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Safety evaluation of the food enzyme containing endo-polygalacturonase and endo-1,3(4)-β-glucanase from the non-genetically modified *Aspergillus fijiensis* strain NZYM-RE

EFSA Panel on Food Contact Materials, Enzymes and Processing Aids (CEP), Vittorio Silano, José Manuel Barat Baviera, Claudia Bolognesi, Pier Sandro Cocconcelli, Riccardo Crebelli, David Michael Gott, Konrad Grob, Evgenia Lampi, Marcel Mengelers, Alicja Mortensen, Gilles Rivière, Inger-Lise Steffensen, Christina Tlustos, Henk Van Loveren, Laurence Vernis, Jaime Aguilera, Magdalena Andryszkiewicz, Natalia Kovalkovicova, Daniele Cavanna, Yi Liu, Giulio di Piazza, Rita Ferreira de Sousa and Andrew Chesson

Abstract

The food enzyme has two declared activities, endo-polygalacuronase ((1→4)-α-D-galacturonan glycanohydrolase; EC 3.2.1.15) and endo-1,3(4)-β-glucanase (3-(1→3;1→4)-β-D-glucan 3(4)-glucanohydrolase; EC 3.2.1.6) and is produced with the non-genetically modified *Aspergillus fijiensis* strain NZYM-RE by Novozymes A/S. The food enzyme was considered free from viable cells of the production organism. It is intended to be used in eight food manufacturing processes, i.e. distilled alcohol production, brewing processes, baking processes, cereal-based processes, wine and wine vinegar production, fruit and vegetable processing for juice production, fruit and vegetable processing for products other than juices and refined olive oil production. Since residual amounts of total organic solids (TOS) are removed during distilled alcohol production and refined olive oil production, dietary exposure was not calculated for these two processes. For the remaining six food manufacturing processes, dietary exposure was estimated to be up to 0.553 mg TOS/kg body weight (bw) per day in European populations. Genotoxicity tests did not raise a safety concern. The systemic toxicity was assessed by means of a repeated dose 90-day oral toxicity study in rats. The Panel identified a no observed adverse effect level of 3,677 mg TOS/kg bw per day, the highest dose tested, resulting in a margin of exposure of at least 6,649. A search for similarity of the amino acid sequence of the food enzyme to known allergens was made and nine matches were found. The Panel considered that, under the intended conditions of use (other than distilled alcohol production), the risk of allergic reactions by dietary exposure to this food enzyme, particularly in individuals suffering from the oral allergy syndrome or sensitised to papaya, cannot be excluded. The Panel concluded that this food enzyme does not give rise to safety concerns under the intended conditions of use.

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Keywords: food enzyme, endo-polygalacturonase, endo-1,3(4)-β-glucanase, EC 3.2.1.15, EC 3.2.1.6, *Aspergillus fijiensis*

Requestor: European Commission

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Correspondence: fip@efsa.europa.eu

Panel members: José Manuel Barat Baviera, Claudia Bolognesi, Andrew Chesson, Pier Sandro Cocconcelli, Riccardo Crebelli, David Michael Gott, Konrad Grob, Claude Lambré, Evgenia Lampi, Marcel Mengelers, Alicja Mortensen, Gilles Rivière, Inger-Lise Steffensen, Christina Tlustos, Henk Van Loveren, Laurence Vernis and Holger Zorn.

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1. Introduction

Article 3 of the Regulation (EC) No 1332/2008[1] provides definition 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] established the 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 food enzymes for safety 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.

Two applications have been introduced by the companies "Novozymes A/S" and "Kerry Ingredients & Flavours" for the authorization of the food enzymes polygalacturonase and beta-glucanase from a strain of *Aspergillus aculeatus* (strain NZYM-RE), and endo-1,4-beta xylanase from a genetically modified stain of *Aspergillus niger* (strain CBS 612.94).

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

1.1.2. Terms of Reference

The European Commission requests the European Food Safety Authority to carry out a safety assessments of the food enzymes polygalacturonase and beta-glucanase from a strain of *Aspergillus aculeatus* (strain NZYM-RE), and endo-1,4-beta xylanase from a genetically modified stain of

[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.

[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, pp. 15–24.

Aspergillus niger (strain CBS 612.94) 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 of the safety assessment of the food enzyme polygalacturonase and beta-glucanase from *Aspergillus aculeatus* (strain NZYM-RE).

Recent data identified the production microorganism as *Aspergillus fijiensis* (Section 3.1). Therefore, this name will be used in this opinion instead of *Aspergillus aculeatus*.

2. Data and methodologies

2.1. Data

The applicant has submitted a dossier in support of the application for authorisation of the food enzyme polygalacturonase and beta-glucanase from *Aspergillus aculeatus* (strain NZYM-RE). The dossier was updated on 13 December 2013.

Additional information was requested from the applicant during the assessment process on 28 April 2021 and on 5 July 2022, and was consequently provided (see 'Documentation provided to EFSA').

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 guidance documents of EFSA Scientific Committee.

The current 'Guidance on the submission of a dossier on food enzymes for safety evaluation' (EFSA, 2009a) has been followed for the evaluation of the application with the exception of the exposure assessment, which was carried out in accordance with the updated 'Scientific Guidance for the submission of dossiers on food enzymes' (EFSA CEP Panel, 2021a).

