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SCIENTIFIC OPINION

Safety evaluation of the food enzyme catalase from the non-genetically modified *Aspergillus tubingensis* strain AE-CN

EFSA Panel on Food Contact Materials, Enzymes and Processing Aids (CEP) | Claude Lambré | José Manuel Barat Baviera | Claudia Bolognesi | Pier Sandro Cocconcelli | Riccardo Crebelli | David Michael Gott | Konrad Grob | Evgenia Lampi | Marcel Mengelers | Alicja Mortensen | Gilles Rivière | Inger-Lise Steffensen | Christina Tlustos | Henk Van Loveren | Laurence Vernis | Holger Zorn | Lieve Herman | Yrjö Roos | Magdalena Andryszkiewicz | Ana Criado | Yi Liu | Simone Lunardi | Elsa Nielsen | Karin Norby | Andrew Chesson

Correspondence: fip@efsa.europa.eu

Abstract

The food enzyme catalase (hydrogen-peroxide:hydrogen-peroxide oxidoreductase; EC 1.11.1.6) is produced with the non-genetically modified *Aspergillus tubingensis* strain AE-CN by Amano Enzyme Inc. The absence of viable cells of the production organism in the food enzyme was not demonstrated. The food enzyme is intended to be used in five food manufacturing processes: production of baked products, processing of egg and egg products, production of fruit and vegetable products other than juices, production of cheese and production of fish roes. The dietary exposure to the food enzyme total organic solids (TOS) was estimated to be up to 0.325 mg TOS/kg body weight (bw) per day in European populations. The results of the *in vitro* genotoxicity studies indicated the presence of a clastogenic agent in the food enzyme which could not be dismissed due to limitations in the *in vivo* studies. 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 323 mg TOS/kg bw per day, the highest dose tested. A search for the similarity of the amino acid sequence of the food enzyme to known allergens was made and one match was found. The Panel considered that the risk of allergic reactions by dietary exposure cannot be excluded, but the likelihood is low. Because of the results of the genotoxicity studies, and as the absence of viable cells from the production strain was not demonstrated, the Panel was unable to establish the safety of the food enzyme.

KEYWORDS

Aspergillus niger, catalase, catalase-peroxidase, EC 1.11.1.6, food enzyme, hydrogen-peroxide:hydrogen-peroxide oxidoreductase

CONTENTS

1 | INTRODUCTION

Article 3 of the Regulation (EC) No 1332/2008[1] provides definition for 'food enzyme' and 'food enzyme preparation'.

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

'Food enzyme preparation' means a formulation consisting of one or more food enzymes in which substances such as food additives and/or other food ingredients are incorporated to facilitate their storage, sale, standardisation, dilution or dissolution.

Before January 2009, food enzymes other than those used as food additives were not regulated or were regulated as processing aids under the legislation of the Member States. On 20 January 2009, Regulation (EC) No 1332/2008 on food enzymes came into force. This Regulation applies to enzymes that are added to food to perform a technological function in the manufacture, processing, preparation, treatment, packaging, transport or storage of such food, including enzymes used as processing aids. Regulation (EC) No 1331/2008[2] established the European Union (EU) procedures for the safety assessment and the authorisation procedure of food additives, food enzymes and food flavourings. The use of a food enzyme shall be authorised only if it is demonstrated that:

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

All food enzymes currently on the 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 "DSM Food Specialties B.V." for the authorisation of the food enzyme Carboxypeptidase C from a genetically modified strain of *Aspergillus niger* (strain PEG); "Advanced Enzyme Technologies Ltd." for the authorisation of the food enzymes Maltogenic amylase from a genetically modified strain of *Escherichia coli* (strain BLASC) and Triacylglycerol Lipase from a genetically modified strain of *Aspergillus niger* agg. (strain FL100SC); "Danisco US Inc." for the authorisation of the food enzyme Glucan 1,4-a-maltotetraohydrolase from a genetically modified strain of *Bacillus licheniformis* (strain DP-Dzf24), and "Amano Enzyme Inc." for the authorisation of the food enzyme Catalase from *Aspergillus niger* (strain AE-CN).

