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Safety evaluation of the food enzyme pectinesterase from the genetically modified *Trichoderma reesei* strain RF6201

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Abstract

The food enzyme pectinesterase (pectin pectylhydrolase; EC 3.1.1.11) is produced with the genetically modified *Trichoderma reesei* strain RF6201 by AB Enzymes GmbH. The genetic modifications do not give rise to safety concerns. The food enzyme was considered free from viable cells of the production organism and its DNA. It is intended to be used in five food manufacturing processes: fruit and vegetable processing for juice production, fruit and vegetable processing for products other than juices, production of wine and wine vinegar, coffee demucilation and production of plant extracts as flavouring preparations. Since residual amounts of the total organic solids (TOS) are removed during the coffee demucilation and the production of flavouring extracts, dietary exposure was calculated only for the remaining three food processes. It was estimated to be up to 0.532 mg TOS/kg body weight (bw) per day in European populations. Genotoxicity tests did not indicate 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 1,000 mg TOS/kg bw per day, the highest dose tested, which, when compared with the estimated dietary exposure, resulted in a margin of exposure of at least 1,880. A search for the similarity of the amino acid sequence of the food enzyme to known allergens was made and two matches were found with pollen allergens. The Panel considered that, under the intended conditions of use, the risk of allergic reactions upon dietary exposure, particularly in individuals sensitised to pollen allergens, cannot be excluded. Based on the data provided, the Panel concluded that this food enzyme does not give rise to safety concerns under the intended conditions of use.

Keywords: food enzyme, pectinesterase, pectin pectylhydrolase; EC 3.1.1.11, pectin methylesterase, *Trichoderma reesei*, genetically modified microorganism

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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 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 on food enzymes.

Four applications have been submitted by the companies "Novozymes A/S" and "AB Enzymes GmbH" for the authorisation of the food enzymes Alpha-amylase from a genetically modified strain of *Bacillus licheniformis* (strain NZYM-AV), Beta-glucanase, Xylanase and Cellulase produced by a strain of *Humicola insolens* (strain NZYM-ST), Polygalacturonase from a genetically modified strain of *Trichoderma reesei* (strain RF6197) and Pectin esterase from a genetically modified strain of *Trichoderma reesei* (strain RF6201).

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

[1] Regulation (EC) No 1332/2008 of the European Parliament and of the Council of 16 December 2008 on Food Enzymes and Amending Council Directive 83/417/EEC, Council Regulation (EC) No 1493/1999, Directive 2000/13/EC, Council Directive 2001/112/EC and Regulation (EC) No 258/97. OJ L 354, 31.12.2008, 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, 11.03.2011, p. 15–24.

1.1.2. Terms of Reference

The European Commission requests the European Food Safety Authority to carry out the safety assessment on the food enzymes Alpha-amylase from a genetically modified strain of *Bacillus licheniformis* (strain NZYM-AV); Beta-glucanase, Xylanase and Cellulase produced by a strain of *Humicola insolens* (strain NZYM-ST); Polygalacturonase from a genetically modified strain of *Trichoderma reesei* (strain RF6197) and Pectin esterase from a genetically modified strain of *Trichoderma reesei* (strain RF6201) in accordance with Article 17.3 of Regulation (EC) No 1332/2008 on food enzymes.

1.2. Interpretation of the terms of Reference

The present scientific opinion addresses the European Commission's request to carry out the safety assessment of food enzyme pectinesterase from the genetically modified *Trichoderma reesei* strain RF6201.

2. Data and methodologies

2.1. Data

The applicant has submitted a dossier in support of the application for authorisation of the food enzyme pectinesterase from a genetically modified *Trichoderma reesei* (strain RF6201).

Additional information was requested from the applicant during the assessment process on 25 February 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 guidance documents of the EFSA Scientific Committee.

The 'Guidance on the submission of a dossier on food enzymes for safety evaluation' (EFSA, 2009a) 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).

3. Assessment

IUBMB nomenclature	Pectinesterase
Systematic name	Pectin pectylhydrolase
Synonyms	Pectin methylesterase; pectin methoxylase; pectin demethoxylase
IUBMB No	EC 3.1.1.11
CAS No	9025-98-3
EINECS No	232-807-0

Pectinesterases catalyse the de-esterification of pectin, resulting in the generation of pectic acid and methanol. The food enzyme under assessment is intended to be used in five food manufacturing processes: fruit and vegetable processing for juice production, fruit and vegetable processing for products other than juices, production of wine and wine vinegar, coffee demucilation and production of plant extracts as flavouring preparations.

