

ADOPTED: 5 May 2021

doi: 10.2903/j.efsa.2021.6635

## Safety evaluation of the food enzyme $\beta$ -amylase from *Bacillus flexus* strain AE-BAF

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### Abstract

The food enzyme  $\beta$ -amylase (4- $\alpha$ -D-glucan maltohydrolase, EC 3.2.1.2) is produced with the non-genetically modified *Bacillus flexus* strain AE-BAF by Amano Enzyme Inc. The production strain has been shown to qualify for Qualified Presumption of Safety (QPS) status. The food enzyme is intended to be used in baking and brewing processes, and in starch processing for the production of glucose syrups and other starch hydrolysates. Since residual amounts of total organic solids (TOS) are removed by the purification steps applied during the production of glucose syrups, dietary exposure was not calculated for this food process. Based on the maximum use levels recommended by the applicant for the baking and brewing processes and individual data from the EFSA Comprehensive European Food Database, dietary exposure was estimated to be up to 2 mg TOS/kg body weight (bw) per day in European populations. Toxicological studies were not considered necessary given the QPS status of the production strain and the nature of the manufacturing process. Similarity of the amino acid sequence to those of known allergens was searched and no 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, but the likelihood for this to occur is considered to be low. Based on the data provided, in particular, the QPS status of the production strain and that no issues of concern arose from the production process, the Panel concluded that the food enzyme  $\beta$ -amylase produced with *B. flexus* strain AE-BAF does not give rise to safety concerns under the intended conditions of use.

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**Keywords:** Food enzyme,  $\beta$ -amylase, 4- $\alpha$ -D-glucan maltohydrolase, EC 3.2.1.2, saccharogen amylase, *Bacillus flexus*

**Requestor:** European Commission

**Question number:** EFSA-Q-2015-00691

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**Declarations of interest:** The declarations of interest of all scientific experts active in EFSA's work are available at <https://ess.efsa.europa.eu/doi/doiweb/doisearch>.

**Suggested citation:** EFSA CEP Panel (EFSA Panel on Food Contact Materials, Enzymes and Processing Aids), Silano V, Barat Baviera JM, Bolognesi C, Cocconcelli PS, Crebelli R, Gott DM, Grob K, Lambré C, Lampi E, Mengelers M, Mortensen A, Rivière G, Steffensen IL, Tlustos C, Van Loveren H, Vernis L, Zorn H, Herman L, Aguilera J, Andryszkiewicz M, Arcella D, Kovalkovicova N, Liu Y and Chesson A, 2021. Scientific Opinion on the safety evaluation of the food enzyme  $\beta$ -amylase from *Bacillus flexus* strain AE-BAF. EFSA Journal 2021;19(6):6635, 15 pp. <https://doi.org/10.2903/j.efsa.2021.6635>

**ISSN:** 1831-4732

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



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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 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 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 Union list may be placed on the market as such and used in foods, in accordance with the specifications and conditions of use provided for in Article 7 (2) of Regulation (EC) No 1332/2008 on food enzymes.

Five applications have been introduced by the company "Amano Enzyme Inc." for the authorisation of the food enzymes Beta-amylase from *Bacillus flexus* (strain AE-BAF), Triacylglycerol lipase from *Mucor javanicus* (strain AE-LM), Beta-glucanase from *Cellulosimicrobium cellulans* (strain AE-TN), Laccase from *Trametes hirsuta* (strain AE-OR) and Protein-glutaminase from *Chrysobacterium proteolyticum* (strain AE-PG).

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 requests the European Food Safety Authority to carry out the safety assessments on the food enzymes Beta-amylase from *Bacillus flexus* (strain AE-BAF), Triacylglycerol lipase from *Mucor javanicus* (strain AE-LM), Beta-glucanase from *Cellulosimicrobium cellulans* (strain AE-TN), Laccase from *Trametes hirsuta* (strain AE-OR) and Protein-glutaminase from *Chrysobacterium proteolyticum* (strain AE-PG) 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  $\beta$ -amylase from *B. flexus* strain AE-BAF.

## 2. Data and methodologies

### 2.1. Data

The applicant has submitted a dossier in support of the application for authorisation of the food enzyme  $\beta$ -amylase from *B. flexus* strain AE-BAF. The dossier was updated on 26 October 2014.

