SCIENTIFIC OPINION

EFSA Journal

ADOPTED: 15 November 2022

doi: 10.2903/j.efsa.2022.7676

Safety evaluation of the food enzyme containing cellulase, endo-1,3(4)-β-glucanase and endo-1,4-β-xylanase activities from the non-genetically modified *Trichoderma reesei* strain AR-256

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Abstract

The food enzyme containing cellulase (EC 3.2.1.4), endo-1,3(4)-β-glucanase (EC 3.2.1.6) and endo-1,4-β-xylanase (EC 3.2.1.8) is produced with the non-genetically modified *Trichoderma reesei* strain AR-256 by AB-Enzymes GmbH. The food enzyme is considered free from viable cells of the production organism. It is intended to be used in seven food manufacturing processes: baking processes, cereal-based processes, brewing processes, fruit and vegetable processing for juice production, wine and wine vinegar production, distilled alcohol production and grain treatment for production of starch and gluten fractions. Since the residual amounts of total organic solids (TOS) are removed during grain treatment and distilled alcohol production, dietary exposure was estimated for the remaining five processes and amounted up to 3.92 mg TOS/kg body weight (bw) per day. The toxicity studies were carried out with an endo-1,4-β-xylanase from *T. reesei* [redacted], considered by the Panel as a suitable substitute, because the genetic differences between the strains are well characterised and of no concern. Additionally, several strains derived from the production strain are considered safe by EFSA and the manufacturing of both food enzymes is similar. Genotoxicity tests did not indicate a safety concern. The systemic toxicity was assessed by a repeated dose 90-day oral toxicity rat study. The no observed adverse effect level of 939 mg TOS/kg bw per day, the highest dose tested, compared with the estimated dietary exposure, resulted in a margin of exposure above 239. In the search for the similarity of the amino acid sequences to known allergens, one match (salmon) was found. The Panel considered that, under the intended conditions of use (except distilled alcohol production), the risk of allergic reactions by dietary exposure cannot be excluded, in particular for individuals sensitised to salmon. The Panel concluded that this food enzyme does not give rise to safety concerns, under the intended conditions of use.

Keywords: cellulase, endo-1,3(4)-β-glucanase, endo-1,4-β-xylanase, EC 3.2.1.4, EC 3.2.1.6, EC 3.2.1.8, *Trichoderma reesei*

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Question number: EFSA-Q-2021-00545

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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 the European Commission.

Declarations of interest: If you wish to access the declaration of interests of any expert contributing to an EFSA scientific assessment, please contact interestmanagement@efsa.europa.eu.

Acknowledgements: The Panel wishes to thank the following for the support provided to this scientific output: Erik Boinowitz and Helen Regina.

Suggested citation: EFSA CEP Panel (EFSA Panel on Food Contact Materials, Enzymes and Processing Aids), Lambré C, Barat Baviera JM, Bolognesi C, Cocconcelli PS, Crebelli R, Gott DM, Grob K, Lampi E, Mengelers M, Mortensen A, Rivière G, Steffensen I-L, Tlustos C, Van Loveren H, Vernis L, Zorn H, Glandorf B, Herman L, Roos Y, Kovalkovičová N, Liu Y, Lunardi S, Di Piazza G, de Sousa RF and Chesson A, 2022. Scientific Opinion on the safety evaluation of the food enzyme containing cellulase, endo-1,3(4)-β-glucanase and endo-1,4-β-xylanase activities from the non-genetically modified *Trichoderma reesei* strain AR-256. EFSA Journal 2022;20(12):7676, 18 pp. https://doi.org/10.2903/j.efsa.2022.7676

ISSN: 1831-4732

The EFSA Journal is a publication of the European Food Safety Authority, a European agency funded by the European Union.

† Deceased

Table of contents

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 micro-organisms or products thereof including a product obtained by a fermentation process using micro-organisms: (i) containing one or more enzymes capable of catalysing a specific biochemical reaction; and (ii) added to food for a technological purpose at any stage of the manufacturing, processing, preparation, treatment, packaging, transport or storage of foods.

'Food enzyme preparation' means a formulation consisting of one or more food enzymes in which substances such as food additives and/or other food ingredients are incorporated to 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[1] 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 European Union 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 CEF Panel, 2009) 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 European Union (EU) Community 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[1] on food enzymes.

