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Safety evaluation of the food enzyme α-amylase from the non-genetically modified *Aspergillus niger* strain AS 29-286

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Abstract

The food enzyme α-amylase (4-α-D-glucan glucanohydrolase; EC 3.2.1.1) is produced with the non-genetically modified *Aspergillus niger* strain AS 29-286 by Shin Nihon Chemical Co., Ltd. The food enzyme is considered free from viable cells of the production organism. It is intended to be used in seven food manufacturing processes: baking processes, fruit and vegetable processing for juice production, fruit and vegetable processing for products other than juices, distilled alcohol production, starch processing for the production of maltodextrins, brewing processes and non-wine vinegar production. Since residual amounts of total organic solids (TOS) are removed during distilled alcohol production and starch processing for the production of maltodextrins, dietary exposure was calculated only for the remaining five food manufacturing processes. It was estimated to be up to 2.158 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 1,774 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 822. A search for the similarity of the amino acid sequence of the food enzyme to known allergens was made and four matches with respiratory allergens were found. The Panel considered that, under the intended conditions of use, the risk of allergic reactions upon dietary exposure cannot be excluded, but the likelihood is 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.

Keywords: food enzyme, α-amylase, 4-α-D-glucan glucanohydrolase, EC 3.2.1.1, glycogenase, *Aspergillus niger*

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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 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[2] established the European Union (EU) procedures for the safety assessment and the authorisation procedure of food additives, food enzymes and food flavourings. The use of a food enzyme shall be authorised only if it is demonstrated that:

- it does not pose a safety concern to the health of the consumer at the level of use proposed;
- there is a reasonable technological need;
- its use does not mislead the consumer.

All food enzymes currently on the European Union market and intended to remain on that market, as well as all new food enzymes, shall be subjected to a safety evaluation by the European Food Safety Authority (EFSA) and approval via an EU Community list.

The 'Guidance on submission of a dossier on food enzymes for safety evaluation' (EFSA, 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 applicant "Shin Nihon Chemical Co." for the authorisation of the food enzymes 3-Phytase from *Aspergillus niger* (strain PHY93-08), Alpha-amylase from *Aspergillus niger* (strain AS 29-286), Invertase and Exo-beta-glucosidase from *Aspergillus niger* (strain IN 319), Alpha-galactosidase from *Aspergillus niger* (strain AGS614) and Lactase from *Aspergillus oryzae* (strain GL 470).

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 five applications fall within the scope of the food enzyme Regulation and contain all the elements required under Chapter II of that Regulation.

1.1.2. Terms of Reference

The European Commission requests the European Food Safety Authority to carry out the safety assessments on the following food enzymes 3-Phytase from *Aspergillus niger* (strain PHY93-08), Alpha-

[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, pp. 15–24.

amylase from *Aspergillus niger* (strain AS 29-286), Invertase and Exo-beta-glucosidase from *Aspergillus niger* (strain IN 319), Alpha-galactosidase from *Aspergillus niger* (strain AGS614) and Lactase from *Aspergillus oryzae* (strain GL 470) 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 on the food enzyme alpha-amylase from *Aspergillus niger* (strain AS 29-286).

2. Data and methodologies

2.1. Data

The applicant has submitted a dossier in support of the application for authorisation of the food enzyme α-amylase from *A. niger* strain AS 29-286.

Additional information was requested from the applicant during the assessment process on 17 March 2022 and received on 17 November 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 existing guidance documents of 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	α-Amylase
Systematic name	4-α-D-glucan glucanohydrolase
Synonyms	Glycogenase, endoamylase, Taka-amylase
IUBMB No	EC 3.2.1.1
CAS No	9000-90-2
EINECS No	232-565-6

α-Amylases catalyse the hydrolysis of 1,4-α-glucosidic linkages in starch (amylose and amylopectin), glycogen and related polysaccharides and oligosaccharides, resulting in the generation of soluble dextrins and other oligosaccharides. The food enzyme is intended to be used in seven food manufacturing processes: baking processes, fruit and vegetable processing for juice production, fruit and vegetable processing for products other than juices, distilled alcohol production, starch processing for the production of maltodextrins, brewing processes and non-wine vinegar production.

3.1. Source of the food enzyme

The α-amylase is produced with the non-genetically modified filamentous fungus *A. niger* strain AS 29-286, which is deposited at the [redacted] with deposit number [redacted].[4] The production strain was identified as *A. niger* [redacted]

[4] Technical dossier/Annex 1.2.