Additional information was requested from the applicant during the assessment process on 2 October 2020 and was consequently provided (see 'Documentation provided to EFSA').

### 2.2. Methodologies

The assessment was conducted in line with the principles described in the EFSA 'Guidance on transparency in the scientific aspects of risk assessment' (EFSA, 2009b) and following the relevant existing guidances of EFSA Scientific Committees.

The current 'Guidance on the submission of a dossier on food enzymes for safety evaluation' (EFSA, 2009a) has been followed for the evaluation of the application with the exception of the exposure assessment, which was carried out in accordance to the methodology described in the CEF Panel 'Statement on the exposure assessment of food enzymes' (EFSA CEF Panel, 2016).

## 3. Assessment

<b>IUBMB nomenclature</b>	$\beta$ -amylase
<b>Systematic name</b>	4- $\alpha$ -D-glucan maltohydrolase
<b>Synonyms</b>	Saccharogen amylase; glycogenase
<b>IUBMB No</b>	3.2.1.2
<b>CAS No</b>	9000-91-3
<b>EINECS No</b>	232-566-1

$\beta$ -Amylase catalyses the hydrolysis of 1,4- $\alpha$ -D-glucosidic linkages in amylose and amylopectin, and releases successively maltose units from the non-reducing ends of the glucan chains. The enzyme is intended to be used in baking and brewing processes, and in starch processing for the production of glucose syrups and other starch hydrolysates.

### 3.1. Source of the food enzyme

The  $\beta$ -amylase is produced with the bacterium *B. flexus* strain AE-BAF, which is deposited at the Biological Resource Center (NBRC, Japan), with deposit number NITE SD 00399.<sup>4</sup>

The production strain was identified as *B. flexus* by whole genome sequence (WGS) analysis with an average nucleotide identity (ANI) of 99.113% to the type strain.<sup>5</sup>

*B. flexus* is included in the list of organisms for which the Qualified Presumption of Safety (QPS) may be applied, provided that the absence of toxigenic activity and acquired antimicrobial resistance genes is verified for the specific strain used.<sup>6</sup> The production strain was shown not to be cytotoxic in

<sup>4</sup> Additional data March 2021/Appendix 1.

<sup>5</sup> Technical dossier/pg. 32/Additional data March 2021/Appendix 2.

<sup>6</sup> <https://zenodo.org/record/3336268#.X8pXR2hKiUn>

Vero cells<sup>7</sup> and susceptible to the battery of antibiotics recommended by the EFSA guidance (EFSA CEP Panel, 2019). Minimum Inhibitory Concentration values were always below the cut-off values established by EFSA.<sup>8</sup> The WGS was checked for the presence of antimicrobial resistance genes based on 70% similarity and 60% coverage, using the NCBI Bacterial Antimicrobial Resistance Reference Gene and the ResFinder databases. Seven genes were found, but no hits of concern were identified. Therefore, the production strain was considered to qualify for the QPS status.

### 3.2. Production of the food enzyme

The food enzyme is manufactured according to the Food Hygiene Regulation (EC) No 852/2004<sup>9</sup>, with food safety procedures based on Hazard Analysis and Critical Control Points, and in accordance with current Good Manufacturing Practice.<sup>10</sup>

The production strain is grown as a pure culture using a typical industrial medium in a submerged batch fermentation system with conventional process controls in place. After completion of the fermentation, the solid biomass is removed from the fermentation broth by filtration, leaving a supernatant containing the food enzyme. The filtrate containing the enzyme is then further purified and concentrated, including an ultrafiltration step in which enzyme protein is retained, while most of the low molecular weight material passes the filtration membrane and is discarded.<sup>11</sup> 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>12</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  $\beta$ -amylase is a single polypeptide chain of 545 amino acids.<sup>13</sup> The molecular mass of the mature protein, derived from the amino acid sequence, was calculated to be 57.6 kDa.<sup>14</sup> The food enzyme was analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). A consistent protein pattern was observed across all batches with a single major protein band corresponding to an apparent molecular mass of about 60 kDa, consistent with the expected mass of the enzyme.<sup>15</sup> The food enzyme was also examined by size exclusion chromatography. The chromatograms of three food enzyme batches showed a consistent pattern with a major peak accompanied by some minor peaks.<sup>16</sup> No other activities were reported.<sup>17</sup>

