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Safety evaluation of the food enzyme glucose oxidase from the genetically modified *Aspergillus niger* strain DP-Aze23

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Abstract

The food enzyme glucose oxidase (β-D-glucose:oxygen 1-oxidoreductase; EC 1.1.3.4) is produced with the genetically modified *Aspergillus niger* strain DP-Aze23 by Danisco US, Inc. The genetic modifications do not give rise to safety concerns. The food enzyme is considered free from viable cells of the production organism and its DNA. It is intended to be used in baking processes, cereal-based processes and egg processing. Based on the maximum use levels, dietary exposure to the food enzyme-total organic solids (TOS) was estimated to be up to 0.05 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 19.55 mg TOS/kg bw per day, the highest dose tested, which, when compared with the estimated dietary exposure, results in a margin of exposure above 380. A search for similarity of the amino acid sequence of the food enzyme to known allergens was made and one match was found. The Panel considered that, under the intended conditions of use, the risk of allergic sensitisation and elicitation reactions by dietary exposure cannot be excluded, but the likelihood for this to occur is considered to be low. Based on the data provided, the Panel concluded that this food enzyme does not give rise to safety concerns, under the intended conditions of use.

Keywords: food enzyme, glucose oxidase, β-D-glucose:oxygen 1-oxidoreductase, EC 1.1.3.4, D-glucose oxidase, *Aspergillus niger*, genetically modified microorganism

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1. Introduction

Article 3 of the Regulation (EC) No 1332/2008[1] provides definitions 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 European Union 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 company "Danisco US Inc." for the authorisation of the food enzymes Beta-galactosidase from a genetically modified strain of *Aspergillus oryzae* (DP-Bzg59), Alpha, alpha trehalase from a genetically modified strain of *Trichoderma reesei* (DP-Nzs51), Alpha-amylase from a genetically modified strain of *Bacillus licheniformis* (DP-Dzb45), Glucose oxidase from a genetically modified strain of *Aspergillus niger* (DP-Aze23), and Alpha-amylase from *Geobacillus stearothermophilus* (DP-Gzb47).

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 assessment on the food enzymes Beta-galactosidase from a genetically modified strain of *Aspergillus*

[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, p. 7–15.

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

[3] Commission Regulation (EU) No 234/2011 of 10 March 2011 implementing Regulation (EC) No 1331/2008 of the European Parliament and of the Council establishing a common authorisation procedure for food additives, food enzymes and food flavourings. OJ L 64, 11.03.2011, p. 15–24.

oryzae (DP-Bzg59), Alpha, alpha trehalase from a genetically modified strain of *Trichoderma reesei* (DP-Nzs51), Alpha-amylase from a genetically modified strain of *Bacillus licheniformis* (DP-Dzb45), Glucose oxidase from a genetically modified strain of *Aspergillus niger* (DP-Aze23), and Alpha-amylase from *Geobacillus stearothermophilus* (DP-Gzb47) 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 glucose oxidase from genetically modified *Aspergillus niger* (strain DP-Aze23).

2. Data and methodologies

2.1. Data

The applicant has submitted a dossier in support of the application for authorisation of the food enzyme glucose oxidase from a genetically modified *Aspergillus niger* strain DP-Aze23.

Additional information was requested from the applicant during the assessment process on 1 December 2020 and was received on 24 August 2021 (see 'Documentation provided to EFSA').

2.2. Methodologies

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

The current '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 to the updated 'Scientific Guidance for the submission of dossiers on food enzymes' (EFSA CEP Panel, 2021a).

3. Assessment

IUBMB nomenclature:	Glucose oxidase
Systematic name:	β-D-glucose:oxygen 1-oxidoreductase
Synonyms:	Glucose oxyhydrase; β-D-glucose oxidase; D-glucose oxidase
IUBMB No:	EC 1.1.3.4
CAS No:	9001-37-0
EINECS No:	232-601-0

Glucose oxidases catalyse the oxidation of glucose to D-glucono-1,5-lactone (glucono δ-lactone), thereby reducing molecular oxygen to hydrogen peroxide. The enzyme is intended to be used in baking processes, cereal-based processes and egg processing.

3.1. Source of the food enzyme

The glucose oxidase is produced with *A. niger* strain DP-Aze23 ([redacted])[4] which is deposited in the Westerdijk Fungal Biodiversity Institute (CBS) with the deposit number [redacted].[5] The production strain was identified as *A. niger* using a [redacted].[6]

3.1.1. Characteristics of the parental and recipient microorganisms

The parental microorganism is the filamentous fungus [redacted] *A. niger*, [redacted].