3.2. Production of the food enzyme

The food enzyme is manufactured according to the Food Hygiene Regulation (EC) No 852/2004[5], 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 [redacted][6] 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.[7]

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 α-amylase is a single polypeptide chain of [redacted] amino acids.[8] The molecular mass of the mature protein, calculated from the amino acid sequence, is [redacted] kDa.[9] The food enzyme was analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.[10] A consistent protein pattern was observed across all batches. The gel showed a major band around [redacted] kDa in all batches. The protein profile also included bands of lesser staining intensity. No other enzyme activities were reported.

The in-house determination of α-amylase activity is based on the hydrolysis of soluble starch (reaction conditions: pH 4.5, 40°C, 10 min). The enzymatic activity is determined by measuring the release of reducing sugar by using a modified Fehling–Lehmann–Schoorl assay. The enzyme activity is expressed in starch saccharifying activity units (U)/g. One unit is defined as the amount of enzyme which liberates reducing sugars equivalent to 1 mg of D-glucose per minute under the conditions of the assay.[11]

The food enzyme has a temperature optimum around 65°C (pH 4.5) and a pH optimum around pH 4.5 (40°C). Thermostability was tested after a pre-incubation of the food enzyme for 15 min at different temperatures (pH 5). α-Amylase activity decreased above 50°C, showing no residual activity at 75°C.[12]

3.3.2. Chemical parameters

Data on the chemical parameters of the food enzyme were provided for three batches used for commercialisation and two batches produced for the toxicological tests (Table 1).[13] The mean total organic solids (TOS) of the three food enzyme batches for commercialisation was 16.2% and the mean enzyme activity/TOS ratio was 7.0 U/mg TOS.

[5] 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.
[6] Technical dossier Figure 3.2.1.2.5.2–1 (Confidential) p. 39–40.
[7] Technical dossier Table 3.2.1.2.5.1–1 p. 38 and Annex III (Confidential).
[8] Technical dossier p. 22/Annex VII.1; Additional information April 2023/Attachment 1.
[9] Technical dossier/Additional information November 2022; Additional information April 2023/Attachment 1.
[10] Technical dossier Figure 3.2.1.1.2.1–1 (Confidential) p.23 and Additional information November 2022.
[11] Technical dossier/Annex II.
[12] Technical dossier p. 27–30.
[13] Technical dossier/Annex V (Confidential) and Additional information November 2022.

Table 1: Composition of the food enzyme

Parameters	Unit	Batches				
		1	2	3	4[(a)]	5[(b)]
α-Amylase activity	U/g[(c)]	1,170	1,120	1,100	1,100	1,470
Protein	%	11.4	11	11.1	11.9	–
Ash	%	0.7	0.8	1.1	1.3	0.3
Water	%	83.0	82.8	82.9	81.0	83.7
Total organic solids (TOS)[(d)]	%	16.3	16.4	16.0	17.7	16.0
Activity/TOS	U/mg TOS	7.2	6.8	6.9	6.2	9.2

TOS: total organic solids.
(a): Batch used for the Ames test, chromosomal aberration test, *in vivo* micronucleus test and the repeated dose 90-day oral toxicity study.
(b): Batch used for *in vitro* mammalian cell micronucleus test
(c): UNIT: Starch saccharifying activity unit (see Section 3.3.1).
(d): TOS calculated as 100% – % water – % ash.

3.3.3. Purity

The lead content in the three commercial batches and in the batches used for toxicological studies was below 0.05 mg/kg[14,15] 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).[16] No antimicrobial activity was detected in any of the tested batches.[16]

Strains of *Aspergillus*, in common with most filamentous fungi, have the capacity to produce a range of secondary metabolites (Frisvad et al., 2018). The presence of aflatoxins B1, B2, G1 and G2, ochratoxin A, zearalenone, T2-toxin, fumonisins B1 and B2 and sterigmatocystin was examined in three food enzyme batches and all were found below the limit of quantification (LoQ) of the applied methods.[17,18] Adverse effects caused by the possible presence of other secondary metabolites 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 [redacted] No colonies were produced. A positive control was included.[19]

3.4. Toxicological data

A battery of toxicological tests, including a bacterial reverse mutation test (Ames test), an *in vitro* mammalian chromosomal aberration test, an *in vivo* and an *in vitro* micronucleus test and a repeated dose 90-day oral toxicity study in rats, were provided.