3.1. Source of the food enzyme

The pectinesterase is produced with *T. reesei* strain RF6201, which is deposited in the Westerdijk Fungal Biodiversity Institute culture collection (CBS, the Netherlands) with the deposit number [redacted].[4] The production strain was identified as *T. reesei* [redacted]

[4] Technical dossier/Volume III/Appendix 11.

[redacted][5].

3.1.1. Characteristics of the parental and recipient microorganisms

The parental strain was [redacted][6]. [redacted][7].

3.1.2. Characteristics of introduced sequences

The sequence encoding the pectinesterase ([redacted][8].

3.1.3. Description of the genetic modification process

The purpose of genetic modification was to enable the production strain to synthesise pectinesterase [redacted][9]. [redacted][10].

3.1.4. Safety aspects of the genetic modification

The technical dossier contains all necessary information on the recipient microorganism, the donor organism and the genetic modification process.

The production strain *T. reesei* RF6201 differs from the recipient strain in its capacity to produce the pectinesterase [redacted][11].

No issues of concern arising from the genetic modifications were identified by the Panel.

[5] Technical Dossier/Additional information July 2022/Annex 1.
[6] Technical Dossier/Volume III/GM part and Appendix 2.
[7] Technical Dossier/Volume III/Appendix 1 and Additional information July 2022/RF6201 Additional information Annex CONFIDENTIAL.
[8] Technical Dossier/Volume III/GM part and Appendices 7 and 8.
[9] Technical Dossier/Volume III/GM part and Appendix 9.
[10] Technical Dossier/Volume III/Appendix 10.
[11] Technical dossier/Volume III/Appendix 12.

3.2. Production of the food enzyme

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 a submerged, [redacted] fermentation system 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 an ultrafiltration step 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

The pectinesterase is a single polypeptide chain of [redacted] amino acids.[16] The molecular mass of the mature protein, calculated from the amino acid sequence, is around [redacted] kDa. The food enzyme was analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).[17] A consistent protein pattern was observed across all batches. The gel showed three major protein bands of about [redacted] kDa, corresponding to differently glycosylated forms of the enzyme. The food enzyme was tested for β-glucanase, cellulase and xylanase activities and all were detected.[18] No other enzyme activities were reported.

The in-house determination of pectinesterase activity is based on the hydrolysis of citrus pectin (reaction conditions: pH 4.5, 30°C, 8 min). The enzymatic activity is determined by measuring the released free carboxylic groups that are titrated with sodium hydroxide. Pectinesterase activity is expressed in pectin esterase units (PE)/g. One unit is defined as the amount of enzyme that will release 1 μmol of acid groups per minute under the conditions of the assay.[19]

The food enzyme has a temperature optimum around 40°C (pH 4.5) and a pH optimum around pH 4.5 (30°C). Thermostability was tested after a pre-incubation of the food enzyme at 85°C for different time periods (pH 4.5). No activity was detected after 2 min pre-incubation.[20]

3.3.2. Chemical parameters

Data on the chemical parameters of the food enzyme were provided for three batches used for commercialisation and one batch produced for the toxicological tests (Table 1).[21] The mean total organic solids (TOS) of the three food enzyme batches for commercialisation was 25.8% and the mean enzyme activity/TOS ratio was 404 PE/mg TOS.

[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 pg. 49 and Volume II/Annex 9.
[14] Technical Dossier/Volume I pg. 40–49 and Volume II/Annex 10.
[15] Technical Dossier/Volume I pg. 41, 44 and Volume II/Annexes 11, 12, 13 and 14.
[16] Technical Dossier/Volume I pg. 31–31 and Volume II/Annex 4B.
[17] Technical Dossier/Volume I pg. 30–32 and Volume II/Annex 4A and Additional information July 2022/Annex 7.
[18] Technical Dossier/Volume I pg. 30, 33.
[19] Technical Dossier/Volume I pg. 32–33 and Volume II/Annex 3.
[20] Technical Dossier/Volume I pg. 33–34 and Volume II/Annex 5.
[21] Technical Dossier/Volume I pg. 29, 66–67 and Volume II/Annexes 1, 2 and 16 and Additional information July 2022/Annexes 8, 9.