Following the requirements of Article 12.1 of Commission Regulation (EU) 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 contains 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 food enzymes Carboxypeptidase C from a genetically modified strain of *Aspergillus niger* (strain PEG), Maltogenic amylase from a genetically modified strain of *Escherichia coli* (strain BLASC), Triacylglycerol Lipase from a genetically modified strain of *Aspergillus niger* agg. (strain FL100SC), Glucan 1,4-α-maltotetraohydrolase from a genetically modified strain of *Bacillus*

[1]Regulation (EC) No 1332/2008 of the European Parliament and of the Council of 16 December 2008 on Food Enzymes and Amending Council Directive 83/417/EEC, Council Regulation (EC) No. 1493/1999, Directive 2000/13/EC, Council Directive 2001/112/EC and Regulation (EC) No 258/97. OJ L 354, 31.12.2008, pp. 7–15.

[2]Regulation (EC) No. 1331/2008 of the European Parliament and of the Council of 16 December 2008 establishing a common authorisation procedure for food additives, food enzymes and food flavourings. OJ L 354, 31.12.2008, pp. 1–6.

[3]Commission Regulation (EU) No. 234/2011 of 10 March 2011 implementing Regulation (EC) No 1331/2008 of the European Parliament and of the Council establishing a common authorisation procedure for food additives, food enzymes and food flavourings. OJ L 64, 11.3.2011, pp. 15–24.

licheniformis (strain DP-Dzf24) and Catalase from *Aspergillus niger* (strain AE-CN) 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 catalase from *A. niger* (strain AE-CN).

Recent data identified the production microorganism as *A. tubingensis* (Section 3.1). Therefore, this name will be used in this opinion instead of *A. niger*.

2 | DATA AND METHODOLOGIES

2.1 | Data

The applicant has submitted a dossier in support of the application for authorisation of the food enzyme catalase from *A. niger* (strain AE-CN). The dossier was updated on 3 December 2015.

Additional information was requested from the applicant during the assessment process on 30 September 2021 and 13 December 2021 and received on 11 October 2021 and 21 March 2023, respectively (see 'Documentation provided to EFSA').

Following the reception of additional data by EFSA on 11 October 2021, EFSA requested a clarification teleconference on 22 October 2021.

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 et al., 2021).

3 | ASSESSMENT

IUBMB nomenclature	Catalase
Systematic name	Hydrogen-peroxide:hydrogen-peroxide oxidoreductase
Synonyms	Catalase-peroxidase
IUBMB No	EC 1.11.1.6
CAS No	9001-05-2
EINECS No	232-577-1

Catalases catalyse the decomposition of hydrogen peroxide, converting it to water and oxygen. The enzyme under assessment is intended to be used in five food manufacturing processes: production of baked products, processing of egg and egg products, production of fruit and vegetable products other than juices, production of cheese and production of fish roes.

3.1 | Source of the food enzyme

The catalase is produced with the non-genetically modified filamentous fungus *A. tubingensis* strain AE-CN, which is deposited at the National Institute of Technology and Evaluation (NITE) Biological Resource Center (Japan), with the deposit number [redacted][4] The production strain was identified as *A. tubingensis* [redacted][5]

[4]Technical dossier/Additional data March 2023/Annex 2.

[5]Technical dossier/Additional data March 2023/Annex 1.

3.2 | Production of the food enzyme

The food enzyme is manufactured according to the Food Hygiene Regulation (EC) No 852/2004[6], with food safety procedures based on Hazard Analysis and Critical Control Points, and in accordance with current Good Manufacturing Practice.[7]

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 and release of the intracellular enzyme [redacted], the solid biomass is removed from the fermentation broth by filtration. The filtrate containing the enzyme is then further purified and concentrated, including an ultrafiltration step in which enzyme protein is retained, while most of the low molecular mass material passes the filtration membrane and is discarded.[8] 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.[9]

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 catalase is a single polypeptide chain of [redacted] amino acids.[10] The molecular mass of the mature protein, calculated from the amino acid sequence, is [redacted] kDa.[10] The food enzyme was analysed by size exclusion chromatography. The chromatograms of the three food enzyme batches for commercialisation show similar patterns with two major peaks.[11] Apart from the presence of glucose oxidase no other enzymatic activities were reported.[12]

The in-house determination of catalase activity is based on the decomposition of hydrogen peroxide (reaction conditions: [redacted]). The enzymatic activity is determined by an iodometric determination of residual hydrogen peroxide. The enzyme activity is expressed in catalase activity unit (CAU)/mL. One CAU is defined as that amount of catalase that decomposes 1 µmol of hydrogen peroxide in 1 min under the conditions of the assay.[13]

The food enzyme has a temperature optimum between 40°C and 60°C (pH 7.0) and a pH optimum between pH 6.0 and 8.0 (30°C). Thermostability was tested after pre-incubation of the food enzyme for 5 min at different temperatures (pH 7.0). Catalase activity decreased above 70°C, showing no residual activity above 80°C.[14]

3.3.2 | Chemical parameters

Data on the chemical parameters of the food enzyme were provided for three batches used for commercialisation (batches 1–3) and four batches used for toxicological testing (batches 4–7) (Table 1).[15] The mean total organic solids (TOS) of the three food enzyme batches for commercialisation was 27.6% and the mean catalase activity/TOS ratio was 5186 U/mg TOS.