The following three applications have been submitted for the authorization of food enzymes:

1) From "Amano Enzyme Inc." for Alpha-glucosidase from *Aspergillus niger* (strain AE-TGU);
2) From the Association of Manufacturers and Formulators of Enzyme Products (AMFEP) for Endo-1,3(4)-beta-glucanase, Endo-1,4-beta-xylanase and Cellulase from *Talaromyces emersonii*;
3) From AMFEP for Cellulase, Endo-1,3(4)-beta-glucanase and Endo-1,4-beta-xylanase obtained from *Trichoderma reesei*.

Following the requirements of Article 12.1 of Regulation (EC) No 234/2011[3] implementing Regulation (EC) No 1331/2008[2], the Commission has verified that the three applications fall within the scope of the food enzyme Regulation and contains all the elements required under Chapter II of that Regulation.

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

1.1.2. Terms of Reference

The European Commission requests the European Food Safety Authority to carry out safety assessments on the food enzymes Alpha-glucosidase from *Aspergillus niger* (strain AE-TGU), Endo-1,3(4)-beta-glucanase, Endo-1,4-beta-xylanase and Cellulase from *Talaromyces emersonii*, and Cellulase, Endo-1,3(4)-beta-glucanase and Endo-1,4-beta-xylanase obtained from *Trichoderma reesei* in accordance with Article 17.3 of Regulation (EC) No 1332/2008[1] on food enzymes.

1.2. Interpretation of the Terms of Reference

The present scientific opinion addresses the European Commission's request to carry out the safety assessment of the food enzyme complex containing cellulase, endo-1,3(4)-β-glucanase and endo-1,4-β-xylanase from the non-genetically modified *T. reesei* strain AR-256 submitted by AB-Enzymes GmbH.

The application was submitted initially as a joint dossier[4] and identified as the EFSA-Q-2014-00804-806. During the risk assessment phase, it was found that the technical dossier is too generic to be evaluated. A solution was found on 16 March 2020 via an ad hoc meeting between EFSA, the European Commission and representatives from the Association of Manufacturers and Formulators of Enzyme Products (AMFEP).[5] It was agreed that joint dossier will be split into 13 individual data packages.

The current opinion addresses one data package originating from the joint dossier EFSA-Q-2014-00804-806. This data package, now identified as EFSA-Q-2021-00545, concerns the food enzyme complex containing cellulase, endo-1,3(4)-β-glucanase and endo-1,4-β-xylanase that is produced with a strain AR-256 of *Trichoderma reesei*, submitted by AB-Enzymes GmbH.

2. Data and methodologies

2.1. Data

The applicant has submitted a dossier in support of the application for authorisation of the food enzyme cellulase, endo-1,3(4)-β-glucanase and endo-1,4-β-xylanase from *T. reesei*.

Additional information was requested from the applicant during the assessment process on 16 December 2021 and received on 13 July 2022 (see 'Documentation provided to EFSA').

Following the request for additional data sent by EFSA on 16 December 2021, the applicant requested a clarification teleconference on 4 February 2022, after which the applicant provided additional data on 13 July 2022.

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, 2009) and following the relevant guidance documents of the EFSA Scientific Committee.

The 'Guidance on the submission of a dossier on food enzymes for safety evaluation' (EFSA CEF Panel, 2009) as well as the 'Statement on characterisation of microorganisms used for the production of food enzymes' (EFSA CEP Panel, 2019) have 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).

[4] Commission Implementing Regulation (EU) No 562/2012 of 27 June 2012 amending Commission Regulation (EU) No 234/2011 with regard to specific data required for risk assessment of food enzymes Text with EEA relevance. OJ L 168, 28.6.2012, pp. 21–23.

[5] The full detail is available at the https://www.efsa.europa.eu/en/events/event/ad-hoc-meeting-industry-association-amfep-joint-dossiers-food-enzymes.

3. Assessment[6]

The food enzyme under application contains three declared activities:

IUBMB nomenclature	**Cellulase**
Systematic name	1,4-(1,3;1,4)-β-D-glucan-4-glucanohydrolase
Synonyms	carboxymethyl cellulase; β-1-4-glucanase
IUBMB No	3.2.1.4
CAS No	9012-54-8
EINECS No	232-734-4

Cellulases catalyse the hydrolysis of 1-4-β-glycosidic linkages in cellulose and other β-glucans resulting in the generation of shorter β-D-glucan chains.

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; laminarinase; laminaranase; β-1,3-glucanase
IUBMB No	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 mixed-linked β-D-glucans, resulting in the generation of partially hydrolysed β-D-glucans.