The in-house determination of  $\beta$ -amylase activity is based on titration of reducing groups released during the hydrolysis of starch (reaction conditions: pH 5.0, 37°C, 10 min). The enzyme activity is expressed in Unit/g or mL. One starch saccharifying activity unit is the amount of enzyme that catalysis the increase of reducing activity equivalent to 1 mg glucose per minute.<sup>18</sup>

The food enzyme has a temperature optimum around 55°C (pH 5.0) and a pH optimum around pH 8.0 (37°C). Thermostability was tested after a pre-incubation of the food enzyme for 10 min at different temperatures (pH 5.0). The enzyme activity decreased above 55°C, showing no residual activity above 65°C.<sup>19</sup>

<sup>7</sup> Additional data March 2021/Appendix 3.

<sup>8</sup> Additional data March 2021/Appendix 4.

<sup>9</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>10</sup> Technical dossier/Annex 4.

<sup>11</sup> Technical dossier/pg. 34-41/Annex 5.

<sup>12</sup> Technical dossier/Annex 6/Additional data March 2021.

<sup>13</sup> Technical dossier/pg. 27.

<sup>14</sup> Additional data March 2021.

<sup>15</sup> Technical dossier/pg. 27/Additional data March 2021/Appendix 5.

<sup>16</sup> Technical dossier/pg. 26.

<sup>17</sup> Technical dossier/pg. 28-29.

<sup>18</sup> Technical dossier/Annex 2.

<sup>19</sup> Technical dossier/pg. 29–30.

### 3.3.2. Chemical parameters

Data on the chemical parameters of the food enzyme preparation were provided for three batches used for commercialisation (1–3) and two batches produced for the toxicological tests (4 and 5) (Table 1).<sup>20</sup> The mean total organic solids (TOS) of the three food enzyme batches for commercialisation was 8.2% and the mean enzyme activity/TOS ratio was 18.6 U/mg TOS.

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

Parameters	Unit	Batches				
		1	2	3	4 <sup>(a)</sup>	5 <sup>(b)</sup>
<b><math>\beta</math>-Amylase activity</b>	U/g batch <sup>(c)</sup>	1,710	1,430	1,450	3,260	2,463
<b>Protein</b>	%	4.7	1.9	3.1	3.2	–
<b>Ash</b>	%	1.3	0.6	1.2	1.0	0.8
<b>Water</b>	%	5.3	3.4	4.7	4.6	90.5
<b>Dextrin (diluent)</b>	%	84.3	87.7	86.8	57.3	–
<b>Total organic solids (TOS)<sup>(d)</sup></b>	%	9.1	8.3	7.3	37.1	8.7
<b>Activity/mg TOS</b>	U/mg TOS	18.8	17.2	19.9	8.8	28.3

(a): Batch used for the genotoxicity studies.

(b): Batch used for the repeated dose 90-day oral toxicity study in rats.

(c): U: UNIT/g (see Section 3.3.1).

(d): TOS calculated as 100% – % water – % ash – % diluent.

### 3.3.3. Purity

The lead content in the three commercial batches and in the two batches used for toxicological studies was below 5 mg/kg,<sup>21, 22</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 preparation complies with the microbiological criteria (for total coliforms, *Escherichia coli* and *Salmonella*)<sup>23</sup> as laid down in the general specifications for enzymes used in food processing (FAO/WHO, 2006). No antimicrobial activity was detected in any of these batches (FAO/WHO, 2006).<sup>23</sup>

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

## 3.4. Toxicological data

As the production strain qualifies for the QPS status and no issues of concern arose from the production process, toxicological tests are no longer required. However, the applicant following the guidance available at the time of the submission of the application provided a bacterial gene mutation assay (Ames test) and an *in vitro* mammalian chromosomal aberration test performed with the food enzyme under assessment (batch 4, Table 1). The applicant also provided a repeated dose 90-day oral toxicity study performed with a different batch (batch 5, Table 1). These were assessed and reported as supporting evidence.