3.3.3 | Purity

The lead content in the three commercial batches was below 5 mg/kg, which complies with the specification for lead as laid down in the general specifications for enzymes used in food processing (FAO/WHO, 2006).[16] In addition, the levels of mercury were below the limit of quantification (LOQ) of the employed methodology. For arsenic and cadmium, the average concentrations determined in the commercial batches were 0.16 and 0.2 mg/kg, respectively.[17,18] The Panel considered these concentrations as of no concern.

[6]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.
[7]Technical dossier 2nd submission/pg. 50/Annexes: 4_1 and 4_2.
[8]Technical dossier 2nd submission/pg. 50–57/Annex 5.
[9]Technical dossier 2nd submission/Annex 6 and Additional data March 2023.
[10]Technical dossier/Additional data March 2023.
[11]Technical dossier 2nd submission/pg. 37.
[12]Technical dossier 2nd submission/pg. 41/Additional data September 2015/Annex 3.
[13]Technical dossier 2nd submission/pg. 40/Annex 2.
[14]Technical dossier 2nd submission/pg. 41–43.
[15]Technical dossier 2nd submission/pg. 36, 75/Annexes: 1,3, 8, 9, 10 and 11/Additional data September 2015/Annex 4.
[16]Technical dossier 2nd submission/pg. 38/Annexes: 1 and 3.
[17]Technical dossier 2nd submission/pg. 38/Annexes: 1 and 3.
[18]LoQs: Pb = 0.05 mg/kg; As = 0.002 mg/kg; Cd and Hg = 0.001 mg/kg each.

TABLE 1 Composition of the food enzyme preparation.

		Batches						
Parameters	**Unit**	**1**	**2**	**3**	**4**[a]	**5**[b]	**6**[c]	**7**[d]
Catalase activity[e]	U/g[f]	1,650,000	1,430,000	1,210,000	72,000	1,210,000	1,760,000	1,490,000
Protein	%	32.9	25.2	23.7	2.19	NA[g]	NA	NA
Ash	%	1.2	1.5	1.6	0.4	1.6	2.0	2.1
Water	%	4.1	4.5	4.5	93.3	5.2	4.0	3.7
Dextrin (excipient)	%	61.8	67.8	70.1	–	NA	61.7	65.0
Total organic solids (TOS)[h]	%	32.9	26.2	23.8	6.3	93.2	32.3	29.2
Catalase activity/TOS	U/mg TOS	5015	5458	5084	1143	1298	5449	5103

[a]Batch used for the Ames test.
[b]Batch used for chromosomal aberration and *in vivo* micronucleus tests.
[c]Batch used for the repeated dose 90-day oral toxicity study in rats.
[d]Batch used for the *in vitro* mammalian cell micronucleus test and *in vivo* Comet assay.
[e]CAU: catalase activity units (see Section 3.3.1).
[f]U/g: glucose oxidase units (see Section 3.3.1).
[g]NA: not analysed.
[h]TOS calculated as 100% – % water – % ash – % excipient.

The food enzyme preparation complies with the microbiological criteria for total coliforms, *Escherichia coli* and *Salmonella* as laid down in the general specifications for enzymes used in food processing (FAO/WHO, 2006).[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 aflatoxin B1, B2, G1 and G2, deoxynivalenol, ochratoxin A, sterigmatocystin, T2-toxin and HT2-toxin, and zearalenone was examined in the three food enzyme batches, and all were below the limits of detection (LoD) of the applied methods.[16,19] Adverse effects caused by the possible presence of other secondary metabolites were addressed by the toxicological examination of the food enzyme–TOS.