IUBMB nomenclature	**Endo-1,4-β-xylanase**
Systematic name	4-β-D-xylan xylanohydrolase
Synonyms	endo-(1→4)-β-xylan 4-xylanohydrolase; xylanase; β-1,4-xylanase; β-xylanase
IUBMB No	3.2.1.8
CAS No	9025-57-4
EINECS No	232-800-2

Endo-1,4-β xylanases catalyse the random hydrolysis of 1,4-β-D-xylosidic linkages in xylans (including arabinoxylans), resulting in the generation of (1-4)-β-D-xylan oligosaccharides of different lengths.

The food enzyme is intended to be used in seven food manufacturing processes: baking processes, cereal-based processes, brewing processes, fruit and vegetable processing for juice production, wine and wine vinegar production, grain treatment for the production of starch and gluten fractions, and distilled alcohol production.

3.1. Source of the food enzyme[7]

The cellulase, endo-1,3(4)-β-glucanase and endo-1,4-β-xylanase are produced with the non-genetically modified filamentous fungus *T. reesei* strain AR-256 ([redacted]), which is deposited [redacted], with the deposit number [redacted].[8] The production strain was identified as *T. reesei* [redacted].[9]

The production strain *T. reesei* AR-256 was derived from [redacted].[10]

[6] Technical dossier/Volume I/p. 3, 5, 10–11.
[7] Technical dossier/Volume I/p. 17–19; Technical dossier/Volume II.
[8] Technical dossier/Volume II/Annex 7.
[9] Technical dossier/Volume II/Annex 2.
[10] Technical dossier/Volume II/Annex 1.

3.2. Production of the food enzyme[11]

The food enzyme is manufactured according to the Food Hygiene Regulation (EC) No 852/2004[12], with food safety procedures based on Hazard Analysis and Critical Control Points, and in accordance with current Good Manufacturing Practice.[13]

The production strain is grown as a pure culture using a typical industrial medium in [redacted] with conventional process controls in place. After completion of the fermentation, the solid biomass is removed from the fermentation broth by filtration. The filtrate containing the enzyme is then further purified and concentrated, including [redacted] in which enzyme protein is retained, while most of the low molecular mass material passes the filtration membrane and is discarded.[14] 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.[15]

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[16]

The cellulase is composed of two isoenzymes of [redacted] and [redacted] amino acids, with molecular masses of [redacted] and [redacted] kDa,[16] respectively. The endo-1,3(4)-β-glucanase has two isoenzymes of [redacted] and [redacted] amino acids, with molecular masses of [redacted] and [redacted] kDa.[16] The endo-1,4-β-xylanase has two isoenzymes of [redacted] and [redacted] amino acids, with molecular masses of [redacted] and [redacted] kDa.[16,17] The food enzyme was analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).[18] A consistent protein pattern was observed across all batches with gels showing multiple bands of different intensities migrating between the marker proteins of 20 and 70 kDa.[18] No other enzymatic activities were reported.[19]

The in-house determination of cellulase activity is based on hydrolysis of carboxymethyl cellulose (reaction conditions: pH [redacted], [redacted]°C, [redacted] min). The enzymatic activity is determined by measuring the reduction of viscosity. The cellulase activity is quantified relative to an internal enzyme standard and expressed in Cellulase Unit/mg (CU/mg).[20]

The in-house determination of endo-1,3(4)-β-glucanase activity is based on hydrolysis of hydroxyethyl cellulose (reaction conditions: pH [redacted], [redacted]°C, [redacted] min). The enzymatic activity is determined by measuring the release of reducing sugars spectrophotometrically at [redacted] nm. The activity is expressed in endo-1,3(4)-β-glucanase units/mg (ECU/mg). One ECU is defined as the amount of enzyme producing 1 nmoL of reducing sugars per second under the conditions of the assay.[20]

The in-house determination of endo-1,4-β-xylanase activity is based on the hydrolysis of a birch wood xylan (reaction conditions: pH [redacted], [redacted]°C, [redacted] min). The enzymatic activity is determined by measuring the increase in reducing groups spectrophotometrically at [redacted] nm following the addition of hydroxybenzoic acid hydrazide. The activity is expressed in xylanase units/mg (XylH/mg). One XylH unit is defined as the amount of enzyme releasing 1 μmoL of xylose equivalent per minute under the conditions of the assay.[20]

The cellulase has a temperature optimum around [redacted]°C (pH [redacted]) and a pH optimum around pH [redacted] ([redacted]°C).[21] Thermostability was tested after a pre-incubation for 60 min at different temperatures

[11] Technical dossier/Volume I/p. 3–4, 19–27; Technical dossier/Volume I/Annex 9; Annex 10; Annex 11; Annex 12.
[12] 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.
[13] Technical dossier/Volume I/p. 3–4, 19, 26.
[14] Technical dossier/Volume I/p. 3–4, 20–26; Technical dossier/Volume I/Annex 10.
[15] Technical dossier/Volume I/Annex 9; Annex 11; Annex 12.
[16] Technical dossier/Additional data, 13 July 2022.
[17] Technical dossier/Volume I/Annex 2.
[18] Technical dossier/Volume I/Annex 1.
[19] Technical dossier/Volume I/p. 3, 17.
[20] Technical dossier/Volume I/Annex 5.
[21] Technical dossier/Volume I/p. 15–16; Technical dossier/Volume I/Annex 6.

