH (Freiburg, Germany) on 29 June 2015 and by the Technical University of Denmark (Søborg, Denmark) on 15 June 2015, respectively.
3) European Commission clarification to the Terms of Reference regarding the name of the production strain information received by June 2016.
4) Additional information was received from Puratos N.V. on May 2017.

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[7] Regulation (EU) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers, amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) No 608/2004.

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Abbreviations

bw	body weight
CAS	Chemical Abstracts Service
CFU	colony forming units
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
HACCP	Hazard Analysis and Critical Control Points
IUBMB	International Union of Biochemistry and Molecular Biology
JECFA	Joint FAO/WHO Expert Committee on Food Additives
kDa	kiloDalton
LOD	limits of detection
MNPE	micronucleated polychromatic erythrocytes
MOE	Margin of Exposure
NE	normochromatic erythrocyte
NOAEL	no observed adverse effect level
OECD	Organisation for Economic Cooperation and Development
PE	polychromatic erythrocytes
PHA	phytohaemagglutinin
QPS	Qualified Presumption of Safety
RNA	ribonucleic acid
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SXU	Skalar xylanase units
TOS	total organic solids
WBC	white blood cell
WHO	World Health Organization

Appendix A – Population groups considered for the exposure assessment

Population	Age range	Countries with food consumption surveys covering more than 1 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	0.752
A.01.03	Grain milling products (unspecified)	1	1	0.752
A.01.03.001	Wheat milling products (unspecified)	1	1	0.752
A.01.03.001.001	Wheat flour, brown	1	1	0.752
A.01.03.001.002	Wheat flour, Durum	1	1	0.752
A.01.03.001.003	Wheat flour, white	1	1	0.752
A.01.03.001.004	Wheat flour, wholemeal	1	1	0.752
A.01.03.001.005	Graham flour	1	1	0.752
A.01.03.001.006	Wheat flour, gluten free	1	1	0.752
A.01.03.001.014	Wheat starch	1.2	1	0.752
A.01.03.002	Rye milling products (unspecified)	1	1	0.752
A.01.03.002.001	Rye flour, gluten free	1	1	0.752
A.01.03.002.002	Rye flour, light	1	1	0.752
A.01.03.002.003	Rye flour, medium	1	1	0.752
A.01.03.002.004	Rye flour, wholemeal	1	1	0.752
A.01.03.003	Buckwheat milling products (unspecified)	1	1	0.752
A.01.03.003.001	Buckwheat flour	1	1	0.752
A.01.03.004	Corn milling products (unspecified)	1	1	0.752
A.01.03.004.001	Corn flour	1	1	0.752
A.01.03.004.003	Corn starch	1.3	1	0.752
A.01.03.005	Oat milling products (unspecified)	1	1	0.752
A.01.03.005.002	Oat flour	1	1	0.752
A.01.03.005.004	Oat starch	1.2	1	0.752
A.01.03.006	Rice milling products (unspecified)	1	1	0.752
A.01.03.006.001	Rice flour	1	1	0.752
A.01.03.006.002	Rice flour white	1	1	0.752
A.01.03.006.003	Rice flour, instant	1	1	0.752
A.01.03.006.004	Rice starch	1.2	1	0.752
A.01.03.007	Spelt milling products	1	1	0.752
A.01.03.008	Other milling products (unspecified)	1	1	0.752
A.01.03.008.001	Amaranth flour	1	1	0.752
A.01.03.008.002	Barley flour	1	1	0.752
A.01.03.008.003	Chapatti flour	1	1	0.752
A.01.03.008.004	Flour mix, wheat/rye/barley/oats	1	1	0.752
A.01.03.008.005	Millet flour	1	1	0.752
A.01.03.008.007	Sorghum flour	1	1	0.752
A.01.04	Bread and rolls (unspecified)	1	0.7	0.752
A.01.04.001	Wheat bread and rolls	1	0.7	0.752
A.01.04.002	Rye bread and rolls	1	0.7	0.752
A.01.04.003	Mixed wheat and rye bread and rolls	1	0.7	0.752
A.01.04.004	Multigrain bread and rolls	1	0.7	0.752
A.01.04.005	Unleavened bread, crisp bread and rusk (unspecified)	1	0.8	0.752
A.01.04.005.001	Crisp bread, rye wholemeal	1	0.9	0.752
A.01.04.005.002	Crisp bread, rye, light	1	0.9	0.752
A.01.04.005.003	Crisp bread, wheat, wholemeal	1	0.9	0.752