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

3.3.4 | Viable cells of the production strain

The absence of viable cells of the production strain in the food enzyme was not demonstrated in three independent batches analysed in triplicate. [redacted] Colonies were detected [redacted] but not all were discriminated from the production strain. No adequate positive control was included.[20]

3.4 | Toxicological data

A battery of toxicological tests has been provided, including a bacterial reverse mutation test (Ames test), an *in vitro* mammalian chromosomal aberration test, an *in vivo* micronucleus test, an *in vitro* mammalian cell micronucleus test, an *in vivo* Comet assay and a repeated dose 90-day oral toxicity study in rats. The batches 6 and 7 (Table 1) used in these studies had similar protein patterns as the batches used for commercialisation and a similar value for activity/TOS. Batch 4 did not contain a diluent, while in batch 5 the amount of diluent present is unknown. However, both batches had lower activity/TOS values and were considered suitable as test items.

3.4.1 | Genotoxicity

3.4.1.1 | *Bacterial reverse mutation test*

A bacterial reverse mutation assay (Ames test) was performed according to a method which was in compliance with the Organisation for Economic Co-operation and Development (OECD) Test Guideline 471 (OECD, 1997a). Four strains of Salmonella Typhimurium (TA98, TA100, TA1535 and TA1537) and *E. coli* WP2uvrA were used in the presence or absence of metabolic activation (S9-mix), applying the pre-incubation method. In two dose finding tests, four concentrations (1.6, 6.3,

[19]LoQs: aflatoxins (B1, B2, G1, G2) = 0.5 μg/kg each; ochratoxin A = 0.5 μg/kg; sterigmatocystin = 20 μg/kg; T2-toxin and deoxynivalenol = 100 μg/kg; zearalenone = 50 μg/kg; HT2-toxin = 250 μg/kg.
[20]Technical dossier/Additional data March 2023/Annex 3.

25 and 100 µL/plate) were tested, with and without S9-mix and with duplicate plating. In the first test, an inhibition of bacterial growth was observed, which was attributed to glucose oxidase contained in the catalase stock solution. The second test was carried out using minimal fructose agar plates containing fructose instead of the glucose.

Based on the results of the dose finding experiments, five concentrations of the food enzyme (6.3, 12.5, 25, 50 and 100 µL/plate, corresponding to 397, 787, 1575, 3150 and 6300 µg TOS/plate) were applied in the main test, using minimal fructose agar plates with triplicate plating. No cytotoxicity was observed at any concentration level of the test substance. 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 catalase 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 fibroblasts (CHL/UI) according to the OECD Test Guideline 473 (OECD, 1997b) and following GLP.[21]

In the cell-growth inhibition test, cells were exposed to 12 concentrations of the food enzyme, ranging from 2.44 to 5000 µg/mL with and without metabolic activation (S9-mix). Based on the test results, the cells were exposed to the food enzyme from 0.254 to 1670 µg/mL of food enzyme (corresponding to 0.237 to 1558 µg TOS/mL) in a short-term treatment (6 h followed by 18 h recovery period) without S9-mix, and at 185, 556, 1670 and 5000 µg/mL (corresponding to 173 to 4665 µg TOS/mL) with S9-mix. The frequency of structural and numerical chromosomal aberrations in treated cultures was comparable to the values detected in negative controls for short-term test with S9-mix, except for the highest concentration tested (4665 µg TOS/mL), where a significant increase of structural chromosomal aberrations was seen. In a confirmatory test, a statistically significant increase was seen only at the second highest concentration (3107 µg TOS/mL). Both these results were accompanied by high cytotoxicity of 60% and 70%, respectively. The short-term treatment without S9-mix gave mixed non-concentration-related results across the nine concentrations tested, both with respect to chromosomal aberrations and cytotoxicity, but none of the chromosomal aberration results were significantly increased over the control. A continuous treatment (24 h without recovery) in the absence of S9-mix was foreseen, but not performed, as the short-term treatment with S9-mix showed positive results.

The Panel concluded that the results on the structural and numerical chromosomal aberrations obtained with the catalase under the test conditions employed in this study were inconclusive due to equivocal results in the experiments with short-term treatment and the lack of extended treatment without S9-mix.

3.4.1.3 | *In vitro mammalian cell micronucleus test*

The *in vitro* mammalian cell micronucleus test was carried out according to the OECD Test Guideline 487 (OECD, 2016a) and following GLP.[22] Two separate experiments were performed with duplicate cultures of human lymphoblastoid TK6 cell line. The cell cultures were treated with the food enzyme with or without metabolic activation (S9-mix).