(pH 4.5). Cellulase activity was stable up to [redacted]°C, but then decreased sharply, showing no residual activity above [redacted]°C.[22]

The endo-1,3(4)-β-glucanase has a temperature optimum around [redacted]°C (pH [redacted]) and a pH optimum around pH [redacted] ([redacted]°C). Activity was lost after 1 min at [redacted]°C.[23]

The endo-1,4-β-xylanase has a temperature optimum around [redacted]°C (pH [redacted]) and a pH optimum around pH [redacted] ([redacted]°C). Activity was lost after 1 min at [redacted]°C.[23]

3.3.2. Chemical parameters[24]

Data on the chemical parameters of the food enzyme were provided for three batches used for commercialisation (Table 1). The mean total organic solids (TOS) was 80.2%, the mean enzyme activity/TOS ratio was 22.6 CU/mg TOS (cellulase), 134.1 ECU/mg TOS (endo-1,3(4)-β-glucanase) and 2.9 XylH/mg TOS (endo-1,4-β-xylanase).[25]

Table 1: Composition of the food enzyme[(e)]

Parameters	Unit	Batches		
		1	2	3
Cellulase activity	CU/mg[(a)]	19,400	17,100	17,900
Endo-1,3(4)-β-glucanase activity	ECU/mg[(b)]	112,000	106,000	104,000
Endo-1,4-β-xylanase activity	XylH/mg[(c)]	2,600	2,100	2,150
Protein	%	65.3	65.9	63.3
Ash	%	1.0	0.7	< 0.1
Water	%	7.3	7.0	6.4
[redacted]	%	14.8	12.9	9.1
Total organic solids (TOS)[(d)]	%	76.9	79.4	84.4
Cellulase activity/TOS	CU/mg TOS	25.2	21.5	21.2
Endo-1,3(4)-β-glucanase activity/TOS	ECU/mg TOS	145.6	133.5	123.2
Endo-1,4-β-xylanase activity/TOS	XylH/mg TOS	3.38	2.64	2.55

(a): CU: Cellulase Unit (see Section 3.3.1).
(b): ECU: Endo-1,3(4)-β-glucanase unit (see Section 3.3.1).
(c): XylH: Endo-1,4-β-xylanase Unit (see Section 3.3.1).
(d): TOS calculated as 100% – % water – % ash – % drying aid.
(e): Technical dossier/Volume I/p. 13; Technical dossier/Volume I/Annex 3.

3.3.3. Purity[26]

The lead content in the three commercial batches was below 5 mg/kg,[26] which complies with the specification for lead as laid down in the general specifications for enzymes used in food processing (FAO/WHO, 2006). In addition, the levels of mercury, cadmium and arsenic were below the limits of quantification (LOQ) of the employed method in all commercial batches except one batch (with 0.07 mg/kg cadmium).[27,28] The Panel considered this concentration as not of concern.

The food enzyme preparation complies with the microbiological criteria (for total coliforms, *Escherichia coli* and *Salmonella*)[29] 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.[30]

Strains of *Trichoderma*, in common with most filamentous fungi, have the capacity to produce a range of secondary metabolites (Frisvad et al., 2018). The presence of T-2 toxin and HT-2 toxin was examined in the three commercial food enzyme batches and was below the limit of detection (LOD) of

[22] Technical dossier/Volume I/Annex 6.
[23] Technical dossier/Additional data, 13 July 2022/Annex 1.
[24] Technical dossier/Volume I/p. 13; Technical dossier/Volume I/Annex 3; Annex 4; Annex 5.
[25] Technical dossier/Volume I/p. 13.
[26] Technical dossier/Volume I/p. 3, 14; Technical dossier/Volume I/Annex 3; Annex 4.
[27] LOQs: Pb = 0.025 mg/kg; As = 0.25 mg/kg; Cd = 0.025 mg/kg; Hg = 0.025 mg/kg.
[28] Technical dossier/Volume I/Annex 3; Annex 4.
[29] Technical dossier/Volume I/p. 3; Technical dossier/Volume I/Annex 3; Annex 4.
[30] Technical dossier/Volume I/p. 14; Technical dossier/Volume I/Annex 3.

the applied method.[31,32] Any adverse effects caused by the possible presence of other secondary metabolites are 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 of the production strain[33]

The absence of viable cells of the production strain in the food enzyme was demonstrated in the three independent batches of liquid concentrates analysed. [redacted] and no colonies were produced.[34] A positive control was included.