Table 1: Composition of the food enzyme

Parameters	Unit	Batches			
		1	2	3	4[(a)]
Pectinesterase activity	PE/g[(b)]	104,000	103,000	106,000	382,000
Protein	%	19.8	19.4	20.0	65.3
Ash	%	0.2	0.2	0.2	0.8
Water	%	74.0	74.5	73.4	5.0
Total organic solids (TOS)[(c)]	%	25.8	25.3	26.4	94.2
Activity/TOS	PE/mg TOS	403	407	402	405

(a): Batch used for the toxicological studies.
(b): PE: Pectin Esterase unit (see Section 3.3.1).
(c): TOS calculated as 100%–% water–% ash.

3.3.3. Purity

The lead content in the three commercial batches and in the batch used for toxicological studies was below 5 mg/kg,[22,23] which complies with the specification for lead as laid down in the general specifications for enzymes used in food processing (FAO/WHO, 2006).

The food enzyme preparation 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).[22] No antimicrobial activity was detected in any of the tested batches.[22]

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 T2-toxin and HT2-toxin was examined in the four food enzyme batches and all were below the limit of quantification (LoQ) of the applied methods.[22,24] 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 and DNA of the production strain

The absence of viable cells of the production strain in the food enzyme was demonstrated [redacted][25][redacted]. No colonies of the production strain were detected. A positive control was included.

The absence of recombinant DNA in the food enzyme was demonstrated [redacted][26].

3.4. Toxicological data

A battery of toxicological tests, including a bacterial reverse mutation test (Ames test), an *in vitro* mammalian chromosomal aberration test and a repeated dose 90-day oral toxicity study in rats, was provided. The batch 4 (Table 1) used in these studies has a comparable activity/TOS value to those of the commercial batches and was considered suitable as a test item.

[22] Technical Dossier/Volume I pg. 30–31 and Volume II/Annexes 1 and 2 and Additional information July 2022/Annexes 8, 9.
[23] LoQ: Pb = 0.05 mg/kg.
[24] LoQs: T2 and HT2-toxin = 10 μg/kg each.
[25] Technical Dossier/Additional information July 2022/Annexes 4 and 5.
[26] Technical Dossier/Additional information July 2022/Annex 6.

3.4.1. Genotoxicity

3.4.1.1. Bacterial reverse mutation test

A bacterial reverse mutation assay (Ames test) was performed according to Organisation for Economic Co-operation and Development (OECD) Test Guideline 471 (OECD, 1997a) and following good laboratory practice (GLP).[27]

Five strains of Salmonella Typhimurium (TA98, TA100, TA102, TA1535 and TA1537) were used in the presence or absence of metabolic activation, applying the standard plate incorporation method (experiment I) and the preincubation method (experiment II). The first experiment used eight concentrations of the food enzyme (3, 10, 33, 100, 333, 1,000, 2,500 and 5,000 μg TOS/plate) and the second experiment six concentrations of the food enzyme (33, 100, 333, 1,000, 2,500 and 5,000 μg TOS/plate).

No cytotoxicity was observed at any concentration tested. 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 pectinesterase did not induce gene mutations under the test conditions employed in this study.

3.4.1.2. *In vitro* mammalian chromosomal aberration test

The *in vitro* mammalian chromosomal aberration test was carried out in Chinese hamster V79 lung cells according to OECD Test Guideline 473 (OECD, 1997b) and following GLP.[28]

The dose-finding study was performed at concentrations ranging from 331.9 to 5,310 μg/mL, and no inhibition of cell growth of 50% or more was observed. Based on these results, the cells were exposed to the food enzyme at 1,327, 2,655 and 5,310 μg/mL (corresponding to 1,250, 2,500 and 5,000 μg TOS/mL) in a short-term treatment (4 h followed by 14 h recovery period) with and without metabolic activation (S9-mix), and in a continuous treatment (18 h) in the absence of S9-mix.

No cytotoxicity was observed at any concentration tested. The frequency of structural and numerical chromosomal aberrations in treated cultures was comparable to the values detected in negative controls and within the range of the laboratory historical control data.

The Panel concluded that the food enzyme pectinesterase does not induce chromosome aberrations under the test conditions employed for this study.