In a range finding test, cytotoxicity of 50% or higher (cell growth inhibition rate) was seen at ≥ 39.1 µg TOS/mL in the short-term treatment (4 h exposure and 20 h recovery period) with metabolic activation (S9-mix), at ≥ 156 µg TOS/mL in the short-term treatment without S9-mix, and at ≥ 19.5 µg TOS/mL in the long-term treatment (24 h continuous exposure); precipitation was observed at ≥ 313 µg TOS/mL under all treatment conditions. Based on the results from the dose range finding test, cells were exposed to the food enzyme and scored for the frequency of bi-nucleated cells with micronuclei (MNBN) at concentrations of 60, 70, 80, 90 or 100 µg TOS/mL in a short-term treatment with S9-mix, at 35, 40 and 45 µg TOS/mL in a short-term treatment without S9-mix, and at concentrations of 0.0781, 0.156, 0.313 and 0.625 µg TOS/mL in a long-term treatment (24 h exposure) without S9-mix.

Cytotoxicity was observed in the short-term treatment at ≥ 80 µg TOS/mL with S9-mix (relative population doubling (RPD) = 51%) and at 45 µg TOS/mL without S9-mix (RPD = 47%). In the long-term treatment, cytotoxicity was observed at 0.625 µg TOS/mL with a cytotoxicity of 42%.

The frequency of MNBN was statistically significantly different to the negative controls only at concentrations of 90 and 100 µg TOS/mL tested with S9-mix (1.6% and 2.0% vs. 0.9%) in the short-term treatment; the increased frequency was outside the 95% of the historical control range.

The Panel concluded that the food enzyme catalase did induce an increase in the frequency of MNBNs only in the presence of metabolic activation under the test conditions applied in this study.

3.4.1.4 | *In vivo micronucleus test*

The *in vivo* mammalian erythrocyte micronucleus test was carried out in Sprague–Dawley Crl:CD(SD) rats according to the OECD Test Guideline 474 (OECD, 1997c) and following GLP.[23]

The food enzyme was tested for its ability to induce micronuclei in the polychromatic erythrocytes (PCEs) of the bone marrow of treated rats to either reject or confirm a clastogenic potential of the food enzyme *in vitro*. Based on a range finding study, where no clinical signs of toxicity and no difference in findings between male and female animals were

[21]Technical dossier 2nd Submission/p. 10, Annex 9.

[22]Technical dossier/Additional data March 2023/Annex 6–1.

[23]Technical dossier 2nd submission/p. 9, Annex 10.

observed, the enzyme concentrate was administered by gavage in the main assay for two consecutive days at 500, 1000 and 2000 mg/kg body weight (bw) per day (corresponding to 467 to 1866 mg TOS/kg bw per day) to groups of five male rats. No treatment-related clinical signs were noted in any animal treated with the food enzyme.

There were no significant differences in bw between treated and control animals at study termination.

Rats treated with the food enzyme exhibited %PCE values and mean frequencies of micronucleated polychromatic erythrocytes (MNPCE) that were similar to and not statistically different from those seen in concurrent vehicle controls for all dose groups. The Panel considered the study as inconclusive, because no data on bone marrow exposure were provided and the highest dose administered was not a maximum tolerated dose (MTD) or achievable dose.

3.4.1.5 | *In vivo comet assay*

An *in vivo* Comet assay in liver and glandular stomach of rats was performed as a follow-up to the increased frequency of MNBNs observed *in vitro* in the short-term treatment with S9-mix. The study was conducted in accordance with the OECD Test Guideline 489 (OECD, 2016b) and following GLP.[24]

In the study, five male Sprague–Dawley rats (Crl:CD(SD)) were dosed once daily for two consecutive days by oral gavage with 500, 1000 and 2000 mg/kg bw per day of test item, corresponding to 145, 290 and 580 mg TOS/kg bw per day. Five rats of the positive control group were dosed once daily with 200 mg ethyl methane sulfonate (EMS)/kg bw per day for two consecutive days.

No mortality, treatment-related clinical signs or changes in bw were observed in any animal group.

No statistically significant increase in mean tail intensity values (% DNA in tail) for animals treated with the food enzyme were observed in liver and glandular stomach of any treated group compared to the concurrent vehicle control group. The positive control (EMS) showed a statistically significant increase in the mean tail intensity in liver and glandular stomach, within the 95% control limit of the distribution of the historical positive control database.

The Panel considered the results on liver as inconclusive, because the exposure was not demonstrated and the highest dose administered was not a MTD or achievable dose.