### 3.4.1. Genotoxicity

#### 3.4.1.1. Bacterial reverse mutation test

A bacterial reverse mutation assay (Ames test) was performed according to the Japanese guidelines, which are equivalent to the OECD Test Guideline 471 (Japanese Ministry of Health and Welfare (JMHW): Notification 1604, 1999) and following Japanese Good Laboratory Practice (GLP).<sup>24</sup> Four strains of *Salmonella* Typhimurium (TA98, TA100, TA1535 and TA1537) and *E. coli* WP2 *uvrA* were used in the presence or absence of metabolic activation (S9-mix), applying the preincubation method. Two separate experiments (the dose-finding and the main test) were performed in duplicate.

<sup>20</sup> Technical dossier/pg. 26, 52/Annexes: 3, 8, 9 and 10/Additional data March 2021.

<sup>21</sup> Technical dossier/pg. 8, 27, 52/Annex 3.

<sup>22</sup> LoD: Pb = 0.01 mg/kg.

<sup>23</sup> Technical dossier: pg. 8, 27, 52/Annex 3.

<sup>24</sup> Technical dossier/pg. 49-52/Annex 8.

The dose-finding test was carried out using seven concentrations of the food enzyme (from 1.22 to 5,000  $\mu\text{g}/\text{plate}$ , corresponding to 0.45 to 1,855  $\mu\text{g TOS}/\text{mL}$ ) in the presence and absence of S9-mix. Based on the results of the range finding test, the main test was carried out at six concentrations, setting the maximum level at 1,250  $\mu\text{g}/\text{plate}$  for the *S. Typhimurium* strains without S9-mix. In the presence of S9-mix strains TA1535 and TA1537 were similarly treated with six concentrations up to 1,250  $\mu\text{g}/\text{plate}$ , (corresponding to 464  $\mu\text{g TOS}/\text{mL}$ ), and up to 5,000  $\mu\text{g}/\text{plate}$  for strains TA98 and TA100 (corresponding to 1,855  $\mu\text{g TOS}/\text{mL}$ ). *E. coli* WP2 *uvrA* was treated at 156, 313, 625, 1,250, 2,500 and 5,000  $\mu\text{g}/\text{plate}$  (corresponding to 58, 116, 232, 464, 928 and 1,855  $\mu\text{g TOS}/\text{mL}$ ) in the presence and absence of S9-mix. Growth inhibition was observed at 625  $\mu\text{g}/\text{plate}$  and above for *S. Typhimurium* TA1537 with and without S9-mix, at 1,250  $\mu\text{g}/\text{plate}$  and above for *S. Typhimurium* TA100, TA1535 and TA98 without S9-mix and for *S. Typhimurium* TA1535 with S9-mix, at 2,500  $\mu\text{g}/\text{plate}$  and above for *S. Typhimurium* TA100 and TA98 with S9-mix, and at 5,000  $\mu\text{g}/\text{plate}$  for *E. coli* WP2 *uvrA* without metabolic activation. Upon treatment with the food enzyme, there was no significant increase in revertant colony numbers above the control values in any strain with or without S9-mix.

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

#### 3.4.1.2. *In vitro* mammalian chromosomal aberration test

The *in vitro* mammalian chromosomal aberration test was carried out in Chinese Hamster Lung fibroblast (CHL/IU) cells according to the Japanese guidelines, which are equivalent to the OECD Test Guideline 473 (JMHW: Notification 1604, 1999 and Notification 29, 1996) and following Japanese GLP.<sup>25</sup> The cell-growth inhibition test was performed at concentrations ranging from 39 to 5,000  $\mu\text{g}/\text{mL}$ , and inhibition of cell growth by 50% or more was observed at 2,500  $\mu\text{g}/\text{mL}$  and above. Based on these results, in the short-term treatment (6 h followed by 18 h recovery period) the cell cultures were exposed in duplicate to the food enzyme at 1,020, 1,280 and 1,600  $\mu\text{g}/\text{mL}$  (corresponding to 378, 475 and 594  $\mu\text{g TOS}/\text{mL}$ ) with metabolic activation (S9-mix) and at 1,020, 1,280, 1,600 and 2,000  $\mu\text{g}/\text{mL}$  (corresponding to 378, 475, 594 and 742  $\mu\text{g TOS}/\text{mL}$ ) without S9-mix. In the continuous treatment (24 and 48 h) the cells were exposed to the food enzyme at 1,020, 1,280, 1,600, 2,000 and 2,500  $\mu\text{g}/\text{mL}$  (corresponding to 378, 475, 594, 742 and 928  $\mu\text{g TOS}/\text{mL}$ ) in the absence of S9-mix. The frequency of structural and numerical chromosomal aberrations in treated cultures was comparable to the values detected in negative controls in all experiments.