The batches 4 and 5 (Table 1) used in these studies have similar protein patterns and activity/TOS values as the batches used for commercialisation and are considered suitable as test items.

[14] LoQs: Pb = 0.05 mg/kg.
[15] Technical dossier/ Annex IV & Table 3.2.1.2.5.3–1 Dossier document p. 41 (Confidential) and Additional information November 2022/Attachment B.
[16] Technical dossier/ Annex IV & Table 3.2.1.2.5.3–1 Dossier document p. 41 (Confidential).
[17] LoQ: aflatoxins B1, B2, G1 and G2 = 0.5 μg/kg each; ochratoxin A = 0.5 μg/kg; zearalenone = 100 μg/kg; sterigmatocystin = 100 μg/kg; T2-toxin = 100 μg/kg; fumonisins B1 and B2 = 500 μg/kg each.
[18] Technical dossier/Annex IV, Annex I and Table 3.2.1.2.5.3–3 Technical dossier document p.42 (Confidential) and Additional information November 2022/Attachment C.
[19] Technical dossier/Additional information November 2022.

3.4.1. Genotoxicity

3.4.1.1. Bacterial reverse mutation test

A bacterial reverse mutation test (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).[20]

Four strains of *Salmonella* Typhimurium (TA98, TA100, TA1535 and TA1537) and *Escherichia coli* WP2*uvr*A(pKM101) were used with or without metabolic activation (S9-mix).

A dose-finding test and three main experiments were conducted in triplicate. The dose-finding study was carried out with eight concentrations of food enzyme ranging from 0.0503 to 110 U/plate (corresponding to 0.008, 0.024, 0.072, 0.216, 0.65, 1.95, 5.87 and 17.6 mg TOS/plate) applying the plate incorporation method. An increase of revertant colony numbers in the *Salmonella* strains and a decrease of revertant colony numbers in *E. coli* WP2*uvr*A were reported at the highest concentrations tested with and without S9-mix. In addition, the number of revertant colony numbers in strain TA1535 treated with negative control solution was outside the range of historical controls. A first experiment was performed with *Salmonella* strain TA1535 and with *E. coli* WP2*uvr*A using seven concentrations of food enzyme from 1.71 to 110 U/plate (corresponding to 0.27, 0.55, 1.1, 2.2, 4.4, 8.8 and 17.6 mg TOS/plate) with the plate incorporation method. An increase of revertant colonies of more than two times compared to control in strain TA1535 and a decrease in *E. coli* WP2*uvr*A were reported.

The increase of revertant colony numbers in *Salmonella* strains was attributed by the study authors to the presence of amino acids, such as histidine, in the medium. Consequently, a second main experiment was carried out with the four strains of *Salmonella* T and *E. coli*, at eight concentrations of food enzyme ranging from 0.0503 to 110 U/plate (corresponding to 0.08, 0.024, 0.072, 0.216, 0.65, 1.95, 5.87 and 17.6 mg TOS/plate) applying the treat and wash method to exclude the effect of free amino acids. No increase of revertant colony numbers was observed. These results were reproduced in a confirmatory experiment following the same experimental conditions.

The Panel concluded that the food enzyme α-amylase 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.[21] A dose finding test and a main experiment were performed with duplicate cultures of Chinese hamster lung fibroblast cell line.

The cell cultures were treated with the food enzyme either with or without metabolic activation (S9-mix) in a short-term treatment (6 h followed by 18 h recovery period) and a long-term 24-h continuous treatment.

In a preliminary cytotoxicity test, the 50% cell growth inhibition concentrations (IC50) were calculated to be 103, 85.7 and 67.6 U/mL (corresponding to 16.5, 13.6 and 10.8 mg TOS/mL) for the short-term treatment without S9-mix, with S9-mix and for the long-term treatment, respectively.

In the main experiment, cells were exposed to the food enzyme and scored for chromosomal aberrations at concentrations 23.8, 39.6, 66 and 110 U/mL (corresponding to 3.8, 6.3, 10.6 and 17.6 mg TOS/mL), in the short-term treatment without S9-mix and in the long-term treatment. Concentrations of food enzyme of 39.6, 66 and 110 U/mL (corresponding to 6.3, 10.6 and 17.6 mg TOS/mL) were applied in the short-term treatment with S9-mix.

