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## Safety evaluation of the food enzyme mannan endo-1,4- $\beta$ -mannosidase from the genetically modified *Trichoderma reesei* strain RF6232

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### Abstract

The food enzyme mannan endo-1,4- $\beta$ -mannosidase (1,4- $\beta$ -D-mannan mannanohydrolase; EC 3.2.1.78) is produced with the genetically modified *Trichoderma reesei* strain RF6232 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 recombinant DNA. It is intended to be used in coffee processing, fruit and vegetable processing for juice production and for edible oil production. Since residual amounts of total organic solids (TOS) are removed during refined edible oil production by repeated washing, dietary exposure was calculated only for the remaining two food manufacturing processes. Dietary exposure to the food enzyme–TOS was estimated to be up to 0.09 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 a repeated dose 90-day oral toxicity study in rats. The Panel identified a no observed adverse effect level of 100 mg TOS/kg bw per day, the lowest dose tested. This results in a margin of exposure above 1,100. A search for similarity of the amino acid sequence of the food enzyme to known allergens was made and one match was found. The Panel considered that, under the intended conditions of use, the risk of allergic sensitisation and elicitation reactions by dietary exposure cannot be excluded, in particular for individuals allergic to avocado, but the likelihood for this to occur is considered to be low. 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.

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**Keywords:** food enzyme, mannan endo-1,4- $\beta$ -mannosidase, 1,4- $\beta$ -D-mannan mannanohydrolase,  $\beta$ -mannanase, EC 3.2.1.78, *Trichoderma reesei*, genetically modified microorganism

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## 1. Introduction

Article 3 of the Regulation (EC) No 1332/2008<sup>1</sup> 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<sup>2</sup> 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, 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 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 on food enzymes.

Five applications have been introduced by the companies "Novozymes A/S", "AB Enzymes GmbH", "Ajinomoto Europe SAS" and "Nagase (Europa) GmbH" for the authorisation of the food enzymes Beta-galactosidase from a genetically modified strain of *Bacillus licheniformis* (strain NZYM-BT), Mannan endo-1,4-beta-mannosidase ( $\beta$ -mannanase) from a genetically modified strain of *Trichoderma reesei* (strain RF6232), Transglutaminase from *Streptovercillium mobaraense* (strain S-8112), Maltogenic amylase from a genetically modified strain of *Bacillus subtilis* (strain NZYM-SM) and Glucanase from *Streptomyces violaceoruber* (strain pGlu).

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

<sup>1</sup> 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.

<sup>2</sup> 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.

<sup>3</sup> 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 (EC) requests the European Food Safety Authority (EFSA) to carry out the safety assessments on the food enzymes Beta-galactosidase from a genetically modified strain of *Bacillus licheniformis* (strain NZYM-BT), Mannan endo-1,4-beta-mannosidase ( $\beta$ -mannanase) from a genetically modified strain of *Trichoderma reesei* (strain RF6232), Transglutaminase from *Streptovorticillium mobaraense* (strain S-8112), Maltogenic amylase from a genetically modified strain of *Bacillus subtilis* (strain NZYM-SM) and Glucanase from *Streptomyces violaceoruber* (strain pGlu) 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 mannan endo-1,4-beta-mannosidase ( $\beta$ -mannanase) from a genetically modified *Trichoderma reesei* (strain RF6232).

## 2. Data and methodologies

### 2.1. Data

The applicant has submitted a dossier in support of the application for authorisation of the food enzyme mannan endo-1,4-beta-mannosidase ( $\beta$ -mannanase) from a genetically modified *Trichoderma reesei* (strain RF6232).

Additional information was requested from the applicant during the assessment process on 14 April 2020 and 5 October 2020 and received on 7 July 2020 and 12 February 2021, respectively. Additional information was spontaneously provided on 3 May 2022 (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	Mannan endo-1,4- $\beta$ -mannosidase
Systematic name	1,4- $\beta$ -D-mannan mannanohydrolase
Synonyms	$\beta$ -mannanase; endo- $\beta$ -1,4-mannase; endo- $\beta$ -mannanase; $\beta$ -D-mannanase
IUBMB no	3.2.1.78
CAS no	37288-54-3
EINECS no	253-446-5

Mannan endo-1,4- $\beta$ -mannosidases catalyse the random hydrolysis of 1,4- $\beta$ -D-glycosidic linkages in mannans, galactomannans and glucomannans, resulting in the generation of  $\beta$ -1,4-manno oligosaccharides. The food enzyme under this assessment is intended to be used in coffee processing, fruit and vegetable processing for juice production and for edible oil production.