3. Assessment

The food enzyme under application contains two declared activities:

IUBMB nomenclature	Endo-polygalacturonase
Systematic name	(1→4)-α-D-galacturonan glycanohydrolase
Synonyms	Pectinase, pectin hydrolase, endo-D-galacturonase
IUBMB No	EC 3.2.1.15
CAS No	9032-75-1
EINECS No	232-885-6

Endo-polygalacturonases catalyse the random hydrolysis of 1,4-α-D-galactosiduronic linkages of pectin and other galacturonans, resulting in the generation of partially hydrolysed galacturonans.

IUBMB nomenclature	Endo-1,3(4)-β-glucanase
Systematic name	3-(1→3;1→4)-β-D-glucan 3(4)-glucanohydrolase
Synonyms	Endo-1,3-β-D-glucanase; laminaranase; beta-1,3-glucanase
IUBMB No	EC 3.2.1.6
CAS No	62213–14-3
EINECS No	263-462-4

Endo-1,3(4)-β-glucanases catalyse the hydrolysis of 1,3- or 1,4-β-glycosidic linkages in β-D-glucans resulting in the generation of partially hydrolysed β-D-glucans.

The food enzyme is intended to be used in eight food manufacturing processes, i.e. distilled alcohol production, brewing processes, baking processes, cereal-based processes, wine and wine vinegar production, fruit and vegetable processing for juice production, fruit and vegetable processing for products other than juices and refined olive oil production.

3.1. Source of the food enzyme

The food enzyme is produced with the non-genetically modified filamentous fungus *Aspergillus fijiensis* strain NZYM-RE, which is deposited in the Westerdijk Fungal Biodiversity Institute culture collection (the Netherlands) with deposit number [redacted].[4] The production strain was derived from [redacted].[5]

The production strain was identified as *A. fijiensis* [redacted].[6]

3.2. Production of the food enzyme

The food enzyme is manufactured according to the Food Hygiene Regulation (EC) No 852/2004[7], with food safety procedures based on hazard analysis and critical control points, and in accordance with current good manufacturing practice.[8]

The production strain is grown as a pure culture using a typical industrial medium in a submerged, fed-batch fermentation system with conventional process controls in place. After completion of the fermentation, the solid biomass is removed from the fermentation broth by filtration leaving a supernatant containing the food enzyme. The filtrate containing the enzyme is then further purified and concentrated, including an ultrafiltration step in which the enzyme proteins are retained, while most of the low molecular mass material passes the filtration membrane and is discarded.[9] The applicant provided information on the identity of the substances used to control the fermentation and in the subsequent downstream processing of the food enzyme.[10]

The Panel considered that sufficient information has been provided on the manufacturing process and the quality assurance system implemented by the applicant to exclude issues of concern.

3.3. Characteristics of the food enzyme

3.3.1. Properties of the food enzyme

The endo-polygalacturonase and the endo-1,3(4)-β-glucanase are single polypeptide chains of [redacted] and [redacted] amino acids, respectively. The molecular masses of the mature proteins, derived from the amino acid sequences, were calculated to be [redacted] and [redacted] kDa, respectively.[11] The food enzyme was analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A consistent protein pattern was observed across all batches. The observed complexity of the protein profiles reflects the fact that the food enzyme is derived from a wild-type fungal strain.[12] Pectin lyase, pectin esterase and cellulase activities were found in all batches. No other enzymatic activities were reported.[13]

The in-house determination of endo-polygalacturonase activity is based on the hydrolysis of polygalacturonic acid with a consequent increase in reducing groups. 3,5-Dinitrosalicylic acid (DNS) is added, which complexes with the reducing carbohydrates, producing colour that is measured spectrophotometrically at 540 nm (reaction conditions: pH 4.5, 40°C, 10 min). Enzyme activity is expressed as polygalacturonase units (PGNU)/g. One PGNU is defined as the amount of enzyme producing reducing groups equivalent to 1 mg of galacturonic acid under the conditions of the assay.[14]

The in-house determination of endo-1,3(4)-β-glucanase activity is based on the hydrolysis of a β-glucan with a consequent increase in reducing sugars The reaction is stopped by adding *p*-hydroxybenzoic acid hydrazide (PAHBAH) and bismuth (III)-tartrate, which complexes with the

[4] Technical dossier/Annex 3/Additional data October 2021/Annex 1.
[5] Technical dossier/ Additional data October 2021.
[6] Technical dossier/Annex 3/Additional data October 2021/Annex 2.
[7] 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.
[8] Technical dossier/Annex 4.
[9] Technical dossier/pg. 55–62.
[10] Technical dossier/Annex 5.
[11] Technical dossier/pg. 37/Additional data August 2015/Additional data October 2021/Annex 4.
[12] Technical dossier/pg. 39.
[13] Technical dossier/pg. 50/Annexes: 2.04, 2.05, 2.06.
[14] Technical dossier/pg. 43-45/Annex 2.01.

reducing carbohydrates, producing a colour. The enzymatic activity is then determined by spectrophotometry at 405 nm (reaction conditions: pH 5.0, 50°C, 20 min). Enzyme activity is expressed as Fungal Beta Glucanase Units (FBG)/g. One FBG unit is defined as the amount of enzyme liberating reducing carbohydrates at a rate corresponding to 1 μmol glucose per minute under conditions of the assay.[15]