A statistically significant concentration-related increase of cells with structural chromosomal aberrations was reported at the two highest concentrations, both in the short-term treatment without S9-mix and in the long-term treatment. A statistically significant increase was observed only at the highest concentration tested in the short-term treatment with S9-mix. The incidences of polyploid cells in all treated groups were comparable with those of negative controls.

The Panel concluded that the food enzyme α-amylase did induce an increase in the frequency of structural aberrations under the test conditions applied in this study, but only at concentrations exceeding the maximum recommended by OECD Guidelines.

[20] Technical dossier/Annex VI/1. Ames Test.
[21] Technical dossier/Annex VI/2. Chromosomal Aberration Test.

3.4.1.3. *In vivo* mammalian erythrocyte micronucleus test

The *in vivo* micronucleus test was carried out according to OECD Test Guideline 474 (OECD, 1997c) and following GLP.[22]

Five males per group were treated with one oral administration by gavage per day for two consecutive days (24 h interval) of the food enzyme at doses of 2,750, 5,500 and 11,000 U/kg body weight (bw) corresponding to 440, 880 and 1,760 mg TOS/kg bw. Controls received the vehicle (water for injection). Bone marrow was sampled 24 h after the final dosing. No clinical signs of toxicity or adverse effects on body weight were observed in the food enzyme groups. No statistically significant increases in the frequency of micronucleated immature erythrocytes in the treated samples were observed in comparison to the controls and no statistically significant difference in the ratio of immature erythrocytes was observed in comparison to total number of erythrocytes.

The study presents deviations from the OECD Test Guideline 474: no data on bone marrow exposure were shown. In addition, the recommended limit dose was not reached with the highest administered dose. Therefore, the Panel considered the study as inconclusive.

3.4.1.4. In vitro mammalian micronucleus test

The *in vitro* mammalian cell micronucleus test was carried out according to OECD Test Guideline 487 (OECD, 2016) and following GLP.[23]

In a cytotoxicity test where cultures of human peripheral blood lymphocytes were treated with the food enzyme up to 5,000 μg TOS/mL, cytotoxicity of more than 50% was not observed in a short-term treatment (3 h treatment followed by 21 h recovery) either with or without metabolic activation (S9-mix), or in a long-term treatment (24 h treatment followed by 24 h recovery) without S9-mix.

In the main experiment, duplicate cultures were treated with the food enzyme and scored for binucleated cells with micronuclei (MNBN) in the short-term treatments, with or without S9-mix, and in a long-term treatment (24 h treatment followed by 24 h recovery) without S9-mix at concentrations of 1,000, 2,000 and 5,000 μg TOS/mL.

The frequency of MNBN was not statistically significantly different compared to the negative controls at any concentrations tested in the short-term nor the long-term treatments. All results except for one culture in the vehicle control in the long-term treatment and one culture in the mid concentration in the short-term treatment, with S9-mix, were slightly above the 95% historical control range, but these observations were not considered to be of biological relevance.

The Panel concluded that the food enzyme α-amylase did not induce an increase in the frequency of MNBNs under the test conditions applied in this study.

3.4.1.5. Conclusion on genotoxicity studies

Based on the negative results obtained with the Ames test and with the *in vitro* micronucleus test in human peripheral lymphocytes, the Panel concluded that there is no concern for genotoxicity of the food enzyme. The Panel considered that the positive results reported in the chromosomal aberration test with transformed rodent cell line were overruled by those obtained with the primary human cell culture.

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 guidelines of the Japanese Ministry of Health and Welfare (1996) and following GLP.[24] The study is in accordance with OECD Test Guideline 408 (OECD, 1998) with the following deviations: detailed clinical observations and functional observations were not performed, urea was not determined, epididymides were not weighed, the regions of the brain examined were not specified and it was not specified whether the examination of the small intestine included the Peyer's patches. The Panel considered that these deviations are minor and do not impact on the evaluation of the study.

Groups of 10 male and 10 female Sprague–Dawley (Crj:CD(SD)) rats received by gavage the food enzyme in doses of 110, 1,100 or 11,000 U/kg bw per day corresponding to 17.7, 177 or 1,774 mg TOS/kg bw per day. Controls received the vehicle (water for injection).