The Panel concluded that food enzyme did not induce chromosomal 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 the Japanese guidelines, which are equivalent to the OECD Test Guideline 408 (JMHW: Notification 24, 1989; Notification 655, 1999 and Notification 29, 1996) and following GLP.<sup>26</sup> Groups of 10 male and 10 female Sprague–Dawley SPF (CrI:CD(SD)) rats received by gavage 2,500, 5,000 and 10,000 mg food enzyme/kg bw per day, corresponding to 218, 435 and 870 mg TOS/kg bw per day. Controls received the vehicle (water for injection).

No mortality was observed.

Feed intake was lower throughout the study in low-dose males, the difference to controls being statistically significant on days 18, 28–60, 67 and 91. As the differences were recorded in absence of dose-response relationship and likely reflect normal biological variation, the Panel considered this finding not toxicologically relevant.

Clinical chemistry investigation revealed statistically significantly lower concentrations of triglycerides in low-dose males, phospholipids in low-dose and mid-dose males and total protein in mid-dose females.

In urinalysis, statistically significant decrease in urine volume and excretion of potassium in low-dose males was observed.

All the changes in clinical chemistry parameters and urinalysis were considered by the Panel as not toxicologically relevant, because the differences were without an apparent dose-dependency.

<sup>25</sup> Technical dossier/pg. 49-52/Annex 9.

<sup>26</sup> Technical report/pg. 49-52/Annexes 10, 10\_Appendix 1, 10\_Appendix 2.

There was a statistically significant decrease in absolute and relative weights of the pituitary and relative weight of the liver in mid-dose females. As these changes were not dose-related, lacked histopathological correlation and were restricted to one sex, they were considered incidental and, therefore, not toxicologically relevant.

No other statistically significant differences to controls were observed.

The Panel identified the no observed adverse effect level (NOAEL) at 870 mg TOS/kg bw per day, the highest dose tested.

### 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  $\beta$ -amylase produced with the non-genetically modified *B. flexus* strain AE-BAF 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, no match was found.<sup>27</sup>

No information is available on oral and respiratory sensitisation or elicitation reactions of this  $\beta$ -amylase.

$\beta$ -Amylase (from barley) is described as an occupational respiratory allergen: sensitisation to barley can induce asthma in bakers and millers (Sandiford et al., 1994; Tatham and Shewry, 2008). The wheat  $\beta$ -amylase has been found to bind IgE of wheat allergic patients (Hofer et al., 2018).

Several studies have shown that adults with occupational asthma to a food enzyme (as described for  $\alpha$ -amylase from *A. oryzae*) may be able to ingest the corresponding allergen without acquiring clinical symptoms of food allergy (Cullinan et al., 1997; Poulsen, 2004; Armentia et al., 2009). Taking into account the wide use of  $\alpha$ -amylase as food enzyme, only a low number of case reports of allergic reactions upon oral exposure to  $\alpha$ -amylase in individuals respiratory sensitised to  $\alpha$ -amylase have been described in the literature (Losada et al., 1992; Quirce et al., 1992; Baur and Czuppon, 1995; Kanny and Moneret-Vautrin, 1995; Moreno-Ancillo et al., 2004).

According to the information provided, substances or products that may cause allergies or intolerances (Regulation (EU) No 1169/2011<sup>28</sup>) are used as raw materials (gluten meal, soybean meal, soybean oil). In addition, yeast extract and corn steep liquor, known allergens, are also present in the media fed to the microorganisms. However, during the fermentation process, these products will be degraded and utilised by the microorganisms for cell growth, cell maintenance and production of enzyme protein. In addition, the microbial 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 could not be excluded for starch processing for the production of glucose syrups and other starch hydrolysates, baking and brewing processes, but the likelihood of such reactions to occur was considered to be low.