The optimum temperature is around 55°C (pH 4.5) for the endo-polygalacturonase and around 50°C (pH 5.0) for endo-1,3(4)-β-glucanase. The optimum pH is around pH 6.0 (37°C) for polygalacturonase and around pH 3.0 (30°C) for endo-1,3(4)-β-glucanase.[16] Thermostability was tested after a pre-incubation of the food enzyme for 30 min at different temperatures (pH 4.5 and pH 5.0). Both activities decreased at temperatures greater than 70°C, with no residual activity detected at 80°C.[17]

3.3.2. Chemical parameters

Data on the chemical parameters of the food enzyme were provided for three batches used for commercialisation (batches 1–3) and one batch used for some of the toxicological tests (Table 1).[18] The mean total organic solids (TOS) of the three food enzyme batches for commercialisation is 25.1% and the mean enzyme activity/TOS ratio is 99.6 PGNU/mg TOS (endo-polygalacturonase) and 1.3 FBG/mg TOS (endo-1,3(4)-β-glucanase).

Table 1: Composition of the food enzyme

Parameters	Unit	Batches			
		1[(a)]	2	3	4[(b)]
Endo-polygalacturonase	PGNU/g[(c)] batch	25,000	24,500	25,600	28,400
Endo-1,3(4)-β-glucanase	FBG/g batch[(d)]	302	316	326	950
Protein	%	17.1	18.0	18.1	49.1
Ash	%	0.9	0.9	0.9	15.2
Water	%	73.6	73.9	74.4	3.1
Total organic solids (TOS)[(e)]	%	25.5	25.2	24.7	81.7
Endo-polygalacturonase activity/mg TOS	PGNU/mg TOS	98.0	97.2	103.6	34.8
Endo-1,3(4)-β-glucanase/mg TOS	FBG/mg TOS	1.2	1.3	1.3	1.2

(a): Batch used for *in vitro* mammalian cell micronucleus test.
(b): Batch used for Ames test, *in vivo* chromosome aberration test and repeated dose 90-day oral toxicity study in rats.
(c): PGNU/g: see Section 3.3.1.
(d): FBG/g: see Section 3.3.1.
(e): TOS calculated as 100%–% water–% ash.

3.3.3. Purity

The lead content in the three commercial batches was below 0.5 mg/kg which complies with the specification for lead as laid down in the general specifications for enzymes used in food processing (FAO/WHO, 2006). The lead content in the batch used for toxicological studies (batch 4) was analysed in 1983 and found to be below 8 mg/kg, which complied with FAO/WHO specification recommended at that time.[19] In addition, the levels of arsenic, cadmium and mercury in all batches were below the limits of detection (LOD) of the employed methods.[20,21]

The food enzyme complies with the microbiological criteria (for total coliforms, *Escherichia coli* and *Salmonella*) as laid down in the general specifications for enzymes used in food processing (FAO/WHO, 2006). No antimicrobial activity was detected in any of the tested batches.[22]

[15] Technical dossier/pg. 43-45/Annex 2.03.
[16] Technical dossier/pg. 46-48/Annexes: 7.01, 7.02.
[17] Technical dossier/pg. 49.
[18] Technical dossier/pg. 38, 71/Additional data October 2021/Annex 7.
[19] Additional data October 2021.
[20] Technical dossier/pg. 39-40/Additional data October 2021/Annex 7.
[21] LODs: Commercial batches: Pb = 0.5 mg/kg; As = 0.1 mg/kg; Cd = 0.05 mg/kg; Hg = 0.03 mg/kg; Batch used for toxicological studies: Pb = 1 mg/kg; As = 0.3 mg/kg; Cd = 0.05 mg/kg; Hg = 0.05 mg/kg.
[22] Technical dossier/pg. 42, 71/Annexes: 1.07–1.11/Additional data October 2021/Annex 7.

Strains of *Aspergillus*, in common with most filamentous fungi, have the capacity to produce a range of secondary metabolites (Parenicová et al., 2001; Frisvad et al., 2018). The presence of secalonic acid was examined in three food enzyme batches and was below the LOD of the applied analytical method.[23,24] The possible presence of other secondary metabolites of concern is addressed by the toxicological examination of the food enzyme TOS.

The Panel considered that the information provided on the purity of the food enzyme is sufficient.

3.3.4. Viable cells and DNA of the production strain

The absence of the production strain in the food enzyme was demonstrated [redacted] No colonies were produced. [redacted][25]

3.4. Toxicological data

A battery of toxicological tests, including a bacterial gene mutation assay (Ames test), an *in vitro* micronucleus assay, an *in vivo* chromosome aberration test in rats and a repeated dose 90-day oral toxicity study in rats, has been provided. Food enzyme batches 1 and 4 (Table 1) were used in these studies. Batch 1 was one of the commercial batches analysed, while batch 4 had a lower specific activity as it was less pure than the commercial batches. Both were considered suitable as test items.