Conclusions on genotoxicity:

The food enzyme catalase was tested in a battery of *in vitro* and *in vivo* genotoxicity studies. The test item in the presence or absence of S9 mix did not induce gene mutations in bacteria (four strains of *S.* Typhimurium, TA1535, TA1537, TA98 and TA100 and one strain of *E. coli*, WP2 *uvrA*). An *in vitro* mammalian chromosomal aberration study, carried out in Chinese hamster lung fibroblasts, provided an indication of increased structural chromosomal aberrations associated with high cell toxicity only in the presence of metabolic activation. An *in vitro* micronucleus test, carried out in human lymphoblastoid TK6 cell line, showed a statistically significant increase of micronucleated cells at the highest concentrations tested in the presence of metabolic activation, confirming the results of the chromosomal aberration study. Negative results were obtained with an *in vivo* mammalian erythrocyte micronucleus test, which were considered inconclusive owing to the absence of data on bone marrow exposure. The negative results obtained with the comet assay in liver did not allow the concern for genotoxicity observed *in vitro* in the presence of metabolic activation to be excluded, because exposure was not demonstrated and the highest dose administered was not a MTD or achievable dose.

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

The repeated dose 90-day oral toxicity study was performed following GLP and in accordance with the guidelines of the Japanese Ministry of Health and Welfare (1996 and 1999).[25] The study is in accordance with the OECD Test Guideline 408 (OECD, 1998) with the following deviation: urea was not determined. The Panel considered that this deviation is minor and does not impact on the evaluation of the study.

Groups of 12 male and 12 female Sprague–Dawley (Crl:CD(SD)) rats received by gavage the food enzyme in doses of 250, 500 or 1000 mg/kg bw per day, corresponding to 81, 162 or 323 mg TOS/kg bw per day. Controls received the vehicle (water for injection).

No mortality was observed.

The feed consumption was statistically significantly increased on day 14 of administration in high-dose males (+4%) and on day 56 in high-dose females (+17%). The Panel considered the changes as not toxicologically relevant, as they were only recorded sporadically, there was no statistically significant change in the final feed consumption and there were no statistically significant changes in the bw or the bw gain.

The haematological investigation revealed a statistically significant increase in monocyte count in high-dose females (+86%). The Panel considered the change as not toxicologically relevant, as it was only observed in one sex, the change was small, there were no changes in other relevant parameters (total leukocyte count) and the value was within the historical control data from the laboratory.

[24]Technical dossier/Additional data March 2023/ Annex 6–2.

[25]Technical dossier/Additional data March 2023/Annexes 5–1, 5–2, 5–3 and 5–4.

The clinical chemistry investigation revealed a statistically significant decrease in alanine aminotransferase activity (ALT) in high-dose males (–21%) and in blood urea nitrogen (BUN) in low- and high-dose males (–12% in both cases). The Panel considered the changes as not toxicologically relevant, as they were only observed in one sex (both), there was no dose–response relationship (both), the changes were small (both), and there were no histopathological changes in the liver or the kidneys.

The urinalysis revealed a statistically significant decrease in urinary sodium (U-Na) and urinary chloride (U-Cl) in high-dose males (–23% and –20%, respectively) and females (–31% and –26%, respectively). The Panel considered the changes as not toxicologically relevant, as there were no changes in other relevant parameters (plasmatic Na and Cl) and there were no histopathological changes in the kidneys.

Statistically significant changes in organ weights detected were increases in the absolute weights of salivary glands in low- and high-dose females (+8% and +9%, respectively), and in the liver, the spleen, kidneys and adrenals in high-dose females (+11%, +19%, +12% and +11%, respectively). The Panel considered the changes as not toxicologically relevant, as they were only observed in one sex (all), there was no dose–response relationship (salivary glands) and there were no histopathological changes in the salivary glands, liver, spleen, kidneys or adrenals.

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

The Panel identified a no observed adverse effect level (NOAEL) of 323 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 that may be used in the final formulation.

The potential allergenicity of the catalase produced with *A. tubingensis* strain AE-CN 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. The matching allergen was Pen c 30.0101, a catalase from *Penicillium citrinum*.[26] It is a respiratory allergen.

In addition, catalase from the fungus *Metarhizium anisopliae* has been found to react with IgE in sera from asthmatic patients (Ward et al., 2009). However, several studies have shown that adults sensitised to an enzyme through the respiratory tract can commonly ingest the corresponding respiratory allergens without acquiring clinical symptoms of food allergy (Armentia et al., 2009; Brisman, 2002; Cullinan et al., 1997; Poulsen, 2004).

No information was available on oral and respiratory sensitisation or elicitation reactions of this catalase.