FoodEx code	FoodEx category	Conversion factor from FoodEx food group to raw material[(a)]	Recipe fraction	mg TOS/kg flour
A.01.04.005.004	Crisp bread, wheat, light	1	0.9	0.752
A.01.04.005.005	Rusk, light	1	0.9	0.752
A.01.04.005.006	Rusk, wholemeal	1	0.9	0.752
A.01.04.005.007	Pita bread	1	0.7	0.752
A.01.04.005.008	Matzo	1	0.9	0.752
A.01.04.005.009	Tortilla	1	0.7	0.752
A.01.04.006	Other bread	1	0.7	0.752
A.01.04.007	Bread products	1	0.7	0.752
A.01.07	Fine bakery wares (unspecified)	1	0.5	0.752
A.01.07.001	Pastries and cakes (unspecified)	1	0.5	0.752
A.01.07.001.001	Beignets	1	0.15	0.752
A.01.07.001.002	Buns	1	0.7	0.752
A.01.07.001.003	Cake from batter	1	0.25	0.752
A.01.07.001.004	Cheese cream cake	1	0.24	0.752
A.01.07.001.005	Cheese cream sponge cake	1	0.24	0.752
A.01.07.001.006	Chocolate cake	1	0.24	0.752
A.01.07.001.007	Chocolate cake with fruits	1	0.24	0.752
A.01.07.001.008	Cream cake	1	0.24	0.752
A.01.07.001.009	Cream cheese cake	1	0.24	0.752
A.01.07.001.010	Cream custard cake	1	0.24	0.752
A.01.07.001.011	Cream custard sponge cake	1	0.24	0.752
A.01.07.001.012	Croissant	1	0.5	0.752
A.01.07.001.013	Croissant, filled with chocolate	1	0.5	0.752
A.01.07.001.014	Croissant, filled with cream	1	0.5	0.752
A.01.07.001.015	Croissant, filled with jam	1	0.5	0.752
A.01.07.001.016	Croquembouche	1	0.15	0.752
A.01.07.001.017	Doughnuts	1	0.24	0.752
A.01.07.001.018	Clair	1	0.15	0.752
A.01.07.001.019	Flan	1	0.5	0.752
A.01.07.001.020	Fruit cake	1	0.6	0.752
A.01.07.001.021	Fruit pie	1	0.15	0.752
A.01.07.001.022	Cheese pie	1	0.15	0.752
A.01.07.001.023	Fruit tart	1	0.15	0.752
A.01.07.001.024	Gingerbread	1	0.6	0.752
A.01.07.001.025	Gougère	1	0.15	0.752
A.01.07.001.026	Kringles	1	0.25	0.752
A.01.07.001.027	Nut cream cake	1	0.24	0.752
A.01.07.001.028	Pancakes	1	0.25	0.752
A.01.07.001.029	Profiterole	1	0.15	0.752
A.01.07.001.030	Pyramid cake	1	0.25	0.752
A.01.07.001.031	Rhubarb flan	1	0.15	0.752
A.01.07.001.032	Scone	1	0.5	0.752
A.01.07.001.033	Sponge dough	1	0.25	0.752
A.01.07.001.034	Sponge cake	1	0.25	0.752
A.01.07.001.035	Sponge cake roll	1	0.25	0.752
A.01.07.001.036	Muffins	1	0.25	0.752
A.01.07.001.037	Waffles	1	0.25	0.752

FoodEx code	FoodEx category	Conversion factor from FoodEx food group to raw material[(a)]	Recipe fraction	mg TOS/kg flour
A.01.07.001.038	Apple strudel	1	0.15	0.752
A.01.07.001.039	Cream-cheese strudel	1	0.24	0.752
A.01.07.001.040	Cheese pastry goods from puff pastry	1	0.15	0.752
A.01.07.001.041	Croissant from puff pastry	1	0.6	0.752
A.01.07.001.042	Brioche	1	0.5	0.752
A.01.07.001.044	Lebkè	1	0.6	0.752
A.01.07.001.045	Dumpling	1	0.5	0.752
A.01.07.001.046	Cake marbled, with chocolate	1	0.5	0.752
A.01.07.001.047	Marzipan pie	1	0.25	0.752
A.01.07.001.048	Baklava	1	0.15	0.752
A.01.07.002	Biscuits (cookies)	1	0.9	0.752
A.01.07.002.001	Biscuits, sweet, plain	1	0.9	0.752
A.01.07.002.002	Biscuits, chocolate filling	1	0.81	0.752
A.01.07.002.003	Biscuits, cream filling	1	0.81	0.752
A.01.07.002.004	Biscuits, fruit filling	1	0.81	0.752
A.01.07.002.005	Biscuits, vanilla filling	1	0.81	0.752
A.01.07.002.006	Butter biscuits	1	0.81	0.752
A.01.07.002.007	Biscuit, iced	1	0.81	0.752
A.01.07.002.008	Speculaas	1	0.9	0.752
A.01.07.002.009	Biscuits, sweet, wheat wholemeal	1	0.9	0.752
A.01.07.002.010	Biscuits, oat meal	1	0.9	0.752
A.01.07.002.011	Biscuits, spelt meal	1	0.9	0.752
A.01.07.002.012	Biscuits, salty	1	0.9	0.752
A.01.07.002.013	Biscuits, salty, with cheese	1	0.81	0.752
A.01.07.002.014	Sticks, salty	1	0.81	0.752
A.17.03.003	Biscuits, rusks and cookies for children	1	0.9	0.752
A.18.04.001	Find bakery products for diabetics	1	0.5	0.752
A.19.01.002	Pizza and pizza-like pies	1	0.3	0.752

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.2017.5008/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.