3.4.1. Genotoxicity

3.4.1.1. Genotoxicity *in vitro*

3.4.1.1.1. Bacterial reverse mutation test

A bacterial reverse mutation assay (Ames test) was performed according to the Organisation for Economic Co-operation and Development (OECD) Test Guideline 471 (OECD, 1983) and compliant with the current OECD (2020) guideline, and following good laboratory practice (GLP).[26]

Four strains of Salmonella Typhimurium (TA 1535, TA 1537, TA 98 and TA 100) and *Escherichia coli* WP2uvrA pKM101 (CM891) were used in the presence or absence of metabolic activation (S9-mix), applying the treat and plate method.[27]

Two separate experiments in triplicate were carried out using five concentrations of the food enzyme (from 100 to 10,000 μg food enzyme/mL, corresponding to 81.7, 269.61, 817, 2,696.1 and 8,170 μg TOS/mL). No cytotoxicity was observed at any concentration level of the test substance. Upon treatment with the food enzyme, there was no significant increase in revertant colony numbers above the control values in any strain with or without S9-mix.

The Panel concluded that the food enzyme did not induce gene mutations under the test conditions employed in this study.

3.4.1.1.2. *In vitro* mammalian cell micronucleus test

An *in vitro* micronucleus assay was carried out according to the OECD Test Guideline 487 (OECD, 2010) and following GLP.

Two experiments were carried out in duplicate in human peripheral blood lymphocyte. Based on the results of a range-finding study, in the short-term treatment (3 h followed by 21 h recovery period), the cell cultures were exposed to the food enzyme at 500, 3,000, 4,000 and 5,000 μg TOS/mL in the presence of metabolic activation (S9-mix) and 500, 2,000 and 3,000 μg TOS/mL without S9-mix. In the continuous treatment (24 h followed by 24 h recovery period) in the absence of S9-mix, the cell cultures were treated at 200, 400 and 700 μg TOS/mL.

Cytotoxicity, measured as decrease of the replication index, was observed at the highest concentrations tested (64% and 67% in the short-term treatment in the presence and absence of S9-mix, respectively, and 52% in the continuous treatment). The frequency of binucleated cells with

[23] Technical dossier/pg. 40/Annex 1.05/Additional data June 2014/Additional data August 2015 Additional data October 2021/ Annex 7.
[24] LOD: secalonic acid = 0.0003 mg/kg.
[25] Additional data October 2021/Annex 3.
[26] Technical dossier/Annex 6.01.
[27] Technical dossier/pg. 72/Annex 6.01.

micronuclei (MNBN) was comparable to the negative controls at all concentrations tested. A single exception to this was observed at an intermediate concentration (3,000 μg TOS/mL) following the short treatment with S9-mix where a small, but statistically significant increase was observed. However, the value was within the 95th percentile of the historical control range, and therefore, it was considered not to be biologically relevant.

The Panel concluded that the food enzyme did not induce an increase in the frequency of MNBN in cultured human peripheral blood lymphocytes under the test conditions employed in this study.

3.4.1.2. Genotoxicity *in vivo*

3.4.1.2.1. *In vivo* chromosome aberration test in rats

The *in vivo* mammalian bone marrow chromosome aberration test in rats was carried out according to the OECD Test Guideline 475 (OECD, 1984) and following GLP.[28]

Fifteen male and 15 female CD rats per group were treated with a single oral administration (gavage) of the food enzyme at doses of 500, 1,600 and 5,000 mg/kg bw, corresponding to 409, 1,308 and 4,085 mg TOS/kg bw (batch 4). In addition, 15 males and 15 females of a negative control group were dosed with distilled water, and 15 males and 15 females of a positive control group were dosed with 20 mg cyclophosphamide/kg bw.

Bone marrow samples were taken 6, 24 and 48 h after dosing, using five males and five females from each treatment group and scheduled sampling time. No mortality or treatment-related clinical signs were observed in any animal group. No statistically significant increases in the frequency of chromosomal aberrations or substantial cytotoxicity were observed in animals treated with the food enzyme, compared with vehicle control values.

The Panel concluded that the food enzyme did not induce structural and numerical chromosomal aberrations in bone marrow when tested up to 5,000 mg/kg bw (corresponding to 4,085 mg TOS/kg bw) under the experimental conditions employed, however, considered this study of limited validity because no evidence on bone marrow exposure were provided.

3.4.1.3. Conclusions on genotoxicity

On the basis of the results of the basic battery of *in vitro* studies, the Panel concluded that there is no concern for genotoxicity for the food enzyme tested.

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

The repeated dose 90-day oral toxicity study was performed following GLP[29] and with the following deviations from OECD TG 408 (1981): no individual data on body weight and on feed consumption as well as no individual and group data on ophthalmological examination were presented in the report. Calcium, phosphorous, creatinine and bilirubin concentrations as well as ornithine decarboxylase and γ-glutamyl transpeptidase activities were not determined, no statistical analyses were conducted for urinalysis data and organ weights, and only visual appraisal was done. Kidneys from low- and mid-dose males and lungs from low- and mid-dose males and females were not examined histopathologically, and no statistical analyses were conducted on incidences of histopathological changes in kidneys. The Panel noted that the 90-day study was conducted under contemporary experimental conditions in early 1980s. Despite the methodological deficiencies in the study conduct, in presentation and reporting of the results, the Panel decided that this study provided sufficient data to assess the systemic toxicity of the enzyme. Groups of 20 male and 20 female Sprague–Dawley rats received the food enzyme at dietary concentrations of 0.5, 1.5 and 5%, corresponding to 368, 1,103 and 3,677 mg TOS/kg bw per day. Controls received the same diet with no food enzyme added.

One control male and one low-dose male died during blood sampling from an orbital sinus in week 13. The Panel considered these deaths as accidental.