3.4. Toxicological data[35]

3.4.1. Choice of test item[36]

No toxicological studies were provided for the food enzyme containing cellulase, endo-1,3(4)-β-glucanase and endo-1,4-β-xylanase produced with *T. reesei* strain AR-256. Instead, the applicant argued that the assessment of this food enzyme could be based on the toxicological data from the endo-1,4-β-xylanase produced with *T. reesei* strain [redacted], previously evaluated by EFSA (EFSA-Q-[redacted]).

The genetically modified *T. reesei* strain [redacted] producing the endo-1,4-β-xylanase was developed [redacted]. *T. reesei* [redacted] only differs from that of *T. reesei* AR-256 [redacted] and all the genetic modifications have been described throughout and raise no concerns. [redacted]. Finally, several other production strains derived from *T. reesei* strain AR-256 by genetic modification were considered safe by EFSA (EFSA CEP Panel, 2020a,b), reducing the possibility that [redacted] would have been rendered less safe by the genetic modification. Therefore, the genetic differences between *T. reesei* strain AR-256 and *T. reesei* strain [redacted] were not expected to result in a different toxigenic potential and consequently the Panel considered *T. reesei* strain [redacted] to be an appropriate substitute for the toxicological evaluation of the food enzyme under assessment.

The batch of food enzyme endo-1,4-β-xylanase from *T. reesei* strain [redacted] used for toxicological studies, was produced according to a standard procedure similar to the one described in Section 3.2 of this opinion. The data provided by the applicant, the raw materials used and the steps involved in the manufacturing of both food enzymes from *T. reesei* strains (AR-256 and [redacted] are similar.[37] Therefore, the compositions of TOS, apart from the enzyme protein itself, are comparable. Taking the molecular and technical data into account, the Panel considered the endo-1,4-β-xylanase produced with *T. reesei* strain [redacted] as a suitable substitute for the cellulase, endo-1,3(4)-β-glucanase and endo-1,4-β-xylanase from *T. reesei* strain AR-256 in the toxicological studies.

A battery of toxicological tests, including a bacterial gene mutation assay (Ames test), an *in vitro* mammalian chromosomal aberration test and a repeated dose 90-day oral toxicity study in rats, has been provided, all made with the substitute food enzyme.

[31] LODs: T2-toxin = 20 μg/kg; HT-2-toxin = 10 μg/kg.
[32] Technical dossier/Volume I/p. 14; Technical dossier/Volume I/Annex 3; Annex 4.
[33] Technical dossier/Additional data, 13 July 2022/Annex 2.
[34] Technical dossier/Volume II/Annex 5; Technical dossier/Additional data, 13 July 2022/Annex 2.
[35] Technical dossier/Volume I/p. 44–47; Technical dossier/Volume I/Annex 13; Annex 14; Annex 15; Annex 17.
[36] Technical dossier/Additional data, 13 July 2022/Annex 3; Appendix 1.
[37] Technical dossier/Volume I/Annex 17.

3.4.2. Genotoxicity

3.4.2.1. 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, 1997a) and following Good Laboratory Practice (GLP).[38]

Four strains of *Salmonella* Typhimurium (TA98, TA100, TA1535 and TA1537) and *Escherichia coli* WP2uvrA(pKM101) were used in the presence or absence of metabolic activation (S9-mix), applying the 'treat and plate' assay. Two separate experiments were carried out in triplicate using six concentrations of the food enzyme, from 17 to 5,000 μg/plate, corresponding to 16.0, 46.9, 156.8, 469.5, 1,667.1 and 4,694.5 μg TOS/plate.

No cytotoxicity was observed at any concentration of the test substance. In the first experiment, a statistically significant increase in revertant colony numbers was observed in strain TA100 in the presence of S9-mix at 5,000 μg/plate (corresponding to 4,694.5 μg TOS/plate). This increase was not reproduced in the second experiment. In all the other strains, the numbers of the revertant colonies were comparable to the values observed in the vehicle control groups, in both the experiments in the presence and absence of S9-mix.