### 3.1. Source of the food enzyme

The mannan endo-1,4- $\beta$ -mannosidase is produced with a genetically modified filamentous fungus *Trichoderma reesei* strain RF6232, which is deposited at the Westerdijk Fungal Biodiversity Institute culture collection (the Netherlands) with the deposit number [REDACTED]<sup>4</sup>.

<sup>4</sup> Technical dossier/Volume II/Annex 6.

### 3.1.1. Characteristics of the parental and recipient microorganisms

The parental strain is the natural isolate *Trichoderma reesei* QM6a. [REDACTED]

<sup>5</sup>

The recipient strain, [REDACTED]

<sup>7</sup>

<sup>8</sup>

### 3.1.2. Characteristics of introduced sequences

### 3.1.3. Description of the genetic modification process

The purpose of the genetic modification [REDACTED]

<sup>9</sup>

### 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 RF6232 differs from recipient strain [REDACTED]

<sup>10</sup>

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

<sup>5</sup> Technical dossier/Volume III/Appendix 1B.

<sup>6</sup> Technical dossier/Volume III/Appendix 2.

<sup>7</sup> Technical dossier/Volume III/Appendix 9.

<sup>8</sup> Technical dossier/Volume III/Appendix 3.

<sup>9</sup> Technical dossier/Volume III/Appendix 10.

<sup>10</sup> 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<sup>11</sup>, with food safety procedures based on hazard analysis and critical control points, and in accordance with current good manufacturing practice.

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 filtrate containing the enzyme. The filtrate containing the enzyme is then further purified and concentrated, including an ultrafiltration step, in which the enzyme protein is retained while most of the low molecular mass material passes the filtration membrane and is discarded.<sup>12</sup> The food enzyme is then dried (see Table 1). 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.<sup>13</sup>

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 mannan endo-1,4- $\beta$ -mannosidase is a single polypeptide chain of [REDACTED] amino acids ([REDACTED] including the signal sequence).<sup>14</sup> The molecular mass of the mature enzyme, derived from the amino acid sequence, was calculated to be [REDACTED] kDa. The food enzyme was analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).<sup>15</sup> A consistent protein pattern was observed across all batches. The gels showed a major band migrating between the marker proteins of [REDACTED] and [REDACTED] kDa attributed to the mannan endo-1,4- $\beta$ -mannosidase together with a number of lesser staining bands. No other enzyme activities were reported.<sup>16</sup>

The in-house determination of mannan endo-1,4- $\beta$ -mannosidase activity is based on hydrolysis of galactomannan (reaction conditions: pH 5.3, 50°C, 5 min). The enzymatic activity is determined colourimetrically by measuring the release of reducing carbohydrates with 3,5-dinitrosalicylic acid. One mannan endo-1,4- $\beta$ -mannosidase unit (MNU) is defined as the amount of enzyme that releases reducing carbohydrates corresponding to 1 nmol mannose per second under the conditions of the assay.<sup>17</sup>

The food enzyme has a temperature optimum of 80°C (pH 5.3) and an activity plateau between pH 3 and pH 6 (50°C). Incubation of the food enzyme at 85°C for 5 min at pH 4.5 resulted in a complete loss of activity.<sup>18</sup>

### 3.3.2. Chemical parameters

Data on the chemical parameters of the dried food enzyme were provided for three batches for commercialisation and one batch used for the toxicological tests (Table 1).<sup>19</sup> The mean total organic solids (TOS) of the three food enzyme batches for commercialisation is 92.5% and the mean enzyme activity/TOS ratio is 1,934 MNU/mg TOS.

<sup>11</sup> 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.

<sup>12</sup> Technical dossier/Section 3.2.1.2.5.

<sup>13</sup> Technical dossier/Volume II/Annex 12 and 14.

<sup>14</sup> Technical dossier/Volume II/Annex 4.

<sup>15</sup> Technical dossier/Volume II/Annex 4.

<sup>16</sup> Technical dossier/Volume I/p. 29, 34.

<sup>17</sup> Technical dossier/Volume II/Annex 3.

<sup>18</sup> Technical dossier/Volume II/Annex 5.