The body weight was statistically significantly decreased in week 3 (−6%), 4 (−6%), 5 (−5%), 6 (−7%), 7 (−3%), 8 (−6%), 9 (−7%), 10 (−7%) and 11 (−7%) in high-dose females. The Panel considered the changes as not toxicologically relevant as they were only observed in one sex, the changes were small and without a statistically significant effect on the final body weight.

The haematological investigation revealed a statistically significant increase in haemoglobin concentration (+5%) and in the monocyte count (+75%) in high-dose males. The Panel considered

[28] Technical dossier/Annex 6.02; Technical dossier/Additional data August 2015.
[29] Technical dossier/Annex 6.03; Technical dossier/Annex 6.02; Technical dossier/Additional data August 2015.

the changes as not toxicologically relevant as they were only observed in one sex (both parameters), the changes were small (both parameters) and the changes were within the historical control values (both parameters).

The clinical chemistry investigation revealed statistically significant decreases in the alanine aminotransferase (ALT) levels in low- and mid-dose males (−24% and −19%, respectively), in the alkaline phosphatase (AP) levels in high-dose males (−20%), in the albumin to globulin (A-G) ratio in mid- and high-dose males (−10% and −10%, respectively) and in total proteins (TP) (−3%) and albumin (Alb) (−8%) in high-dose females, an increase in blood urea nitrogen (BUN) in low- and mid-dose females (+14% and +16%,respectively) and in AP in low-dose females (+25%). The Panel considered these changes as not toxicologically relevant as the changes were small (all parameters), there was no dose–response relationship (ALT, A-G ratio, BUN, AP in females), the changes were only observed in one sex (all parameters except for AP), there was no consistency in the direction of the change between males and females (AP) and all the changes were within the historical control data from the laboratory.[30]

The urinalysis revealed a decrease in pH in low-, mid- and high-dose females (−21%, −16% and −5%, respectively) and a decrease in the 4-h urine volume in low-, mid- and high-dose males (−52%, −54% and −48%, respectively) and in low-, mid- and high-dose females (−15%, −24% and −24%, respectively). The authors of the study report attributed the latter finding to the higher than normal urine volume in the control group. The Panel considered this plausible, but noted that no historical control data were presented to support this explanation. The Panel considered the changes as not toxicologically relevant because there was no dose–response relationship (both parameters) and lack of clinical chemistry correlates.

The microscopic examination revealed changes in the kidneys. Inflammatory foci were observed in high-dose males (1/20 vs. 0/20 in the control group; $p > 0.05$) and in females (1/20, 5/20 and 9/20 in low-, mid- and high-dose groups, respectively, vs. 0/20 in the control group; $p < 0.05$ for mid- and high-dose groups). Focal tubular regeneration was observed in high-dose males (total incidence 7/20 vs. 3/20 in the control group; $p > 0.05$; incidence for severity grades in high-dose males: minimal 5/7 vs. 1/3 in the control group and mild 2/7 vs. 2/3 in the control group) and in the females (total incidence 0/20, 2/20 and 7/20 in low-, mid- and high-dose groups, respectively, vs. 0/20 in the controls; $p < 0.05$ in the high-dose group; incidence for severity grades: minimal 2/2 and 3/7 in the mid- and high-dose groups vs. 0/0 in the controls, and mild 2/7 and moderate 2/7 in the high-dose group vs. 0/0 in both controls). Furthermore, mineral tubular casts were observed at the cortico-medullary junction in the kidneys of females only (total incidence 10/20, 13/20 and 16/20 in low-, mid- and high-dose groups, respectively, vs. 3/20 in the control group; $p < 0.05$ at all doses). The severity of the mineral casts was minimal in 4/10, 3/13 and 1/16 females in the low-, mid- and high-dose groups, respectively, versus 1/3 in the controls ($p > 0.05$) (mild in 4/10, 2/13 and 5/16 females in the low-, mid- and high-dose groups, respectively, vs. 2/3 in controls, moderate in 2/10, 7/13 and 3/16 in the low-, mid- and high-dose groups, respectively, vs. 0/3 in controls, and severe in 1/13 and 7/16 females in mid- and high-dose groups vs. 0/3 in the controls).

The Panel considered the inflammatory cell foci in the kidneys of mid- and high-dose females as related to a tubular damage associated with the mineralised tubular casts. Regarding focal tubular regeneration, the Panel noted that it is a reparative response to a previous damage of renal tubular epithelium. Therefore, this change in the treated females could be related to the presence of tubular damage associated with the presence of mineralised tubular casts. Furthermore, this change can often be seen as a part of spontaneous chronic progressive nephropathy (CPN), a common disease of rats with a distinct male predisposition. The presence of tubular regeneration in the control males supports its spontaneous aetiology. The latter is further supported by a lack of difference in the severity grades between the control and high-dose male groups, a not statically significant increase in the incidence in the high-dose males and absence of any histopathological changes which could be considered related to the test item in this organ. The main finding reported was a dose-dependent increase in the incidence of mineral tubular casts at the cortico-medullary junction, a manifestation of nephrocalcinosis in the females. A decreased calcium to phosphorus ratio (Ca/P) was reported in the batches of the diets given to all groups, both control and treated, which occurred with time for all groups and correlated with the amount of test item added to the diet. Nephrocalcinosis is known to be associated with a decrease in the Ca/P in the diet and to occur more often in females. Therefore, the

[30] Technical dossier/Additional data August 2015.