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

The repeated dose 90-day oral toxicity study was performed in accordance with OECD Test Guideline 408 (OECD, 1998) and following GLP.[29] Groups of 10 male and 10 female RccHan™: WIST (SPF) rats received by gavage the food enzyme in doses equivalent to 100, 300 and 1,000 mg TOS/kg bw per day. Controls received the vehicle (bidistilled water).

One low-dose male was found dead on day 67 of treatment. The necropsy findings indicated misdosing as the cause of death.

The body weight was statistically significantly increased on day 15 (+8%) in mid-dose females and on days 15 (+9%) and 22 (+7%) in low-dose females when compared with controls. The body weight gain was statistically significantly decreased from day 8 onwards, with statistical significance on days 15, 22 and 29 (−15%, −18%, −16%, respectively) of administration in high-dose males. The body weight gain was statistically significantly increased (+58%, +58%, +58%, respectively) on day 15 of administration in all treated females. The Panel considered the changes as not toxicologically relevant, as they were only recorded sporadically and no statistically significant changes in the final body weight and body weight gain were reported.

Functional observational battery tests revealed that locomotor activity was statistically significantly increased in mid-dose males during 0–10 min (+30%) and decreased in high-dose males from 50 to 60 min (−64%) when compared with the controls. The Panel considered the changes as not toxicologically relevant as they were only recorded sporadically, they were only seen in one sex and there was no dose–response relationship (the first interval).

The haematological investigation revealed in high-dose males a statistically significant decrease in relative reticulocyte counts (−17%), in mean high-fluorescence reticulocytes (−41%) and a statistically

[27] Technical dossier/Annex 17.
[28] Technical dossier/Annex 18.
[29] Technical dossier/Annex 19.

significant increase in mean low-fluorescence reticulocytes (+11%). In mid-dose males, a higher methaemoglobin level was noted (+13%). In high-dose females, a statistically significant increase in the relative monocyte counts (+63%) was reported. In mid-dose females, reduced white blood cell count (WBC; −19%) and absolute basophil count (−50%) were noted. Reduced lymphocyte counts were reported in mid- (−25%) and high-dose females (−17%). The Panel considered the changes as not toxicologically relevant as they were only observed in one sex (all parameters), there was no dose–response relationship (methaemoglobin, WBC, absolute basophile and lymphocyte counts), the magnitude of the changes was small (absolute basophil and relative monocyte count), there were no changes in other relevant parameters (for lymphocytes in a total white blood cell count), the changes were within the historical control values and there were no changes in the bone marrow (reticulocytes).

The clinical chemistry investigation revealed a statistically significant decrease in total bilirubin (−21%, −25%, −25%, respectively) in all treated males. A statistically significant increase in sodium (+1%) was reported in high-dose males and in chloride levels in mid- (+1%) and high-dose (+2%) males. In high-dose females, a statistically significant decrease in lactate dehydrogenase (LDH) activity (−33%) and an increase in calcium (+3%) were reported. A statistically significant increase in sodium (+1%, +2%, respectively) and chloride (+3%, +2%, respectively) were observed in mid- and high-dose females. In mid-dose females, a statistically significant decrease in phosphorus (−17%) was noted. The Panel considered the changes as not toxicologically relevant as they were only observed in one sex (bilirubin, LDH, calcium, phosphorus), there was no dose–response relationship (chloride in females, phosphorus) and the changes were within the historical control values (with the exception of the sodium levels in females, which were slightly outside the historical control values, i.e. 148.8 and 149.4 mmol/L vs. 137.8–147.8 mmol/L in the historical controls).

Statistically significant changes in organ weights included an increase in absolute heart weights (+15%), heart-to-body weight ratio (+10%) and heart-to-brain weight ratio (+12%) in high-dose females. In low-dose females, a significantly significant increase in absolute heart weight (+12%) and absolute liver weight (+15%) and a decrease in ovary-to-body weight ratio (−19%) were noted. The Panel considered the changes as not toxicologically relevant as they were only observed in one sex, the changes were small (heart, liver), there was no dose–response relationship (absolute liver weight, relative ovary weight), there were no histopathological changes in the organs.

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

The Panel identified the no observed adverse effect level (NOAEL) of 1,000 mg TOS/kg bw per day, the highest dose tested.