<sup>19</sup> Technical dossier/Volume II/Annex 1 and Annex 15.

**Table 1:** Compositional data of the food enzyme

Parameters	Unit	Batches			
		1	2	3	4 <sup>(a)</sup>
<b>Mannanase activity</b>	MNU/g batch <sup>(b)</sup>	1,660,000	1,930,000	1,780,000	1,940,000
<b>Protein</b>	%	67.3	64.4	60.8	65.7
<b>Ash</b>	%	1.9	1.7	2.0	0.7
<b>Water</b>	%	7.0	5.3	4.6	7.4
<b>Total organic solids (TOS)<sup>(c)</sup></b>	%	91.1	93.0	93.4	91.9
<b>Activity/mg TOS</b>	MNU/mg TOS	1,822	2,075	1,906	2,111

(a): Batch used for the toxicological studies.

(b): MNU/g: mannan endo-1,4- $\beta$ -mannosidase unit/g (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,<sup>20</sup> 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 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).<sup>21</sup> No antimicrobial activity was detected in any of the tested batches (FAO/WHO, 2006).<sup>21</sup>

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 aflatoxin B1, B2, G1 and G2, sterigmatocystin, ochratoxin A, fumonisin B2, zearalenone, deoxynivalenol, T2-toxin and HT2-toxin was examined in the four food enzyme batches shown in Table 1.<sup>21</sup> All were below the LoQ of the applied analytical methods,<sup>22</sup> except for T2-toxin detected at levels up to 24.5  $\mu$ g/kg.<sup>23</sup> Taking into account the proposed use levels and the results of the toxicological examination of the food enzyme–TOS, the concentration of T2-toxin in the food enzyme was not considered to be of concern. 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 viable cells of the production strain in the food enzyme was demonstrated in three independent batches each tested in duplicate. For each batch 2  $\times$  10 g of product was diluted in 90 ml buffer solution. From this, 20 ml was inoculated on non-selective agar medium and incubated at 30°C for 4 days. No colonies were produced.<sup>24</sup>

The absence of recombinant DNA in the food enzyme was demonstrated by polymerase chain reaction (PCR) analysis, performed on 1 mL samples taken from three batches in triplicate. No DNA was detected with primers that would amplify a 929-bp fragment specific for [REDACTED], with a limit of detection of at least 10 ng spiked DNA/ml in each of the samples of the food enzyme.<sup>25</sup>

## 3.4. Toxicological data

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. The test item used in these studies (batch 4, Table 1) is considered suitable, since its composition represents that of the three commercial batches.

<sup>20</sup> Technical dossier/Volume I/p. 30/Volume II/Annexes: 1, 2, 15/Additional data July 2020.

<sup>21</sup> Technical dossier/Volume I/p. 30/Volume II/Annexes: 1, 2, 15.

<sup>22</sup> LoQs: aflatoxins (B1, B2, G1, G2) = 0.05  $\mu$ g/kg each; sterigmatocystin = 10  $\mu$ g/kg; ochratoxin A = 2  $\mu$ g/kg; deoxynivalenol = 100  $\mu$ g/kg; T2-toxin and HT-2 toxin = 20  $\mu$ g/kg each; fumonisin B2 = 10  $\mu$ g/kg; zearalenone = 50  $\mu$ g/kg.

<sup>23</sup> Technical dossier/Volume II/Annex 1.

<sup>24</sup> Technical dossier/Volume III/Annex 20.

<sup>25</sup> Technical dossier/ Spontaneous data May 2022.



### 3.4.1. Genotoxicity

#### 3.4.1.1. 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, 1997a) and following Good Laboratory Practice (GLP).<sup>26</sup> Five strains of *Salmonella* Typhimurium (TA 1535, TA 1537, TA 98, TA 100 and TA 102) were used with or without metabolic activation (S9-mix). Two separate experiments were carried out in triplicate. The first experiment was carried out applying the plate incorporation method, using eight concentrations of the food enzyme: 3, 10, 33, 100, 333, 1,000, 2,500 and 5,000  $\mu$ g TOS/plate. The second experiment was performed applying the pre-incubation method, using six concentrations of the food enzyme 33, 100, 333, 1,000, 2,500 and 5,000  $\mu$ g TOS/plate.