Panel considered that the increase in nephrocalcinosis was attributed to alterations in the Ca/P in the experimental diets.

No other statistically significant or biologically relevant differences to controls were reported.

The Panel identified a no observed adverse effect level (NOAEL) of 3,677 mg TOS/kg bw per day, the highest dose tested.

3.4.3. Allergenicity

The allergenicity assessment considered only the food enzyme and not carriers or other excipients that may be used in the final formulation.

The potential allergenicity of the endo-polygalacturonase and endo-1,3(4)-β-glucanase produced with the non-genetically modified *A. fijiensis* strain NZYM-RE was assessed by comparing their amino acid sequences 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 sliding window of 80 amino acids as the criterion. No match was found for endo-1,3(4)-β-glucanase,[31] but nine matches were found for endo-polygalacturonase.[32] Eight of the nine matches found were with the pollen allergens Sor h 13.0101 (*Sorghum halepense*; Johnson grass), Pla or 2.0101 (*Platanus orientalis*; Oriental plane tree), Cry j 2.0101 (*Cryptomeria japonica*; Japanese cedar), Pla a 2.0101 (*Platanus acerifolia*; London plane tree), Cha o 2.0101 (*Chamaecyparis obtusa*; Japanese cypress), Jun a 2.0101 (*Juniperus ashei*; Mountain cedar), Phl p 13.0101 (*Phleum pratense*; Thimothy) and Ole e 14.0101 (*Olea europea*; Olive), and one match with food allergen Cari p 1.0101 (*Carica papaya*; Papaya).

No information is available on oral and respiratory sensitisation or elicitation reactions of endo-polygalacturonase and endo-1,3(4)-β-glucanase produced with the *A. fijiensis* strain NZYM-RE.

Endo-polygalacturonases are allergens often present in grass and tree pollen. The oral allergy syndrome, i.e. allergic reactions mainly in the mouth, is associated with sensitisation to pollen such as from cedar trees (Terumi Midoro-Horiuti et al., 2003) and grasses. Such reactions are seldomly leading to severe systemic anaphylaxis.

Cari p 1 (Cari p 1.0101) is the main allergen present in *C. papaya*., described as both a food and a respiratory allergen (Sarkar et al., 2018). Several studies reported occupational rhinitis and asthma in workers of industries where papain is handled (Baur and Fruhmann, 1979; Baur et al., 1982; Niinimaki et al., 1993; Soto-Mera et al., 2000; Van Kampen et al., 2005). In other studies, allergy to papaya-derived products unrelated to occupational exposure has also been described. Garcia-Ortega et al. (1991) showed that administration of chymopapain for chemonucleolysis resulted in sensitisation in some patients. Mansfield and Bowers (1983) reported severe systemic allergic reactions mediated by papain-specific IgE in some individuals that ingested papain-containing meat tenderiser. Sensitisation to papaya does not typically occur from eating papaya fruit. However, once sensitised, individuals may suffer allergic reactions following any type of exposure to papaya or papaya-derived products (Morton, 1987).

[redacted], a product that may cause allergies or intolerances (listed in the Regulation (EU) No 1169/2011[33]), is used as a raw material in the media fed to the microorganisms. However, during the fermentation process, this protein source will be degraded and utilised by the microorganism for cell growth, cell maintenance and production of enzyme protein. In addition, the fungal biomass and fermentation solids are removed. Taking into account the fermentation process and downstream processing, the Panel considered that no potentially allergenic residues are present in the food enzyme.

The Panel considered that, under the intended conditions of use, the risk of allergic reactions by dietary exposure to this food enzyme, particularly in individuals suffering from the oral allergy syndrome or sensitised to papaya, cannot be excluded.

[31] Technical dossier/Additional data August 2015/Annex 2/Additional data October 2021/Annexes: 6 and 13.

[32] Additional data October 2021/Annexes: 5 and 12.

[33] 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.

3.5. Dietary exposure

3.5.1. Intended use of the food enzyme

The food enzyme is intended to be used in eight food processes at the recommended use levels summarised in Table 2.[34]

Table 2: Intended uses and recommended use levels of the food enzyme as provided by the applicant

Food manufacturing process	Raw material (RM)	Recommended dosage (mg TOS/kg RM)[(d)]
Distilled alcohol production	Starch	16–80[(b)]
Brewing processes	Cereals	16–**80**[(b)]
Baking processes	Flour	8–**40**[(b)]
Cereal-based processes	Flour	0.8–**4**[(b)]
Wine and wine vinegar production	Grapes	0.2–**2**[(c)]
Fruit and vegetable processing for juice production	Fruits or vegetables	0.1–**0.5**[(c)]
Fruit and vegetable processing for products other than juices (puree only)[(a)]	Fruits or vegetables	1–**10**[(c)]
Refined olive oil production[(a)]	Olive paste	1.6–6[(b)]

(a): The name has been harmonised by EFSA according to the calls-for-data launched on the EFSA website.
(b): Based on the average activity/TOS ratio of 1.25 FBG/mg TOS.
(c): Based on the average activity/TOS ratio of 99.6 PGNU/mg TOS.
(d): The numbers in bold were used for calculation.