## 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.<sup>29</sup>

<sup>27</sup> Technical dossier/pg. 53-55/Annex 11.

<sup>28</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>29</sup> Technical dossier/pg. 42-47/Additional data March 2021/Intended use 12042021.

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

Food manufacturing process <sup>(a)</sup>	Raw material	Recommended dosage of the food enzyme
Baking processes	Flour	45–90 mg TOS/kg flour
Brewing processes	Malt	37–381 mg TOS/kg malt
Starch processing for the production of glucose syrups and other starch hydrolysates	Starch	5–45 mg TOS/kg starch

TOS: total organic solids.

(a): The description provided by the applicant 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.

In baking processes,  $\beta$ -amylase is added to flour during the preparation of dough. It is used to shorten the 1,4-linked chains of the amylopectin molecules during dough handling.

In brewing processes,  $\beta$ -amylase is added during the mashing step. It is used to convert liquefied starch into a maltose-rich solution, improving the amounts of fermentable sugars and thus increasing the brewing yield.

The food enzyme–TOS remains in the dough and beer. Based on data provided on thermostability (see Section 3.3.1), it is expected that the  $\beta$ -amylase is inactivated during baking and brewing processes.

In starch processing for the production of glucose syrups and other starch hydrolysates,  $\beta$ -amylase is added during the saccharification to convert liquefied starch into a maltose-rich solution. The hydrolysis of starch results in faster and improved processing, improved yields of high maltose syrup and hydrolysis of maltotriose to maltose and glucose.

### 3.5.2. Dietary exposure estimation

The technical information and experimental data provided on the removal of food enzyme–TOS during starch processing for the production of glucose syrups and other starch hydrolysates was considered by the Panel as sufficient to exclude these processes from the exposure assessment (Annex B in EFSA CEF Panel, 2016).

The food enzyme–TOS remains in the dough and beer. Therefore, a dietary exposure was calculated only for the baking and brewing processes.

Chronic dietary exposure was calculated by combining the maximum recommended use level provided by the applicant (see Section 3.5.1, Table 2) with the relevant FoodEx categories (Annex B in EFSA CEF Panel, 2016), based on individual consumption data. 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 40 different dietary surveys (covering infants, toddlers, children, adolescents, adults and the elderly), carried out in 23 European countries (Appendix B).

**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 (number of surveys)</b>	0.017–0.279 (12)	0.192–0.610 (16)	0.224–0.576 (19)	0.136–0.345 (20)	0.133–0.584 (22)	0.124–0.343 (21)
<b>Min–max 95th percentile (number of surveys)</b>	0.098–1.071 (10)	0.477–1.131 (14)	0.424–1.119 (19)	0.264–0.719 (19)	0.365–2.018 (22)	0.332–0.988 (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
<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>	
FoodEx categories included in the exposure assessment were assumed to always contain the food enzyme-TOS	+
Exposure to food enzyme-TOS was always calculated based on the recommended maximum use level	+
Selection of broad FoodEx categories for the exposure assessment	+
Use of recipe fractions in disaggregation FoodEx categories	+/-
Use of technical factors in the exposure model	+/-
Exclusion of some processes from the exposure assessment – Starch processing for the production of glucose syrups and other starch hydrolysates	–

+: 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 a considerable overestimation of the exposure.

The exclusion of one food manufacturing process (starch processing for the production of glucose syrups and other starch hydrolysates) 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

Since toxicological tests are no longer considered necessary by the Panel, the margin of exposure was not calculated.

## 4. Conclusion

Based on the data provided, in particular, the QPS status of the production strain and that no issues of concern arose from the production process, the Panel concluded that the food enzyme  $\beta$ -amylase produced with *B. flexus* strain AE-BAF does not give rise to safety concerns under the intended conditions of use.

## 5. Documentation as provided to EFSA

- 1) Application for authorisation of Beta-amylase from *Bacillus flexus* AE-BAF. October 2014. Submitted by Amano Enzyme Inc.
- 2) Additional information. March 2021. Submitted by Amano Enzyme Inc.