No cytotoxicity was observed at any concentration of the test substance. Upon treatment with the food enzyme, there was no biologically relevant increase in the number of revertant colonies above the control values, in any strain tested, with or without S9-mix.

The Panel concluded that the food enzyme mannan endo-1,4- $\beta$ -mannosidase did not induce gene mutations under the test conditions applied in this study.

#### 3.4.1.2. *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.<sup>27</sup> Three separate experiments were performed with Chinese hamster V79 cells. The cell cultures were treated with the food enzyme either with or without metabolic activation (S9-mix) up to 5,000  $\mu$ g TOS/mL.

In the first experiment, cells were exposed to the food enzyme and scored for chromosomal aberrations at 2,721, 4,081 and 5,441  $\mu$ g food enzyme/mL (corresponding to 2,500, 3,750 and 5,000  $\mu$ g TOS/mL) in a short-term treatment (4 h exposure + 18 h recovery period) either with or without S9-mix. No cytotoxicity, evaluated as reduction of cell growth and of mitotic index, was seen at any concentration tested. The frequency of structural chromosomal aberrations was statistically significantly different to the negative controls at concentration of 5,000  $\mu$ g TOS/mL in the short-term treatment without S9-mix.

In the second, confirmatory experiment, cells were exposed to the food enzyme and scored for chromosomal aberrations at a narrower range of concentrations (4,081, 4,761 and 5,441  $\mu$ g food enzyme/mL, corresponding to 3,750, 4,375 and 5,000  $\mu$ g TOS/mL) in a short-term treatment without S9-mix. In this confirmatory experiment, the frequency of structural and numerical aberrations was not statistically significantly different to the negative controls at any concentrations tested.

In the third experiment, cells were exposed to the food enzyme and scored for chromosomal aberrations at concentrations of 4,081, 4,761 and 5,441  $\mu$ g food enzyme/mL (corresponding to 3,750, 4,375 and 5,000  $\mu$ g TOS/mL) in a long-term treatment (18 h continuous exposure) without S9-mix. No cytotoxicity, evaluated as reduction of cell growth and of mitotic index, was seen at any concentration tested. The frequency of structural and numerical aberrations was not statistically significantly different to the negative controls at any concentrations tested.

The Panel concluded that food enzyme mannan endo-1,4- $\beta$ -mannosidase did not induce an increase in the frequency of structural and numerical aberrations under the test conditions applied in 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.<sup>28</sup> Groups of 10 male and 10 female Wistar rats [RccHan<sup>TM</sup>: WIST(SPF)] received the food enzyme in doses of 100, 300 and 1,000 mg TOS/kg body weight (bw) per day by gavage. Controls received the vehicle (twice-distilled water).

One high-dose male and one high-dose female died on day 2 and on day 83, respectively. Both deaths were due to lesions in the respiratory tract, caused by the accidental aspiration of the formulation in the airways. One mid-dose male was sacrificed on day 31 for ethical reasons after a leg injury.

<sup>26</sup> Technical dossier/Volume II/Annex 16.

<sup>27</sup> Technical dossier/Volume II/Annex 17.

<sup>28</sup> Technical dossier/Volume II/Annex 18.

The haematological investigation revealed a statistically significant decrease in the total white blood cell (WBC) count in mid- and high-dose males (–22% and –25%, respectively), a decrease in absolute lymphocyte (LYMPH) count in mid- and high-dose males (–26% and –29%, respectively), a decrease in absolute monocyte (MONO) count in low-, mid- and high-dose males (–24%, –29% and –24%, respectively), a decrease in absolute large unstained cell (LUC) count in high-dose males (–33%), an increase in methaemoglobin (MET-HB) in mid-dose males (+9%), an increase in mean corpuscular haemoglobin (MCH) in mid- and high-dose females (+4% at both dose levels), an increase in mean corpuscular haemoglobin concentration (MCHC) in high-dose females (+2%), an increase in the absolute neutrophil (NEUT) count in high-dose females (+58%), a decrease in MET-HB in mid- and high-dose females (–17% at both dose levels).