In distilled alcohol production, the food enzyme is added to the slurry prior to liquefaction or during pre-saccharification.[35] The endo-1,3(4)-β-glucanase activity is used to break down cell wall polysaccharides in the cereal grain. The food enzyme TOS is removed during the production process, only negligible amount of TOS residue may remain in the final distilled alcohol products (EFSA CEP Panel, 2021b).

In brewing processes, the food enzyme is added to cereals during the mashing step.[36] The endo-1,3(4)-β-glucanase activity is used to hydrolyse β-glucans present in the cell walls, aiding the release of starch and protein and, therefore, increasing the brewing yield by facilitating filtration. The food enzyme TOS remains in the final foods.

In baking processes and cereal-based processes, the food enzyme is added to flour during the preparation of dough.[37] The action of the food enzyme is primarily dependent on its endo-1,3(4)-β-glucanase content, which is used to hydrolyse β-glucans present in flour that can interact with gluten and bind water. Hydrolysis of β-glucans contributes to the reduction of dough viscosity, facilitating the handling and leading to improved crumb structure and increased volume during baking. The food enzyme TOS remains in the final foods.

In wine and wine vinegar production, the food enzyme is added to the grapes at various steps: during crushing and maceration, during fermentation, at the clarification step or during ageing.[38] The endo-polygalacturonase activity present is employed to break down the cell wall polysaccharides in the grape. The food enzyme TOS remains in the final foods.

In fruit and vegetable processing for juice and other products, the food enzyme is added to fruits or vegetables during mash treatment and to the raw juice.[39] The action of the food enzyme is primarily dependent on the endo-polygalacturonase activity to degrade galacturonans in the cell wall. The food enzyme TOS remains in the final foods.

[34] Technical dossier/pg. 46/Additional data October 2021/Answer 11.1.
[35] Technical dossier/pg. 93–94.
[36] Technical dossier/pg. 95–96.
[37] Technical dossier/pg. 97–98.
[38] Technical dossier/pg. 91–92.
[39] Technical dossier/pg. 89–90.

The food enzyme is also used for the production of olive oil. The term 'olive oil' is defined in the Regulation (EU) No 1308/2013[40] as '*composed of refined olive oils and virgin olive oils*'. The term 'virgin olive oils' means '*oils obtained from the fruit of the olive tree solely by mechanical or other physical means under conditions that do not lead to alterations in the oil, which have not undergone any treatment other than washing, decantation, centrifugation or filtration, to the exclusion of oils obtained using solvents or using adjuvants having a chemical or biochemical action, or by re-esterification process and any mixture with oils of other kinds*.'

In accordance with the law, the use of enzymes is not permitted in the production of virgin olive oils in the European Union. Therefore, this assessment is limited to the use of this food enzyme in the production of refined olive oil only.

In the olive oil production process, enzymes (if used) are added to the olive paste in the malaxation step.[41] Chiefly the endo-1,3(4)-β-glucanase activity is used to hydrolyse β-glucans present in the cell walls, facilitating the release of oil retained in the cells and thus increasing extraction yield.

The production processes of olive oil and palm oil are very similar. It has been demonstrated that > 99.8% of the food enzyme TOS could be removed in palm oil.[42] When experimentally analysed, the residue amounts of enzyme in crude palm oil were below the detection limit.[43] Although equivalent analytical data were not available for olive oil, the Panel considered that only negligible amounts of enzyme TOS (< 1%) would remain in refined olive oils.

Based on data provided on thermostability (see Section 3.3.1), it is expected that the endo-polygalacturonase and endo-1,3(4)-β-glucanase would be inactivated by heat during the food processes, but may remain active in juices, depending on the pasteurisation conditions, and in wine.

3.5.2. Dietary exposure estimation

In accordance with the guidance document (EFSA CEP Panel, 2021a), a dietary exposure was calculated only for food manufacturing processes where the food enzyme-TOS remains in the final foods, i.e. brewing processes, baking processes, cereal-based processes, wine and wine vinegar production, fruit and vegetable processing for juice production, fruit and vegetable processing for products other than juices, refined olive oil production.

Chronic exposure to the food enzyme-TOS was calculated by combining the maximum recommended use level with individual consumption data (EFSA CEP Panel, 2021a). The estimation involved selection of relevant food categories and application of technical conversion factors (EFSA CEP Panel, 2021b). Exposure from all FoodEx categories was subsequently summed up, averaged over the total survey period (days) and normalised for body weight. This was done for all individuals across all surveys, resulting in distributions of individual average exposure. Based on these distributions, the mean and 95th percentile exposures were calculated per survey for the total population and per age class. Surveys with only 1 day per subject were excluded and 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, 2011).

Table 3 provides an overview of the derived exposure estimates across all surveys. Detailed mean and 95th percentile exposure to the food enzyme-TOS per age class, country and survey, as well as contribution from each FoodEx category to the total dietary exposure are reported in Appendix A – Tables 1 and 2. For the present assessment, food consumption data were available from 41 dietary surveys (covering infants, toddlers, children, adolescents, adults and the elderly), carried out in 22 European countries (Appendix B). The highest dietary exposure was estimated to be about 0.553 mg TOS/kg bw per day in infants at the 95th percentile.

[40] Regulation (EU) No 1308/2013 of the European Parliament and of the Council of 17 December 2013 establishing a common organisation of the markets in agricultural products and repealing Council Regulations (EEC) No 922/72, (EEC) No 234/79, (EC) No 1037/2001 and (EC) No 1234/2007.
[41] Additional data October 2021/Answer 11.2.
[42] Additional data October 2021/Answer 11.2/Annex 15.
[43] Additional data October 2021/Annex 14. LoD = 10 ng/mL.