3.4.3. Allergenicity

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

The potential allergenicity of the pectinesterase produced with the genetically modified *T. reesei* strain RF6201 was assessed by comparing its amino acid sequence with those of known allergens according to the 'Scientific opinion on the assessment of allergenicity of GM plants and microorganisms and derived food and feed of the Scientific Panel on Genetically Modified Organisms' (EFSA GMO Panel, 2010). Using higher than 35% identity in a sliding window of 80 amino acids as the criterion, two matches were found. The matching allergens were pectin methylesterase from Russian thistle (*Salsola kali*) and Ole e 11 pectinesterase from olive tree (*Olea europaea*), known as respiratory allergens.[30]

No information is available on oral and respiratory sensitisation or elicitation reactions of this pectinesterase.

Pectinesterases present in plant tissues and pollen are reported for their role in allergenicity: the allergen Ole e 11, a pectinesterase from Olive tree (*Olea europaea*), was identified as a source of allergy (Salamanca et al., 2010), as well as Sal k 1, a pectinesterase from Russian thistle (*Salsola kali*) (Barderas et al., 2007). The Panel noted that the oral allergy syndrome, i.e. allergic reactions mainly in the mouth and seldomly leading to anaphylaxis, is associated with sensitisation to olive and Russian thistle pollen.

[30] Technical dossier/ Volume I pg. 67–71 and Volume II/Annex 20 and Additional information July 2022/Annex 10.

The Panel considered that, under the intended conditions of use, the risk of allergic reactions upon dietary exposure to this food enzyme, particularly in individuals sensitised to pollen allergens, cannot be excluded.

3.5. Dietary exposure

3.5.1. Intended use of the food enzyme

The food enzyme is intended to be used in five food 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)	Maximum recommended use level (mg TOS/kg RM)[(b)]
Fruit and vegetable processing for juice production		Fruits and vegetables	**3**
Fruit and vegetable processing for products other than juices	Puree	Fruits and vegetables	**24**
	Fruit firming		12
Production of wine and wine vinegar		Grapes	**1**
Coffee demucilation		Coffee cherries	0.5
Production of plant extracts as flavouring preparations[(d)]		Fruit and vegetables	265

(a): The name has been harmonised 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): The numbers in bold were used for calculation.
(c): Technical dossier/p. 58 and Additional information July 2022/Answers 13, 14 and 15.
(d): Additional data July 2022/Answer 16.

In fruit and vegetable processing, the function of pectinesterase is to aid the depolymerisation of pectin in different raw materials at various points in the production process. For juice production, the food enzyme can be added during the peeling and crushing; to the crush mash of fruits/vegetables (with or without peels) and/or to the pressed juice before clarification and filtration.[31] The disruption of the gel structure reduces the viscosity, thus improving the pressing ability of the pulp and consequently increasing the yield of fruit juices. The enzymatic treatment can reduce haze and enhance colour and aroma. The food enzyme-TOS remains in the juices.

In puree production, the pectinesterase is added to the crushed pulp before pasteurisation.[32] The enzymatic treatment reduces viscosity and improves the consistency of the puree. Treatment with pectinesterase can also improve the firmness of jams, canned and frozen fruit and vegetables products.[34] The food enzyme-TOS remains in these products.

In wine and wine vinegar production, the pectinesterase is often added together with other cell wall hydrolytic enzymes during crushing. It can be added also during maceration and clarification steps. Such enzymatic treatment aids pressing and facilitates the extraction of aromatic compounds.[33] The food enzyme-TOS may remain in wine and wine vinegar.

In coffee bean demucilation, the pectinesterase is added to green coffee cherries during pulping and fermentation to degrade the mucilage.[34] The food enzyme-TOS is removed during the subsequent washing steps (EFSA CEP Panel, 2021b).

The food enzyme is used to obtain aroma concentrates or essential oils for use as flavouring preparations. To produce essential oils, fruit components rich in oil are treated with the pectinesterase to assist the release of aromatic compounds from the raw material. It is expected that the food enzyme-TOS partitions with the water phase. Therefore, they are not carried into the oil phase.[32] The aroma concentrates are primarily used in the reconstitution of juices.

[31] Technical dossier/p. 53.
[32] Technical dossier/p. 54.
[33] Technical dossier/p. 55.
[34] Technical dossier/p. 56.