The Panel noted that the data on total WBC count, absolute LYMPH and LUC counts suggested a treatment-related effect although the dose response was less clear. In addition, even if it is known that males and females do often not react identically with regard to the immune system; in this study, there is a remarkable difference between males and females, with females showing no equivalent effects. An average decrease of total WBC by 22–25% in the mid and high dose, and a decrease in the absolute LYMPH counts by 26 and 29% in the mid and high dose, respectively, present a flag for potential immunosuppression. Without further functional testing of the immune system, such an adverse effect cannot be excluded. The Panel considered other changes as not toxicologically relevant as they were only observed in one sex (MONO, LUC, MCH, MCHC, NEUT), there was no dose–response relationship (MONO, MET-HB, MCH), the changes were small (MONO, MCH, MCHC), there were no changes in other relevant parameters (NEUT in females in the absence of changes in WBC, MCH and MCHC in females in the absence of changes in haemoglobin concentration and red blood cell count) and the values were within the historical control values.

The clinical chemistry investigation revealed a statistically significant increase in glucose (+14%) and in albumin (+5%) in high-dose males, an increase in sodium (+1%) and phosphorus (+25%) in high-dose females and a decrease in creatinine kinase (CK) in mid-dose females (–41%). The Panel considered the changes as not toxicologically relevant as they were only observed in one sex (all parameters), the changes were small (albumin and sodium), there was no dose–response relationship (CK), there were no changes in other relevant parameters (albumin in the absence of changes in total protein) and the values were within the historical control values.

Statistically significant changes in organ weights included an increase in the relative weight of testes (+15%) and of the epididymides (+11%) in mid-dose males. The Panel considered the changes as not toxicologically relevant as there was no dose–response relationship (both parameters) and there were no histopathological changes in the organs.

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

The Panel identified the no observed adverse effect level (NOAEL) of 100 mg TOS/kg bw per day, the lowest dose tested based on the decrease in total leucocyte and absolute lymphocyte counts in males.

### 3.4.3. Allergenicity

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

The potential allergenicity of the mannan endo-1,4- $\beta$ -mannosidase produced with the genetically modified *T. reesei* strain RF6232 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, one match was found.<sup>29</sup> The matching allergen was ██████████, an endochitinase from avocado (*Persea americana*).

No information is available on oral and respiratory sensitisation or elicitation reactions of this mannan endo-1,4- $\beta$ -mannosidase.

A case of anaphylaxis due to  $\alpha$ -mannosidase from *Auricularia* (a basidiomycete) has been reported (Kobayashi et al., 2019), but no allergic reactions upon dietary exposure to any mannan endo-1,4- $\beta$ -mannosidase have been reported in the literature.

<sup>29</sup> Technical dossier/Volume II/Annex 19/Additional data July 2020.

██████████, a product that may cause allergies or intolerances (listed in the Regulation (EU) No 1169/2011<sup>30</sup>) 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 potentially allergenic residues of these foods employed as protein sources are not expected to be present.

The Panel considered that, under the intended conditions of use, the risk of allergic sensitisation and elicitation reactions upon dietary exposure to this food enzyme cannot be excluded, in particular for individuals allergic to avocado, but the risk will not exceed that of avocado.

### 3.5. Dietary exposure

#### 3.5.1. Intended use of the food enzyme

The food enzyme is intended to be used in three 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<sup>31</sup>

Food manufacturing process <sup>(a)</sup>	Raw material (RM)	Maximal recommended dosage of the food enzyme (mg TOS/kg RM) <sup>(b)</sup>
Coffee processing	Coffee extract	<b>11</b>
Fruit and vegetable processing to juice production	Fruit	<b>2</b>
Edible oil production	Oil seed/fruit pulp	26

(a): The description provided by the applicant 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): The numbers in bold were used in calculation.

In coffee processing, mannan endo-1,4- $\beta$ -mannosidase together with pectinases and glucanases can be added to the liquid coffee extract concentrate to hydrolyse haemicellulose, of which mannans form a part, reducing the viscosity of the coffee extract.<sup>32</sup> The pH profile of this mannan endo-1,4- $\beta$ -mannosidase produced by *T. reesei* strain RF6232 makes it suitable also for hydrolysing the galactomannans in liquid coffee extract, inhibiting gel formation during freeze-drying of instant coffee. The food enzyme TOS will remain in the instant coffee.