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

3.4.2.2. *In vitro* mammalian chromosomal aberration test

The *in vitro* mammalian chromosomal aberration test was carried out according to OECD Test Guideline 473 (OECD, 1997b) and following GLP[39] in Chinese Hamster Ovary (CHO) cell cultures with and without metabolic activation (S9-mix).

In the first experiment, the cultures were exposed to 1,250, 2,500 and 5,000 μg of food enzyme/mL, corresponding to 1,174, 2,347 and 4,695 μg TOS/mL, in the presence and absence of S9-mix, during 6 h treatment + 24 h recovery. In a second experiment, the same concentrations were applied for 6 h treatment + 24 h recovery with S9-mix and for 22 h treatment + 24 h recovery without S9-mix, whereas cultures were exposed to 625, 1,250 and 2,500 μg of food enzyme/mL, corresponding to 587, 1,174 and 2,347 μg TOS/mL for 22 h treatment followed by 48 h recovery without S9-mix.

Cell counts below 50% of the control values were observed at the two highest concentrations (2,500 and 5,000 μg of food enzyme/mL) after 22 h treatment + 48 h recovery without S9-mix. For all food enzyme concentrations tested, the frequency of cells with structural chromosomal aberrations was similar to that of negative controls. No significant increases in the frequency of polyploid cells were observed in cultures harvested 48 h after treatment.

The Panel concluded that the food enzyme did not induce an increase in the frequency of structural or numerical chromosome aberrations under the test conditions applied for this study.

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

The repeated dose 90-day oral toxicity study was performed in accordance with the OECD Test Guideline 408 (OECD, 1998) and following GLP.[40] Groups of 10 male and 10 female Sprague–Dawley (Crl:CD(SD)) rats received by gavage the food enzyme in doses of 250, 500 and 1,000 mg/kg per day, corresponding to 235, 470 and 939 mg TOS/kg body weight (bw) per day. Controls received the vehicle (water).

One low-dose female was found dead on day 74 and one mid-dose male was sacrificed on day 90. The Panel considered the deaths to be due to mis-dosing (a gavage error), based on the pulmonary changes recorded at necropsy.

The body weight was statistically significantly decreased on days 7, 14 and 21 in mid-dose males (−8%, −9% and −9%, respectively). The Panel considered the changes as not toxicologically relevant, as they were only recorded sporadically, they were only observed in one sex, there was no dose–response relationship and the changes were without a statistically significant effect on the final mean body weight.

The feed consumption was statistically significantly decreased on days 7 (−14%), 14 (−14%), 21 (−11%) and 35 (−8%) in the mid-dose males, and on days 28 and 84 (−8% and −10%,

[38] Technical dossier/Volume I/Annex 13.
[39] Technical dossier/Volume I/Annex 14.
[40] Technical dossier/Volume I/Annex 15.

respectively) in high-dose males. In all treated females, a statistically significant decrease in feed consumption was recorded on day 7 (−11%, −3% and −14% at low, mid and high doses, respectively) and day 14 (−7%, −9% and −9% at low, mid and high doses, respectively). The Panel considered the changes as not toxicologically relevant, as they were only transitory and the changes were without a statistically significant effect on the final body weights.

The haematological investigation revealed a statistically significant decrease in prothrombin time in high-dose females (−7%) and a decrease in large unstained cells (LUC) in low- and mid-dose females (−33% at both dose levels). The Panel considered the changes as not toxicologically relevant, as they were only observed in one sex (both parameters), the changes were small (both parameters) and there was no dose–response relationship (LUC).

The clinical chemistry investigation revealed a statistically significant decrease in alanine aminotransferase (ALT) level in mid-dose females (−28%) and an increase in phosphate levels in low- and high-dose females (+14% and +10%, respectively). The Panel considered the changes as not toxicologically relevant as they were only observed in one sex (both parameters), the changes were small (ALT) and there was no dose–response relationship (both parameters).

The urinalysis revealed a statistically significant decrease in urinary pH in all treated males (pH of 6.6, 6.8 and 6.5 at the low-, mid- and high-dose groups, respectively, vs. 7.9 in the control group) and an increase in specific gravity in mid-dose females (+1%). The Panel considered the changes as not toxicologically relevant, as they were only observed in one sex (both parameters), the change was small (specific gravity) and there were no histopathological changes in the kidneys.

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

The Panel identified a no observed adverse effect level (NOAEL) of 939 mg TOS/kg bw per day, the highest dose tested.

3.4.4. Allergenicity[41]

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 cellulase, endo-1,3(4)-β-glucanase and endo-1,4-β-xylanase produced with the non-genetically modified *T. reesei* strain AR-256 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, one match was found with one of the two cellulase isoenzymes. The matching allergen was Sal s 6, collagen alpha, from Atlantic salmon (*Salmo salar*),[41] known as a food allergen (Ruethers et al., 2021).