Table 3: Summary of estimated dietary exposure to food enzyme–TOS in six population groups

Population group	Estimated exposure (mg TOS/kg body weight 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 mean (number of surveys)	0.033–0.187 (11)	0.113–0.277 (15)	0.137–0.261 (19)	0.078–0.159 (21)	0.063–0.175 (22)	0.054–0.115 (22)
Min–max 95th (number of surveys)	0.124–0.553 (9)	0.256–0.474 (13)	0.240–0.470 (19)	0.134–0.313 (20)	0.146–0.498 (22)	0.114–0.270 (21)

3.5.3. Uncertainty analysis

In accordance with the guidance provided in the EFSA opinion related to uncertainties in dietary exposure assessment (EFSA, 2006), the following sources of uncertainties have been considered and are summarised in Table 4.

Table 4: Qualitative evaluation of the influence of uncertainties on the dietary exposure estimate

Sources of uncertainties	Direction of impact
Model input data	
Consumption data: different methodologies/representativeness/underreporting/ misreporting/no portion size standard	+/–
Use of data from food consumption surveys 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	
Exposure to food enzyme–TOS was always calculated based on the recommended maximum use level	+
Selection of broad FoodEx categories for the exposure assessment	+
The applicant indicated only puree, but all foods covered by the process 'Fruit and vegetable processing for products other than juices' were included in the calculation	+
Use of recipe fractions in disaggregation FoodEx categories	+/–
Use of technical factors in the exposure model	+/–
Exclusion of two processes from the exposure assessment: distilled alcohol production and refined olive oil production	–

+: 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 on the occurrence and use levels of this specific food enzyme, is likely to have led to overestimation of the exposure.

The exclusion of two food manufacturing processes from the exposure assessment was based on > 99% of TOS removal. This is not expected to have an impact on the overall estimate derived.

3.6. Margin of exposure

A comparison of the NOAEL (3,677 mg TOS/kg bw per day) from the 90-day rat study with the derived exposure estimates of 0.033–0.277 mg TOS/kg bw per day at the mean and from 0.114–0.553 mg TOS/kg bw per day at the 95th percentile, resulted in margin of exposure (MOE) of at least 6,649.

4. Conclusion

Based on the data provided, removal of TOS during two food manufacturing processes and the MoE calculated for the other six processes, the Panel concluded that the food enzyme containing endo-polygalacturonase and endo-1,3(4)-β-glucanase produced with *Aspergillus fijiensis* strain NZYM-RE does not give rise to safety concerns under the intended conditions of use, except for the individuals suffering from oral allergy syndrome or sensitised to papaya.

5. Remarks

In accordance with Regulation (EU) No 1308/2013, no substances other than water are permitted in the production of virgin olive oils. Therefore, the use of this food enzymes in producing virgin olive oils was excluded in this evaluation.

6. Documentation as provided to EFSA

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Additional information. August 2015. Submitted by Novozymes A/S.

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Abbreviations

bw	body weight
CAS	Chemical Abstracts Service
CEF	EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
CEP	EFSA Panel on Food Contact Materials, Enzymes and Processing Aids
EINECS	European Inventory of Existing Commercial Chemical Substances
FAO	Food and Agricultural Organisation of the United Nations
GLP	good laboratory practice
GMM	genetically modified microorganism
GMO	genetically modified organism
IUBMB	International Union of Biochemistry and Molecular Biology
JECFA	Joint FAO/WHO Expert Committee on Food Additives
kDa	kiloDalton
LOD	limit of detection
MNBN	bi-nucleated cells with micronuclei
MOE	margin of exposure
OECD	Organisation for Economic Cooperation and Development
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
WHO	World Health Organisation

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

Information provided in this appendix is shown in an Excel file (downloadable https://efsa.onlinelibrary.wiley.com/doi/10.2903/j.efsa.2022.7648#support-information-section).

The file contains two sheets, corresponding to two tables.

Table 1: Mean and 95th percentile exposure to the food enzyme–TOS per age class, country and survey.

Table 2: Contribution of food categories to the dietary exposure to the food enzyme–TOS per age class, country and survey.

Appendix B – 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, Cyprus, Denmark, Estonia, Finland, France, Germany, Italy, Latvia, Portugal, Slovenia
Toddlers	From 12 months up to and including 35 months of age	Belgium, Bulgaria, Cyprus, Denmark, Estonia, Finland, France, Germany, Hungary, Italy, Latvia, Netherlands, Portugal, Slovenia, Spain
Children	From 36 months up to and including 9 years of age	Austria, Belgium, Bulgaria, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Italy, Latvia, Netherlands, Portugal, Spain, Sweden
Adolescents	From 10 years up to and including 17 years of age	Austria, Belgium, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Italy, Latvia, Netherlands, Portugal, Romania, Slovenia, Spain, Sweden
Adults	From 18 years up to and including 64 years of age	Austria, Belgium, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Netherlands, Portugal, Romania, Slovenia, Spain, Sweden
The elderly[(a)]	From 65 years of age and older	Austria, Belgium, Cyprus, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Netherlands, Portugal, Romania, Slovenia, Spain, Sweden

(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, 2011).