In fruit and vegetable processing, the food enzyme is added to the depectinised fruit juice to aid clarification.<sup>33</sup> Due to the high concentration of mannans, mannan endo-1,4- $\beta$ -mannosidase is useful in the treatment of pineapple fruit for juice production.<sup>34</sup> The food-enzyme TOS will remain in the fruit juice. Although the food enzyme could also be used in the processing of other types of fruit products, the applicant specified that this mannan endo-1,4- $\beta$ -mannosidase is currently used only in fruit juice production.<sup>35</sup>

In edible oil production, the food enzyme is added to oilseeds or fruit pulp during wet processing.<sup>36</sup> The action of mannan endo-1,4- $\beta$ -mannosidase aids the release of the oil contained in the cell wall. The obtained crude oil will be further refined into edible oil for human consumption. The food enzyme-TOS is removed in the final edible oils by repeated washing during degumming (EFSA CEP Panel, 2021b).

<sup>30</sup> 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.

<sup>31</sup> Technical dossier/p. 61 and Additional information July 2020.

<sup>32</sup> Technical dossier/p. 55 and Additional information July 2020.

<sup>33</sup> Technical dossier/p. 56.

<sup>34</sup> Technical dossier/p. 57.

<sup>35</sup> Additional information July 2020.

<sup>36</sup> Technical dossier/p. 59.

### 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, namely, coffee processing and fruit and vegetable processing for juice 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 one 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 0.086 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
<b>Age range</b>	3–11 months	12–35 months	3–9 years	10–17 years	18–64 years	≥ 65 years
<b>Min–max mean</b> (number of surveys)	0.001–0.013 (11)	0.003–0.048 (15)	0–0.027 (19)	0–0.015 (21)	0.002–0.013 (22)	0.001–0.011 (22)
<b>Min–max 95th</b> (number of surveys)	0–0.051 (9)	0.022–0.081 (13)	0.001–0.086 (19)	0.001–0.051 (20)	0.008–0.041 (22)	0.003–0.028 (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
<b>Model input data</b>	
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	+/-
<b>Model assumptions and factors</b>	
Exposure to food enzyme-TOS was always calculated based on the recommended maximum use level	+
Selection of broad FoodEx categories for the exposure assessment	+
Use of recipe fractions in disaggregation FoodEx categories	+/-

Sources of uncertainties	Direction of impact
Use of technical factors in the exposure model	+/-
Exclusion of one process from the exposure assessment: edible 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 one food manufacturing process (fruit and vegetable processing for edible oil production) 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 (100 mg TOS/kg bw per day) from the 90-day rat study with the derived exposure estimates of 0–0.048 mg TOS/kg bw per day at the mean and from 0–0.086 mg TOS/kg bw per day at the 95th percentile, resulted in margin of exposure (MoE) of at least 1,163.

## 4. Conclusions

Based on the data provided, the removal of TOS during edible oil production and the derived margin of exposure for coffee processing and fruit and vegetable processing for juice production, the Panel concluded that the food enzyme mannan endo-1,4- $\beta$ -mannosidase produced with the genetically modified *Trichoderma reesei* strain RF6232 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

Application for authorisation of a Mannan endo-1,4-beta-mannosidase ( $\beta$ -mannanase) from a genetically modified strain of *Trichoderma reesei* in accordance with Regulation (EC) No 1331/2008. January 2015. Submitted by AB Enzymes GmbH.

Additional information. July 2020, February 2021 and May 2022. Submitted by AB Enzymes GmbH.

Additional information on 'The transfer of enzymes into food for fat and oil processing'. October 2017 and February 2018. Provided by the Association of Manufacturers and Formulators of Enzyme Products.

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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
EC	European Commission
EINECS	European Inventory of Existing Commercial Chemical Substances
FAO	Food and Agricultural Organisation of the United Nations
GLP	Good Laboratory Practice
GMO	genetically modified organism
ICP-MS	inductively coupled plasma-mass spectrometry
IUBMB	International Union of Biochemistry and Molecular Biology
JECFA	Joint FAO/WHO Expert Committee on Food Additives
kDa	kiloDalton
LoD	limit of detection
LoQ	limit of quantification
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

Information provided in this appendix is shown in an Excel file (downloadable <https://efsa.onlinelibrary.wiley.com/doi/10.2903/j.efsa.2022.7478#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
<b>Infants</b>	From 12 weeks on up to and including 11 months of age	Bulgaria, Cyprus, Denmark, Estonia, Finland, France, Germany, Italy, Latvia, Portugal, Slovenia
<b>Toddlers</b>	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
<b>Children</b>	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
<b>Adolescents</b>	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
<b>Adults</b>	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
<b>The elderly<sup>(a)</sup></b>	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).