[redacted], a product that may cause allergies or intolerances (Regulation (EU) No 1169/2011[27]), is used as a raw material. In addition, [redacted], a 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 no potentially allergenic residues from these sources are present in the food enzyme.

The Panel considered that the risk of allergic reactions upon dietary exposure to this food enzyme cannot be excluded, 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 five food manufacturing processes at the recommended use levels summarised in Table 2.

In the first three food manufacturing processes shown in Table 2, the raw materials are treated firstly with glucose oxidase. In baking processes, the food enzyme is added to flour together with glucose oxidase.[28] In egg processing, whole egg, egg yolk or egg white are treated with glucose oxidase.[29] In fruit and vegetable processing, glucose oxidase is added to fruit or vegetable concentrate or extracts during fermentation.[30] The action of glucose oxidase generates hydrogen

[26]Technical dossier/Additional data March 2023/Annex 4.

[27]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.

[28]Technical dossier/p. 61.

[29]Technical dossier/p. 63.

[30]Technical dossier/p. 64.

TABLE 2 Intended uses and recommended use levels of the food enzyme as provided by the applicant.[c]

Food manufacturing process[a]	Raw material (RM)	Recommended use level (mg TOS/kg RM)[b]
Production of baked products	Flour	1–**10**
Processing of egg and egg products	Liquid egg, egg yolk, egg white	0.004–**0.03**
Production of fruit and vegetable products other than juices	Fruit and vegetable	1.4–**14**
	Fruit peels slurry	0.3
Production of cheese	Milk	0.0003–**0.001**
Production of fish roes	Fish roes	14–**1413**

Abbreviation: TOS, total organic solids.

[a]The name has been harmonised by EFSA according to the 'Food manufacturing processes and technical data used in the exposure assessment of food enzymes' (EFSA CEP Panel et al., 2023).

[b]The numbers in bold were used for calculation.

[c]Technical dossier/p. 67, Additional data March 2023/Answers 12, 13, 14, 15.

TABLE 3 Summary of the estimated dietary exposure to food enzyme–TOS in six population groups.

	Estimated exposure (mg TOS/kg body weight per day)					
Population group	**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)	0.009–0.186 (12)	0.024–0.134 (15)	0.030–0.139 (19)	0.013–0.075 (21)	0.011–0.040 (22)	0.011–0.046 (23)
Min–max 95th percentile (number of surveys)	0.039–0.325 (11)	0.071–0.321 (14)	0.069–0.277 (19)	0.029–0.160 (20)	0.024–0.093 (22)	0.021–0.093 (22)

Abbreviation: TOS, total organic solids.

peroxide, which is subsequently removed by the catalase. The food enzyme–TOS remains in the final foods (e.g. baked foods, processed egg powder, ready-to-use fruit or vegetable concentrates).

In the production of fruit peel fibres, cheeses and fish roes, the raw materials (fruit peel slurry,[31] milk[32] or fish roes[31]) are first treated with hydrogen peroxide to remove potential microbial contaminations. Catalase is then added to remove excessive hydrogen peroxide. The food enzyme–TOS remains in the fruit fibres and cheeses. In fish processing, fish roe is firstly treated with hydrogen peroxide, washed with salt water, then treated with catalase and washed again with salt water before canning. The washing step is expected to remove the food enzyme–TOS from the processed fish roe. However, in the absence of analytical data[33], the Panel was unable to establish the extent of removal. Consequently, a scenario of not considering any removal was taken to estimate the dietary exposure.

Based on data provided on thermostability (see Section 3.3.1), the catalase may remain active in cheeses, depending on the pasteurisation conditions, but it is expected to be inactivated in the other foods.

3.5.2 | Dietary exposure estimation

Chronic exposure to the food enzyme–TOS was calculated by combining the maximum recommended use level with individual consumption data (EFSA CEP Panel et al., 2021). The estimation involved selection of relevant food categories and application of technical conversion factors (EFSA CEP Panel et al., 2023). 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 48 dietary surveys (covering infants, toddlers, children, adolescents, adults and the elderly), carried out in 26 European countries (Appendix B). The highest dietary exposure was estimated to be 0.325 mg TOS/kg bw per day in infants at the 95th percentile.

[31]Additional data March 2023/Answer 15.

[32]Technical dossier/p. 62.

[33]Additional data March 2023/Answer 13.