## References

- Armentia A, Diaz-Perales A, Castrodeza J, Duenas-Laita A, Palacin A and Fernandez S, 2009. Why can patients with baker's asthma tolerate wheat flour ingestion? Is wheat pollen allergy relevant? *Allergologia Immunopathologia*, 37, 203–204. <https://doi.org/10.1016/j.aller.2009.05.001>
- Baur X and Czuppon AB, 1995. Allergic reaction after eating alpha-amylase (Asp o 2)-containing bread. A case report. *Allergy*, 50, 85–87.
- Cullinan P, Cook A, Jones M, Cannon J, Fitzgerald B and Newman Taylor AJ, 1997. Clinical responses to ingested fungal  $\alpha$ -amylase and hemicellulase in persons sensitized to *Aspergillus fumigatus*? *Allergy*, 52, 346–349.
- EFSA (European Food Safety Authority), 2006. Opinion of the Scientific Committee related to uncertainties in dietary exposure assessment. *EFSA Journal* 2006;5(1):438, 54 pp. <https://doi.org/10.2903/j.efsa.2007.438>
- EFSA (European Food Safety Authority), 2009a. Guidance of EFSA prepared by the Scientific Panel of Food Contact Material, Enzymes, Flavourings and Processing Aids on the Submission of a Dossier on Food Enzymes. *EFSA Journal* 2009;7(5):1305, 26 pp. <https://doi.org/10.2903/j.efsa.2009.1305>
- EFSA (European Food Safety Authority), 2009b. Guidance of the Scientific Committee on transparency in the scientific aspects of risk assessments carried out by EFSA. Part 2: general principles. *EFSA Journal* 2009;7(5):1051, 22 pp. <https://doi.org/10.2903/j.efsa.2009.1051>
- EFSA (European Food Safety Authority), 2011. Use of the EFSA Comprehensive European Food Consumption Database in Exposure Assessment. *EFSA Journal* 2011;9(3):2097, 34 pp. <https://doi.org/10.2903/j.efsa.2011.2097>
- EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids), 2016. Panel statement on the exposure assessment of food enzymes. *EFSA Journal* 2016;14(11):4581, 9 pp. <https://doi.org/10.2903/j.efsa.2016.4581>. Available online: [https://efsa.onlinelibrary.wiley.com/action/downloadSupplement?doi=10.2903%2Fj.efsa.2016.4581&file=efs24581-sup-0001-Annex\\_B.pdf](https://efsa.onlinelibrary.wiley.com/action/downloadSupplement?doi=10.2903%2Fj.efsa.2016.4581&file=efs24581-sup-0001-Annex_B.pdf)
- EFSA CEP Panel (EFSA Panel on Food Contact Materials, Enzymes and Processing Aids), 2019. Statement on the characterisation of microorganisms used for the production of food enzymes. *EFSA Journal* 2019;17(6):5741, 13 pp. <https://doi.org/10.2903/j.efsa.2019.5741>
- EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms), 2010. Scientific Opinion on the assessment of allergenicity of GM plants and microorganisms and derived food and feed. *EFSA Journal* 2010;8(7):1700, 168 pp. <https://doi.org/10.2903/j.efsa.2010.1700>
- FAO/WHO (Food and Agriculture Organization of the United States/World Health Organization), 2006. General specifications and considerations for enzyme preparations used in food processing in Compendium of food additive specifications. 67th meeting. FAO JECFA Monographs 3, pp. 63–67. Available online: <ftp://ftp.fao.org/docrep/fao/009/a0675e/a0675e00.pdf>
- Hofer G, Wieser S, Bogdos MK, Gattinger P, Nakamura R, Ebisawa M, Mäkelä M, Papadopoulos N, Valenta R and Keller W, 2018. Three-dimensional structure of the wheat  $\beta$ -amylase Tri a 17, a clinically relevant food allergen. <https://doi.org/10.1111/all.13696>
- Kanny G and Moneret-Vautrin D-A, 1995. Alpha-amylase contained in bread can induce food allergy. *J Allergy Clin Immunol*, 95, 132–133.
- Losada E, Hinojosa M, Quirce S, Sanchez-Cano M and Moneo I, 1992. Occupational asthma caused by alpha-amylase inhalation: clinical and immunologic findings and bronchial response patterns. *Journal of Allergy and Clinical Immunology*, 89(1 Pt 1), 118–125.
- Moreno-Ancillo A, Domínguez-Noche C, Gil-Adrados AC and Cosmes PM, 2004. Bread eating induced oral angioedema due to alpha-amylase allergy. *Journal of Investigative Allergology and Clinical Immunology*, 14, 346–347.
- Poulsen LK, 2004. Allergy assessment of foods or ingredients derived from biotechnology, gene-modified organisms, or novel foods. *Molecular Nutrition and Food Research*, 48, 413–423. <https://doi.org/10.1002/mnfr.200400029>