Samples of the apple aroma concentrate and orange aroma oil, as well as samples obtained by trichloroacetic acid precipitation were separated by SDS-PAGE and stained with Coomassie Blue.[35] No proteins of the food enzyme were detected by liquid chromatography tandem mass spectrometry.[36] The Panel accepted this evidence as sufficient to support the lack of TOS transfer into the essential oils.

Based on data provided on thermostability (see Section 3.3.1), the pectinesterase is expected to be inactivated by heat in most of the food processes, but may remain active in wine and wine vinegar, and in juices, depending on the pasteurisation conditions.

3.5.2. Dietary exposure estimation

In accordance with the guidance document (EFSA CEP Panel, 2021a), dietary exposure was calculated only for food manufacturing processes where the food enzyme-TOS remains in the final foods: fruit and vegetable processing for juice production, fruit and vegetable processing for products other than juice and production of wine and wine vinegar.

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 43 different dietary surveys (covering infants, toddlers, children, adolescents, adults and the elderly), carried out in 22 European countries (Appendix B). The highest dietary exposure to the food enzyme-TOS was estimated to be 0.532 mg TOS/kg bw per day in infants.

Table 3: Summary of the 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)	0.008–0.316 (12)	0.032–0.203 (15)	0.024–0.207 (19)	0.005–0.111 (21)	0.004–0.054 (22)	0.003–0.060 (23)
Min–max 95th percentile (number of surveys)	0.035–0.532 (11)	0.107–0.499 (14)	0.087–0.432 (19)	0.020–0.245 (20)	0.015–0.136 (22)	0.013–0.134 (22)

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.

[35] Additional data July 2022/Annex 11.
[36] Additional data July 2022/Annex 12.

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	+
Although two different used levels were given for puree and firming, only the higher one was used in the calculation.	+
Selection of broad FoodEx categories for the exposure assessment	+
Use of recipe fractions to disaggregate FoodEx categories	+/–
Use of technical factors in the exposure model	+/–
Exclusion of other processes from the exposure assessment – Production of plant extract as flavouring preparations – Coffee demucilation	–

+: Uncertainty with potential to cause overestimation of exposure.
–: Uncertainty with potential to cause underestimation of exposure.

The conservative approach applied to estimate the dietary exposure to the 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 during these processes and is not expected to have an impact on the overall estimate derived.

3.6. Margin of exposure

A comparison of the NOAEL (1,000 mg TOS/kg bw per day) from the 90-day rat study with the derived exposure estimates of 0.003–0.316 mg TOS/kg bw per day at the mean and from 0.013–0.532 mg TOS/kg bw per day at the 95th percentile resulted in a margin of exposure (MoE) of at least 1,880.

4. Conclusions

Based on the data provided, the removal of TOS during coffee demucilation and the production of flavouring extracts, and the derived margin of exposure for the remaining three food manufacturing processes, the Panel concluded that the food enzyme pectinesterase produced with the genetically modified *Trichoderma reesei* strain RF6201 does not give rise to safety concerns under the intended conditions of use.

The CEP Panel considered the food enzyme free from viable cells of the production organism and recombinant DNA.

5. Documentation as provided to EFSA

1) Dossier "Application for authorisation of a pectinesterase from a genetically modified strain of *Trichoderma reesei* in accordance with Regulation (EC) No 1331/2008". October 2014. Submitted by AB Enzymes GmbH.
2) Summary report on the GMM part for pectinesterase produced by *Trichoderma reseei* strain RF6201 by AB Enzymes. Delivered by Pedersen and Eriksen (Kongens Lyngby, Denmark) on 3 February 2016.
3) Summary report on technical data and dietary exposure related to pectinesterase from a strain of *Trichoderma reesei* (strain RF6201) by AB Enzymes. Delivered by Hylobates Consulting and BiCT (Rome, Italy) on 23 February 2016.
4) Additional information. July 2022. Submitted by AB Enzymes GmbH.

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Abbreviations

bw	body weight
CAS	Chemical Abstracts Service
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
MoE	margin of exposure
OECD	Organisation for Economic Cooperation and Development
PCR	polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TOS	total organic solids
WHO	World Health Organization

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

Appendix A can be found in the online version of this output (in the 'Supporting 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, Spain
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).