No information was available on oral and respiratory sensitisation or elicitation reactions of this cellulase, endo-1,3(4)-β-glucanase and endo-1,4-β-xylanase.[42]

Xylanases, cellulases (Merget et al., 2001) and glucanases (Zober et al., 2002) have all been shown to be respiratory allergens. Studies have shown that adults respiratorily sensitised to an enzyme can commonly ingest the corresponding allergen without acquiring clinical symptoms of food allergy (Cullinan et al., 1997; Brisman, 2002; Poulsen, 2004; Armentia et al., 2009).

[redacted], a product that may cause allergies or intolerances (listed in the Regulation (EU) No 1169/2011[43]) is used as a raw material. However, during the fermentation process, this product will be degraded and utilised by the microorganisms 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 concluded that, under the intended conditions of use, the risk of allergic reactions upon dietary exposure to this food enzyme cannot be excluded (except for distilled alcohol production), in particular for individuals sensitised to salmon.

[41] Technical dossier/Volume I/p. 47–49; Technical dossier/Volume I/Annex 2; Technical dossier/Additional data, 13 July 2022/Annex 4.

[42] Technical dossier/Additional data, 13 July 2022/Annex 4.

[43] 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 seven food manufacturing processes at the recommended use levels summarised in Table 2.

Table 2: Intended uses and recommended use levels of the food enzyme as provided by the applicant[(c)]

Food manufacturing process[(a)]	Raw material (RM)	Recommended use level in mg TOS/kg RM (average – maximum)[(b)]
Baking processes	Flour	4–**15**
Cereal-based processes	Flour	4–**15**
Brewing processes	Cereals	75–**298**
Fruit and vegetable processing for juice production	Fruit and vegetables	22–**90**
Wine and wine vinegar production	Grapes	22–**75**
Grain treatment for the production of starch and gluten fractions	Cereals	45–90
Distilled alcohol production	Cereals	75–186

TOS: total organic solids.
(a): The description has been harmonised by EFSA according to the 'EC working document describing the food processes in which food enzymes are intended to be used' – not yet published at the time of adoption of this opinion.
(b): Numbers in bold were used for calculation.
(c): Technical dossier/Volume I/p. 32; Technical dossier/Additional data, 13 July 2022/Answer to question 4.

In baking processes and cereal-based processes, the food enzyme is added to flour during the preparation of dough or batter.[44] Cellulase, endo-1,3(4)-β-glucanase and endo-1,4-β-xylanase together hydrolyse cellulose, glucans and (arabino)xylans, reducing dough stiffness. The food enzyme–TOS remains in the final foods (e.g. bread, breakfast cereals and pasta).

In brewing processes, the food enzyme is added to cereals during mashing and fermentation.[45] These three enzymes together hydrolyse cellulose, glucans and (arabino)xylans, reducing viscosity and expanding the choice of raw material. The food enzyme–TOS remains in the final foods (e.g. beers and malted beverages).

In fruit and vegetable processing for juice production, the food enzyme is added to fruit and vegetables during mashing.[46] The action of these three enzymes together eases the removal of peels and reduces the viscosity in the juice. The food enzyme–TOS remains in the juices.

In wine and wine vinegar production, the food enzyme is added to grapes during crushing, maceration and juice clarification.[47] The enzymatic reaction facilitates the release of colouring and flavouring substances. The food enzyme–TOS remains in wines and wine vinegars.

In grain treatment for the production of starch and gluten fractions, the food enzyme is added to grain during multiple steps (slurry mixing, liquefaction, pre-saccharification and fermentation).[48] The enzymatic reaction reduces viscosity and increases yield. The food enzyme–TOS is removed from the gluten and starch fractions by repeated washing (EFSA CEP Panel, 2021b).

In distilled alcohol production, the food enzyme is added to the grain together with water during agglomeration.[49] The enzymatic reaction reduces viscosity and increases yield. The food enzyme–TOS is not carried over to the distilled alcohols (EFSA CEP Panel, 2021b).

Based on data provided on thermostability (see Section 3.3.1), it is expected that all three activities are inactivated during the five of the seven food processes in which it remains. The Panel expected

[44] Technical dossier/Volume I/p. 32–33; Additional data, 13 July 2022/Answer to question 4a.
[45] Technical dossier/Volume I/p. 34.
[46] Technical dossier/Volume I/p. 35–36.
[47] Technical dossier/Volume I/p. 39.
[48] Technical dossier/Volume I/p. 37.
[49] Technical dossier/Volume I/p. 38.

that enzymatic activity may remain in wine and fruit and vegetable juices, depending on the pasteurisation conditions.