- Quirce S, Cuevas M, Diez-Gomez M, Fernandez-Rivas M, Hinojosa M, Gonzalez R and Losada E, 1992. Respiratory allergy to Aspergillus-derived enzymes in bakers' asthma. *Journal of Allergy and Clinical Immunology*, 90(6 Pt 1), 970–978.
- Sandiford CP, Tee RT and Newman Taylor AJ, 1994. The role of cereal and fungal amylases in cereal flour hypersensitivity. *Clinical and Experimental Allergy*, 24, 549–557.
- Tatham AS and Shewry PR, 2008. Allergens to wheat and related cereals. *Clinical and Experimental Allergy*, 38, 1712–1726. <https://doi.org/10.1111/j.1365-2222.2008.03101.x>

## Abbreviations

ANI	average nucleotide identity
bw	body weight
CAS	Chemical Abstracts Service
CEF	EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
CEP	EFSA Panel on Food Contact Materials, Enzymes and Processing Aids
EINECS	European Inventory of Existing Commercial Chemical Substances
FAO	Food and Agricultural Organization of the United Nations
GLP	Good Laboratory Practice
GMO	genetically modified organism
IUBMB	International Union of Biochemistry and Molecular Biology
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LoD	limit of detection
NOAEL	no observed adverse effect level
OECD	Organisation for Economic Cooperation and Development
QPS	Qualified Presumption of Safety
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 detail

Information provided in this appendix is shown in an excel file (downloadable <https://efsa.onlinelibrary.wiley.com/doi/10.2903/j.efsa.2021.6635#support-information-section>).

The file contains two sheets, corresponding to two tables.

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

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

## Appendix B – Population groups considered for the exposure assessment

Population	Age range	Countries with food consumption surveys covering more than one day
<b>Infants</b>	From 12 weeks on up to and including 11 months of age	Bulgaria, Denmark, Estonia, Finland, France, Germany, Italy, Latvia, Portugal, United Kingdom
<b>Toddlers</b>	From 12 months up to and including 35 months of age	Belgium, Bulgaria, Denmark, Estonia, Finland, France, Germany, Italy, Latvia, Netherlands, Portugal, Spain, United Kingdom
<b>Children<sup>(a)</sup></b>	From 36 months up to and including 9 years of age	Austria, Belgium, Bulgaria, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Italy, Latvia, Netherlands, Portugal, Spain, Sweden, United Kingdom
<b>Adolescents</b>	From 10 years up to and including 17 years of age	Austria, Belgium, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Italy, Latvia, Netherlands, Portugal, Spain, Sweden, United Kingdom
<b>Adults</b>	From 18 years up to and including 64 years of age	Austria, Belgium, Croatia, Czech Republic, Denmark, Estonia, Finland, France, Germany, Hungary, Ireland, Italy, Latvia, Netherlands, Portugal, Romania, Spain, Sweden, United Kingdom
<b>The elderly<sup>(a)</sup></b>	From 65 years of age and older	Austria, Belgium, Denmark, Estonia, Finland, France, Germany, Hungary, Ireland, Italy, Latvia, Netherlands, Portugal, Romania, Spain, Sweden, United Kingdom

(a): The terms 'children' and 'the elderly' correspond, respectively, to 'other children' and the merge of 'elderly' and 'very elderly' in the Guidance of EFSA on the 'Use of the EFSA Comprehensive European Food Consumption Database in Exposure Assessment' (EFSA, 2011).