[4] Technical dossier/ Additional data August 2021/Annex AE.
[5] Technical dossier/ Additional data August 2021/Annex AF.
[6] Technical dossier/Additional data August 2021/Annex AG.

The recipient strain [redacted][7]

3.1.2. Characteristics of the introduced sequences

[redacted][8][9][10]

3.1.3. Description of the genetic modification process

The purpose of the genetic modification was to enable the production strain to synthesise glucose oxidase from [redacted] in the production strain *A. niger* DP-Aze23.[10]

3.1.4. Safety aspects of the genetic modification

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

The production strain *A. niger* DP-Aze23 differs from the recipient strain [redacted][11][9]

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

3.2. Production of the food enzyme

The food enzyme is manufactured according to the Food Hygiene Regulation (EC) No 852/2004, with food safety procedures based on hazard analysis and critical control points, and in accordance with current good manufacturing practice.[12]

The production strain is grown as a pure culture using a typical industrial medium in a submerged, batch or fed-batch fermentation system with conventional process controls in place. After completion of the fermentation and release of the intracellular enzyme, the solid biomass is removed from the fermentation broth by filtration, leaving a filtrate 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 mass material passes the filtration membrane and

[7] Technical dossier/Confidential/Annex W.
[8] Technical dossier/Additional data August 2021/Annex AJ.
[9] Technical dossier/Confidential/Annex V_updated.
[10] Technical dossier/Confidential/Annex V_updated and Technical dossier/ Additional data August 2021/Annex AJ.
[11] Technical dossier/Confidential/ Annexes V_updated and AB.
[12] Technical dossier/p. 51 and Annex K.

is discarded.[13] 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.[14]

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 glucose oxidase is a single polypeptide chain of [redacted] amino acids. The molecular mass of the mature protein, calculated from the amino acid sequence, was [redacted] kDa.[15] The food enzyme was analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A consistent protein pattern was observed across all batches. The gels showed a single major protein band corresponding to an apparent molecular mass of about [redacted] kDa,[16] consistent with the expected mass of the enzyme. No other enzymatic activities were reported.[17]

The in-house determination of the enzyme activity is based on the oxidation of glucose to gluconic acid (reaction conditions: pH 5.1, 35°C, 15 min). The amount of gluconic acid produced in an aerated, buffered glucose solution is quantified by back titration. The enzyme activity is expressed in glucose oxidase units/g (GOU/g). One unit of glucose oxidase is defined as the amount of enzyme that oxidises 3.0 mg of glucose to gluconic acid under the conditions of the assay.[18]

The food enzyme has a temperature optimum around 40°C (pH 6.0) and a pH optimum around pH 6.0 (35°C).[19] Thermostability was tested after incubation of the food enzyme for 50 min at 70°C (pH 6.0). The enzyme activity decreased by 80% after 20 min of incubation. No enzyme activity was detected at temperatures above 70°C after 50 min of incubation.[20]

3.3.2. Chemical parameters

Data on the chemical parameters of the food enzyme were provided for three batches used for commercialisation and two batches produced for the toxicological tests (Table 1). The mean total organic solids (TOS) of the three food enzyme batches for commercialisation is 11.4% and the mean enzyme activity/TOS ratio is 58.8 GOU/mg TOS.[21]

Table 1: Compositional data of the food enzyme

Parameters	Unit	Batches				
		1	2	3	4[(a)]	5[(b)]
Glucose oxidase activity	GOU/g batch[(c)]	4,998	9,769	5,733	2,423	10,199
Protein	%	4.97	9.09	5.24	3.62	9.87
Ash	%	0.23	0.22	0.28	0.37	–
Water	%	90.90	85.62	88.46	93.12	–
Total organic solids (TOS)[(d)]	%	8.87	14.16	11.26	6.51	14.6
Activity/mg TOS	GOU/mg TOS	56.4	69.0	50.9	37.2	72.0

[(a)]: Batch used for Ames test 1 and 2; *in vivo* micronucleus test and repeated dose 90-day oral toxicity study.
[(b)]: Batch used for Ames test 3 and *in vivo* alkaline Comet assay.
[(c)]: GOU: glucose oxidase units (see Section 3.3.1).
[(d)]: TOS calculated as 100% – % water – % ash.