[22] Technical dossier/Annex VI/3. Micronucleus Test.
[23] Additional data November 2022/Attachment D – In vitro Micronucleus Test Study Report.
[24] Technical dossier/Annex VI/90-Day Study.

One high-dose male was euthanised on day 22 of dosing due to a gavage-related incident. There were no abnormal findings in the macroscopic examination and no histopathological changes in the microscopic examination. Therefore, the Panel considered that the death was not test item related.

The feed consumption was statistically significantly decreased on days 28, 63 and 90 of administration in high-dose males (−14%, −10% and −16%, respectively). The total feed consumption was also decreased in high-dose males (−9%). The Panel considered the changes as not toxicologically relevant as they were sporadic, only observed in one sex and there were no statistically significant changes in body weight and body weight gain.

Feed efficiency was statistically significantly decreased on day 7 of administration in mid-dose males (−11%) and increased on day 77 in high-dose males (+43%). The Panel considered the changes as not toxicologically relevant as they were only recorded sporadically, as they were only observed in one sex and there were no statistically significant changes in the body weight and the body weight gain.

The haematological investigation revealed a statistically significant increase in the blood coagulation parameter activated partial thromboplastin time in high-dose males (+12%). The Panel considered the change as not toxicologically relevant as it was only observed in one sex, the change was small and there were no changes in other related haematological parameters (platelets count, prothrombin time and fibrinogen).

The clinical chemistry investigation revealed a statistically significant increase in calcium in mid-dose females (+4%), in α_1 globulin fraction ratio (A/G ratio) in high-dose females (+13%) and in α_1 globulin concentration in mid- and high-dose females (+13% and + 15%, respectively), and a decrease in alkaline phosphatase activity in high-dose females (−26%). 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 (calcium), the changes were small (all parameters), there were no changes in other relevant parameters (A/G ratio and α_1 globulin concentration) and there were no histopathological changes in the liver.

The urinalysis revealed a statistically significant decrease in total chloride excretion in high-dose males (−30%). The Panel considered the change as not toxicologically relevant as it was only observed in one sex and there were no histopathological changes in the kidneys.

Statistically significant changes in organ weights included a decrease in the absolute pituitary gland weight in low-dose males (−15%) and an increase in the relative liver weight in mid-dose females (+6%). The Panel considered the changes as not toxicologically relevant as they were only observed in one sex (both parameters), there was no dose–response relationship (both parameters), the changes were small (both parameters) and there were no histopathological changes in the pituitary gland or in the liver.

The microscopic examination revealed a statistically significant decreased incidence of mineralisation in the kidneys of high-dose females (0/10 vs. 4/10 in the control group). The Panel considered the change as not toxicologically relevant as the incidence of the finding was decreased.

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

The Panel identified a no observed adverse effect level (NOAEL) of 1,774 mg TOS/kg bw per day, the highest dose tested.

3.4.3. Allergenicity

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

The potential allergenicity of the α-amylase produced with the non-genetically modified *A. niger* strain AS 29-286 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, four matches were found. The matching allergens were [redacted] all known as respiratory allergens.[25]

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

[25] Technical dossier pg. 59-61/Annex VII; Additional information April 2023/Attachment 1.

Both [redacted] are known as occupational respiratory allergens associated with baker's asthma. However, several studies have shown that adults with occupational asthma to a food enzyme (as described for α-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 α-amylase as food enzyme, only a low number of case reports of allergic reactions upon oral exposure to α-amylase in individuals respiratory sensitised to α-amylase have been described in literature (Losada et al., 1992; [redacted]; Baur and Czuppon, 1995; Kanny and Moneret-Vautrin, 1995; Moreno-Ancillo et al., 2004).

[redacted], a product that may cause allergies or intolerances (listed in the Regulation (EU) No 1169/2011[26]) was used as raw material. In addition, [redacted], known source of allergens, is 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 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 proteins are not expected to be present in the food enzyme.

The Panel considered that, under the intended conditions of use, the risk of allergic reactions upon dietary exposure to this food enzyme cannot be excluded (except for distilled alcohol production), but the likelihood is low.

3.5. Dietary exposure

3.5.1. Intended use of the food enzyme

The food enzyme is intended to be used in seven food manufacturing processes at the recommended use levels summarised in Table 2.