3.5.2. Dietary exposure estimation

In accordance with the guidance document (EFSA CEP Panel, 2021a), the dietary exposure was calculated only for food manufacturing processes where the food enzyme–TOS remains in the final foods, i.e., baking processes, cereal-based processes, brewing processes, fruit and vegetable processing for juice production, wine and wine vinegar 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 3.921 mg TOS/kg bw per day in children of 3–9 years of age at the 95th percentile.

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)	0.039–0.643 (11)	0.210–2.269 (15)	0.090–1.286 (19)	0.095–0.763 (21)	0.131–0.633 (22)	0.105–0.528 (22)
Min–max 95th (number of surveys)	0.101–2.383 (9)	1.025–3.811 (13)	0.159–3.921 (19)	0.212–2.448 (20)	0.563–2.009 (22)	0.418–1.426 (21)

TOS: total organic solids.

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	+

Sources of uncertainties	Direction of impact
Selection of broad FoodEx categories for the exposure assessment	+
Use of recipe fractions in disaggregation FoodEx categories	+/–
Use of technical factors in the exposure model	+/–
Exclusion of other processes from the exposure assessment – Grain treatment for the production of starch and gluten fractions – Distilled alcohol production	–

+: uncertainty with potential to cause overestimation of exposure.
–: uncertainty with potential to cause underestimation of exposure.
FoodEx: the food classification and description system; TOS: total organic solids.

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 (grain treatment for the production of starch and gluten fractions, and distilled alcohol production) from the exposure assessment was based on > 99% of TOS removal during these processes (EFSA CEP Panel, 2021b) and was not expected to have an impact on the overall estimate derived by the Panel.

3.6. Margin of exposure

The comparison of the NOAEL (939 mg TOS/kg bw per day) from the 90-day rat study with the derived exposure estimates of 0.039–2.269 mg TOS/kg bw per day at the mean and from 0.101–3.921 mg TOS/kg bw per day at the 95th percentile resulted in margin of exposure (MoE) of at least 239.

4. Conclusions

Based on the data provided, the removal of TOS during grain treatment for the production of starch and gluten fractions and distilled alcohol production, and the derived margin of exposure for the remaining food processes, the Panel concluded that the food enzyme containing cellulase, endo-1,3(4)-β-glucanase and endo-1,4-β-xylanase activities, produced with *T. reesei* strain AR-256, does not give rise to safety concerns under the intended conditions of use.

Documentation as provided to EFSA

Technical dossier 'Application for authorisation of a cellulase, glucanase and xylanase from strain of *Trichoderma reesei* in accordance with Regulation (EC) No 1331/2008'. Joint dossier was originally submitted on 3 October 2014 (EFSA-Q-2014-00804-806). The updated technical dossier was submitted by AB Enzymes GmbH on 30 September 2021.

Additional information. 13 July 2022. Submitted by AB Enzymes GmbH.

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Abbreviations

ALT	alanine aminotransferase
AMFEP	Association of Manufacturers and Formulators of Enzyme Products
bw	body weight
CAS	Chemical Abstracts Service

[redacted]	[redacted]
CHO	Chinese Hamster Ovary cell culture
CU	cellulase unit
ECU	endo-1,3(4)-β-glucanase unit
EFSA CEF Panel	EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
EFSA CEP Panel	EFSA Panel on Food Contact Materials, Enzymes and Processing Aids
EFSA GMO Panel	EFSA Panel on Genetically Modified Organisms
EINECS	European Inventory of Existing Commercial Chemical Substances
FAO	Food and Agricultural Organization of the United Nations
FoodEx	the food classification and description system
GLP	Good Laboratory Practice
GM	genetically modified
GMO	genetically modified organisms
[redacted]	[redacted]
IUBMB	International Union of Biochemistry and Molecular Biology
JECFA	Joint FAO/WHO Expert Committee on Food Additives
kDa	kilo Dalton
LOD	limit of detection
LOQ	limit of quantification
LUC	large unstained cells
MoE	margin of exposure
NOAEL	no observed adverse effect level
non-GM	non-genetically modified
OECD	Organisation for Economic Cooperation and Development
RM	raw material
[redacted]	[redacted]
SDS–PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
[redacted]	[redacted]
TOS	total organic solids
WHO	World Health Organization
XylH	endo-1,4-β-xylanase unit

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.2022.7676#support-information-section).

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: 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).