[13] Technical dossier/p. 51-57 and Annex L.
[14] Technical dossier/Additional data August 2021/Annex AM.
[15] Technical dossier/Additional data August 2021/Annex 1.
[16] Technical dossier/p. 39.
[17] Technical dossier/p. 42.
[18] Technical dossier/Annex D.
[19] Technical dossier/p. 43 and Annex I.
[20] Technical dossier/p. 44 and Annex I.
[21] Technical dossier/Annex S and Additional data August 2021/Annex AN, Annex AP.

3.3.3. Purity

The lead content in the three commercial batches and in both batches used for toxicological studies[22] 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).[23] In addition, arsenic, mercury and cadmium in the batches used for toxicological testing[24] were below the limits of detection (LoD) of the employed methodologies.[25]

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).[24] No antimicrobial activity was detected in any of these batches (FAO/WHO, 2006).[24]

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, ochratoxin A, sterigmatocystin, fumonisin and zearalenone was examined in the three food enzyme batches[26] used for commercialisation and all were below the limit of detection of the applied methods.[27] Adverse effects caused by the potential presence of other secondary metabolites are addressed by the toxicological examination of the food enzyme TOS.

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

3.3.4. Viable cells and DNA of the production strain

The absence of the production strain in the food enzyme was demonstrated in three independent batches analysed in triplicate. [redacted] No growth was observed.[28]

The absence of recombinant DNA in the food enzyme was demonstrated by PCR analysis of three batches in triplicate. [redacted][29]

3.4. Toxicological data

Two batches were used for the toxicological studies, batch 4 and 5 (Table 1). Both were considered as suitable test items, as both had an activity:TOS ratio similar to the commercial batches.

3.4.1. Genotoxicity

3.4.1.1. Bacterial reverse mutation test

Three bacterial reverse mutation studies (Ames test) were performed according to the Organisation for Economic Co-operation and Development (OECD) Test Guideline 471 (OECD, 1997a) and following good laboratory practice (GLP)[30],. Selective agar plates containing glucose were used in the first study. The second study was performed with selective plates replacing glucose with fructose, since glucose is the substrate of the food enzyme. In the first two studies, five strains of *Salmonella* Typhimurium (TA98, TA100, TA102, TA1535 and TA1537) were used in the presence or absence of metabolic activation (S9-mix), applying the 'treat and plate' assay. In the third study, *Escherichia coli* WP2uvrA (pKM101) substituted for TA102.

In the first study, a preliminary test was performed on strain TA98 with and without S9-mix with five concentrations of the food enzyme (batch 4) 50, 160, 500, 1,600 and 5,000 μg of total protein/plate (corresponding to 90, 288, 899, 2,877 and 8,992 μg TOS/plate). The preliminary test showed severe toxicity (measured as the presence of microcolonies) in the treatment without S9-mix, from 899 μg TOS/plate. In the treatment with S9-mix, severe toxicity was observed at all concentrations except for the lowest tested (90 μg TOS/plate). Two separate main experiments were carried out. Nine concentrations from 0.5 to 5,000 μg protein/plate, (0.9–8,992 μg TOS/plate) in experiment 1 and six

[22] LoD: Pb = 5 mg/kg.
[23] Technical dossier/Annex S and Additional data August 2021/Annex AN.
[24] LoDs: As = 3 mg/kg, Hg and Cd = 0.5 mg/kg.
[25] Technical dossier/Annex S.
[26] Technical dossier/ Additional data August 2021/Annex 1, Annex AN and Annex AO.
[27] LoDs: sterigmatocystin and fumonisin = 100 μg/kg each; ochratoxin A = 10 μg/kg; aflatoxin = 5 μg/kg; zearalenone = 25 μg/kg.
[28] Technical dossier/Additional data August 2021/Annex AK.
[29] Technical dossier/Additional data August 2021/Annex AL.
[30] Technical dossier/Annexes O and P.

concentrations from 1.6 to 500 μg protein/plate (2.88–899 μg TOS/plate) in experiment 2 were tested in strains TA102, TA100 and TA1535. Six concentrations from 0.5 to 160 μg protein/plate (0.9–288 μg TOS/plate) were tested in strains TA98 and TA1537. Toxic effects, evident as formation of microcolonies, occurred in all strains at the highest concentrations tested in both experiments, with and without S9-mix. Upon treatment with the food enzyme, an increase of the revertant colony numbers twice above the control values was observed in a single experiment in strain TA1535 in the presence of S9-mix at 500 μg protein/plate (3.17-fold in the presence of toxicity) and in strain TA98 in the absence of S9-mix at 50 μg protein/plate (2.12-fold). These findings were not reproduced in a second experiment. No increase of revertants was reported in any other strain and condition of treatment.