Table 2: Intended uses and recommended use levels of the food enzyme as provided by the applicant.[27]

Food manufacturing process[(a)]	Raw material (RM)	Recommended use level (mg TOS/kg RM)[(b)]
Baking processes	Flour (wheat or rye)	**43.5**
Fruit and vegetable processing for juice production	Fruits and vegetables (apple, grape, carrot, tomato)	**43.5**
Fruit and vegetable processing for products other than juices	Fruits and vegetables (strawberry, apple, banana, carrot, pumpkin, potato)	**43.5**
Distilled alcohol production	Grape, malt, rice, barley	216
Starch processing for the production of maltodextrins	Starch (corn, wheat, potato, cassava)	43.5
Brewing processes for the production of rice wine	Rice	**216**
Non-wine vinegar production	Rice, wheat	**216**

TOS: total organic solids.
(a): The name has been harmonised by EFSA according to the 'EC working document describing the food processes in which food enzymes are intended to be used' – not yet published at the time of adoption of this opinion.
(b): Numbers in bold were used for calculation.

In baking processes, the food enzyme is added to flour during dough preparation.[28] The hydrolysis of starch reduces the viscosity of the dough and the final product has an increased volume. The food enzyme remains in the baked foods.

[26] 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.
[27] Additional information November 2022/Table 2.
[28] Additional information November 2022/Attachment E/Flowchart E-1.

In juice production, the food enzyme is added to fruits and vegetables along with other pectinolytic enzymes during the mash treatment.[29] The α-amylase hydrolyses starch in the pressed juices, improving the filtration rate and preventing haze. The food enzyme remains in the juice.

For the production of other fruit and vegetable products, the food enzyme is added to crushed or milled fruits and vegetables.[30] The hydrolysis of starch reduces viscosity, and the hydrolysates have higher solubility and sweetness in the final products (e.g. jam, puree, paste and sauce). The food enzyme remains in the final products.

In distilled alcohol production, the food enzyme is added to fermented starch (koji) during the saccharification and fermentation steps.[31] The hydrolysis of starch increases the amount of fermentable sugars for higher alcohol yields. The food enzyme–TOS is not carried over to the distilled alcohols (EFSA CEP Panel, 2021b).

In the production of maltodextrins, the food enzyme is added to liquefied starch during the dextrinisation step.[32] The hydrolysis of starch produces oligosaccharides (maltodextrins). The food enzyme–TOS is removed from the final product (maltodextrins) by treatment with activated charcoal or equivalent, and purification using ion-exchange resins (EFSA CEP Panel, 2021b).

During the production of rice wine (sake), the food enzyme is added to steamed or liquefied rice or fermented rice (koji) during saccharification and fermentation step.[33] The α-amylase hydrolyses starch to fermentable sugars. The food enzyme remains in the rice wine.

For the production of non-wine vinegars, the food enzyme is added to steamed grains or fermented rice (koji) during saccharification and alcoholic fermentation but before the acetic acid fermentation step.[34] The hydrolysis of starch increases the amount of fermentable sugars for higher alcohol yields. The hydrolysis of starch is accelerated for improvement of yield. The food enzyme remains in grain vinegar.

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

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: baking processes, fruit and vegetable processing for juice production, fruit and vegetable processing for products other than juices, brewing processes and non-wine vinegar production.

Rice wine (sake) is not a commonly consumed alcoholic beverage in the EU. It is not identifiable as a specific FoodEx category in the Comprehensive European Food Consumption Database.[35] To obtain the consumption data of sake from European consumers, having considered that sake is made by a brewing process, the Panel decided to substitute the consumption data of sake with those of beer.

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 bw. 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 dietary

[29] Additional information November 2022/Attachment E/Flowchart E-2.
[30] Additional information November 2022/Attachment E/Flowchart E-3.
[31] Additional information November 2022/Attachment E/Flowchart E-4.
[32] Additional information November 2022/Attachment E/Flowchart E-8.
[33] Additional information November 2022/Attachment E/Flowchart E-5.
[34] Additional information November 2022/Attachment E/Flowchart E-6 and E-7.
[35] Available online: https://www.efsa.europa.eu/en/data-report/food-consumption-data

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 2.158 mg TOS/kg bw per day in children at the 95th percentile.