TABLE 4 Qualitative evaluation of the influence of uncertainties on the dietary exposure estimate

Sources of uncertainties	Direction of impact
Model input data	
Consumption data: different methodologies/representativeness/underreporting/misreporting/no portion size standard	+/–
Use of data from food consumption surveys of a few days to estimate long-term (chronic) exposure for high percentiles (95th percentile)	+
Possible national differences in categorisation and classification of food	+/–
Model assumptions and factors	
Exposure to food enzyme–TOS was always calculated based on the recommended maximum use level	+
Selection of broad FoodEx categories for the exposure assessment	+
The calculation considered 100% remaining of the food enzyme–TOS in fish roes	+
Use of recipe fractions in disaggregation FoodEx categories	+/–
Use of technical factors in the exposure model	+/–

Abbreviations: +, uncertainty with potential to cause overestimation of exposure; –, uncertainty with potential to cause underestimation of exposure.

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.

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.

3.6 | Margin of exposure

The Panel considered that because of the uncertainty over genotoxicity the calculation of a margin of exposure was not appropriate.

4 | CONCLUSIONS

As the available set of genotoxicity tests was not sufficient to dismiss the indications that the food enzyme may have a clastogenic effect, and because the absence of viable cells from the production strain was not demonstrated, the Panel was not able to establish the safety of the food enzyme catalase produced with the non-genetically modified *A. tubingensis* strain AE-CN.

5 | DOCUMENTATION AS PROVIDED TO EFSA

Application for authorisation of Catalase from *Aspergillus niger* AE-CN in accordance with Regulation (EC) No 1331/2008. February 2015. Submitted by Amano Enzyme Inc.

Additional information. October 2021 and March 2023. Submitted by Amano Enzyme Inc.

ABBREVIATIONS

bw body weight
CAS Chemical Abstracts Service
CEP EFSA Panel on Food Contact Materials, Enzymes and Processing Aids
EINECS European Inventory of Existing Commercial Chemical Substances
FAO Food and Agricultural 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
kDa kilodalton
LoD limit of detection
LOQ limit of quantification
MoE margin of exposure

OECD Organisation for Economic Co-operation and Development
PCR polymerase chain reaction
TOS total organic solids
WHO World Health Organization

CONFLICT OF INTEREST

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

REQUESTOR

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PANEL MEMBERS

José Manuel Barat Baviera, Claudia Bolognesi, Andrew Chesson, Pier Sandro Cocconcelli, Riccardo Crebelli, David Michael Gott, Konrad Grob, Claude Lambré, Evgenia Lampi, Marcel Mengelers, Alicja Mortensen, Gilles Rivière, Inger-Lise Steffensen, Christina Tlustos, Henk Van Loveren, Laurence Vernis, Holger Zorn

NOTE

The full opinion will be published in accordance with Article 12 of Regulation (EC) No 1331/2008 once the decision on confidentiality will be received from the European Commission.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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APPENDIX A

Dietary exposure estimates to the food enzyme–TOS in details

Appendix A can be found in the online version of this output (in the 'Supporting information' section). The file contains two sheets, corresponding to two tables.

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

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

APPENDIX B

Population groups considered for the exposure assessment

Population	Age range	Countries with food consumption surveys covering more than 1 day
Infants	From 12 weeks on up to and including 11 months of age	Bulgaria, Cyprus, Denmark, Estonia, Finland, France, Germany, Italy, Latvia, Portugal, Slovenia, Spain
Toddlers	From 12 months up to and including 35 months of age	Belgium, Bulgaria, Cyprus, Denmark, Estonia, Finland, France, Germany, Hungary, Italy, Latvia, the Netherlands, Portugal, Republic of North Macedonia[a], Serbia[a], 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, the Netherlands, Portugal, Republic of North Macedonia[a], Serbia[a], Spain, Sweden
Adolescents	From 10 years up to and including 17 years of age	Austria, Belgium, Bosnia and Herzegovina[a], Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Italy, Latvia, Montenegro[a], the Netherlands, Portugal, Romania, Serbia[a], Slovenia, Spain, Sweden
Adults	From 18 years up to and including 64 years of age	Austria, Belgium, Bosnia and Herzegovina[a], Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Montenegro[a], the Netherlands, Portugal, Romania, Serbia[a], Slovenia, Spain, Sweden
The elderly[b]	From 65 years of age and older	Austria, Belgium, Cyprus, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Montenegro[a], the Netherlands, Portugal, Romania, Serbia[a], Slovenia, Spain, Sweden

[a]Consumption data from these pre-accession countries are included for testing purpose.

[b]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).