In the preliminary test of the second Ames study, toxicity (measured as reduced growth of bacterial background lawn) was observed in the treatment without S9-mix, above 500 μg protein/plate. In the treatment with S9-mix, toxicity was observed only at the highest concentration tested. Two separate main experiments were carried out using five different concentrations of batch 4 of the food enzyme (from 50 to 5,000 μg protein/plate, corresponding to 90, 288, 899, 2,877 and 8,992 μg TOS/plate). Toxicity occurred in most strains at the higher concentrations tested. Upon treatment with the food enzyme, there was no increase in revertants more than twice the control values in any strain with or without S9-mix.

In the third Ames test, batch 5 was used (Table 1) with four strains of *Salmonella* Typhimurium (TA98, TA100, TA1535 and TA1537) and *Escherichia coli* WP2uvrA(pKM101) in the presence or absence of metabolic activation, applying the 'treat and plate' assay.[31] In a preliminary toxicity test carried out with seven concentrations of food enzyme from 50 to 5,000 μg TOS/plate, no cytotoxicity was observed. Two separate experiments were carried out in triplicate at 50, 158, 500, 1,581 and 5,000 μg TOS/plate. No cytotoxicity was observed at any concentration tested. Upon treatment with the food enzyme, there was no significant increase in revertant colony numbers above the control values in any strain with or without S9-mix.

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

3.4.1.2. In vitro mammalian chromosomal aberration test

The *in vitro* mammalian chromosomal aberration test was carried out in human peripheral blood lymphocytes according to OECD Test Guideline 473 (OECD, 1997b) and following GLP.[32]As the test item oxidises glucose-producing hydrogen peroxide, all the treatments were performed in the presence and absence of catalase.

Two separate experiments were performed in duplicate. In the first experiment, cells were exposed in a short-term treatment (3 h followed by 17-h recovery period) to the food enzyme with and without metabolic activation (S9-mix) in the absence of catalase at concentrations of 156, 313, 625, 1,250, 2,500 and 5,000 μg total protein/mL (corresponding to 280, 563, 1,124, 2,248, 4,496 and 8,992 μg TOS/mL) and at 78.1, 156, 313, 625 and 1,250 μg total protein/mL (corresponding to 140.5, 280, 563, 1,124, 2,248, μg TOS/mL) in the presence of catalase. In the second experiment, cells were exposed in a short-term treatment with S9-mix in the presence and absence of catalase at 78.1, 156, 313, 625 and 1,250 μg protein/mL (corresponding to 140.5, 280, 563, 1,124, 2,248 μg TOS/mL). In a continuous treatment (20 h) without S9-mix in the presence and absence of catalase cells were treated with 19.5, 39.1, 78.1, 156, 313, 625 μg protein/mL (corresponding to 35.1, 70, 140.5, 280, 563, 1,124 μg TOS/mL). In the first experiment, severe cytotoxic effects were observed above 625 μg protein/mL in all treated samples (100% mitotic inhibition), whereas at 625 μg protein/mL, mitotic inhibition was 55% and 62% without S9-mix, and 53% and 61% with S9-mix in the absence and presence of catalase, respectively. In the second experiment, for treatment in the presence of S9-mix at 625 μg protein/mL the mitotic inhibition in the absence and presence of catalase was 53% and 59%, respectively. For treatment without S9-mix, severe toxicity was observed from 313 μg protein/mL (100% mitotic inhibition) and at 156 μg protein/mL in the absence and presence of catalase (mitotic inhibition 66% and 82%, respectively). A statistically significant increase of metaphases with chromosomal aberrations was observed at the highest concentrations analysed (625 and 313 μg protein/mL) after a short-term treatment with and without metabolic activation in the presence or absence of catalase without a clear dose-response effect. In the second experiment, the frequency of

[31] Technical dossier/Additional data August 2021/Annex AP.
[32] Technical dossier/Annex Q.

structural chromosomal aberration was statistically significant increased after a short-term treatment at 625 μg protein/mL in the presence of S9-mix and of catalase.