Table 3: Summary of the estimated dietary exposure to the 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)	0.110–0.619 (12)	0.287–1.459 (15)	0.266–0.813 (19)	0.129–0.501 (21)	0.116–0.396 (22)	0.070–0.307 (23)
Min–max 95th (number of surveys)	0.292–1.603 (11)	0.849–2.111 (14)	0.546–2.158 (19)	0.283–1.400 (20)	0.350–1.244 (22)	0.198–0.881 (22)

TOS: total organic solids.

3.5.3. Uncertainty analysis

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

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

Sources of uncertainties	Direction of impact
Model input data	
Consumption data: different methodologies/representativeness/underreporting/ misreporting/no portion size standard	+/–
Use of data from food consumption surveys of a few days to estimate long-term (chronic) exposure for high percentiles (95th percentile)	+
Possible national differences in categorisation and classification of food	+/–
Model assumptions and factors	
Exposure to food enzyme–TOS was calculated based on the recommended maximum use level	+
Selection of broad FoodEx categories for the exposure assessment	+
Substitution of the consumption data of sake in the EU with those of beer	+
Use of recipe fractions in disaggregation FoodEx categories	+/–
Use of technical factors in the exposure model	+/–
Exclusion of two food manufacturing processes from the exposure estimation – Distilled alcohol production – Starch processing for the production of maltodextrins	–

TOS: total organic solids.
+: uncertainty with potential to cause overestimation of exposure.
–: uncertainty with potential to cause underestimation of exposure.

The conservative approach applied to the exposure estimate to food enzyme–TOS, in particular assumptions made on the occurrence and use levels of this specific food enzyme, is likely to have led to overestimation of the exposure.

The exclusion of two food manufacturing processes from the exposure assessment was based on > 99% of TOS removal 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 (1,774 mg TOS/kg bw per day) from the 90-day rat study with the derived exposure estimates of 0.070 to 1.459 mg TOS/kg bw per day at the mean and from 0.198 to 2.158 mg TOS/kg bw per day at the 95th percentile, resulted in margins of exposure (MoE) of at least 822.

4. Conclusion

Based on the data provided, removal of TOS during distilled alcohol production and starch processing for the production of maltodextrins and the derived margin of exposure for the remaining food manufacturing processes, the Panel concluded that the food enzyme α-amylase produced with *A. niger* strain AS 29-286 does not give rise to safety concerns under the intended conditions of use.

5. Documentation as provided to EFSA

Application for the Authorisation of Alpha-Amylase from *Aspergillus niger* Strain AS 29-286 as a Food Enzyme in the European Union. March 2015. Submitted by Shin Nihon Chemical Co., Ltd.

Additional information. November 2022. Submitted by Intertek Health Sciences Inc.

Additional information. April 2023. Submitted by Intertek Health Sciences Inc.

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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
CFU	colony forming units
DRF	dose-range finding
EINECS	European Inventory of Existing Commercial Chemical Substances
FAO	Food and Agricultural Organization 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
JMHW	Japanese Ministry of Health and Welfare
kDa	kiloDalton
LoQ	limit of quantification
MoE	margin of exposure
NA	not analysed
NITE	National Institute of Technology and Evaluation, Biological Resource Center (Japan)
NOAEL	no observed adverse effect level
OECD	Organisation for Economic Cooperation and Development
RM	raw material
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.2023.8090#support-information-section).

The file contains two sheets, corresponding to two tables.

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

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

Appendix B – Population groups considered for the exposure assessment

Population	Age range	Countries with food consumption surveys covering more than 1 day
Infants	From 12 weeks on up to and including 11 months of age	Bulgaria, Cyprus, Denmark, Estonia, Finland, France, Germany, Italy, Latvia, Portugal, Slovenia, 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, Republic of North Macedonia*, Serbia*, 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, Republic of North Macedonia, Serbia*, Spain, Sweden
Adolescents	From 10 years up to and including 17 years of age	Austria, Belgium, Bosnia and Herzegovina*, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Italy, Latvia, Montenegro*, Netherlands, Portugal, Romania, Serbia*, Slovenia, Spain, Sweden
Adults	From 18 years up to and including 64 years of age	Austria, Belgium, Bosnia and Herzegovina*, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Montenegro*, Netherlands, Portugal, Romania, Serbia*, 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, Montenegro*, Netherlands, Portugal, Romania, Serbia*, Slovenia, Spain, Sweden

*: Consumption data from these pre-accession countries are not reported in Table 3 of this opinion, however, they are included in Appendix A for testing purpose.

(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).