The Panel concluded that the food enzyme induced chromosome aberrations under the test conditions employed for this study.

Following a request by the WG, the applicant provided a re-evaluation of the positive results obtained with the chromosomal aberration test conducted with a specifically prepared buffered solution containing either active or inactive glucose oxidase at different concentrations and sodium chloride: active glucose oxidase (protein concentrations 18.24 mg/mL and 30.94 mg/mL), inactive glucose oxidase (protein concentration 18.13 mg/mL).[33] The three solutions were tested on chromosomal aberration in human peripheral blood lymphocytes up to 5,000 μg/mL for 4 h (followed by 18 h recovery) in the presence and absence of S9-mix and for 22 h continuous treatment in the absence of S9-mix. High toxicity was reported with a reduction of about 80% of the mitotic index at 625 μg/mL after a short-term treatment and at 313 μg/mL after the continuous treatment. Haemolysis was also observed starting at 50 μg/mL after 22 h continuous treatment. An *in vitro* micronucleus test was also conducted by exposing cultures of CHO cells to the test substance for 4 h in the presence and absence of S9-mix. At the time of harvesting (22 h after the treatment), precipitation was observed starting at 1,004 μg/mL. The Panel concluded that the test item is not compatible for *in vitro* testing in either the chromosomal aberration or micronucleus assay.

3.4.1.3. *In vivo* mammalian erythrocyte micronucleus test

The *in vivo* mammalian erythrocyte micronucleus test in mice was carried out according to the OECD Test Guideline 474 (OECD, 1997c) and following GLP.[34] A preliminary toxicity assay was performed on two males and two females SPF BomTac:BRI mice that were dosed by oral gavage with the test item (batch 4, Table 1) at the maximum possible dose, corresponding to 725 mg food enzyme/kg body weight (bw). No toxic effects were detected. In the main experiment, five mice per group (males) were treated with two oral administrations (intragastric gavage) of the food enzyme at doses of 181, 362 and 725 mg protein/kg bw, corresponding to 325.5, 651 and 1,304 mg TOS/kg bw. The two dosages of test item were separated by 24 h. Mice were sacrificed 24 h after the last dosing. No mortalities and clinical signs of toxicity were reported after treatment with the test item. The ratio of polychromatic erythrocytes (PCE) to normochromatic erythrocytes (NCE) was assessed by examination of at least 1,000 erythrocytes per animal. For each animal, 2,000 PCE were scored for the presence of micronuclei (micronucleated polychromatic erythrocytes (MNPCE)). The incidence of normochromatic erythrocytes with micronuclei (MNNCE) was also noted. No statistically significant increases in the frequency of MNPCE and no substantial decrease in the proportion of immature erythrocytes were observed in animals treated with the food enzyme, compared with control values.

The Panel concluded that the food enzyme did not induce micronuclei in bone marrow when tested up to the maximum possible dose (725 mg protein/kg bw, corresponding to 1,304 mg TOS/kg bw) under the experimental conditions employed.

3.4.1.4. *In vivo* mammalian alkaline Comet assay

The *in vivo* mammalian alkaline Comet assay was carried out on liver and duodenum of Wistar Han rats according to the OECD Test Guideline 489 (OECD, 2016) and following GLP.[35] A preliminary toxicity assay was performed on three males and three females that were treated for two consecutive days by oral gavage at 2,000 mg/kg bw of the test item (batch 5, Table 1), corresponding to 283.2 mg TOS/kg bw. No toxic effects were detected. Because there were no substantial differences in toxicity between sexes, only male animals were used in the main study. In the main study, male animals were dosed with the test item at 500, 1,000 and 2,000 mg/kg bw (corresponding to 70.8, 141.6 and 283.2 mg TOS/kg bw) for two consecutive days. No clinical signs of toxicity or changes in clinical chemistry parameters were observed in any of the animals. There were no test item-related microscopic findings in the liver or duodenum from the treated animals. No statistically significant increase in the mean % tail DNA was observed in liver cells of test item-treated animals compared to the control animals. A statistically significant increase in the mean % tail DNA (1.7-fold with respect to the mean value in controls) was reported only in the 2,000 mg/kg test item-treated group for duodenum cells compared to the vehicle-treated. This increase at the limit dose was attributed, by the

[33] Technical dossier/Additional data August 2021/Annex AQ.
[34] Technical dossier/Annex Z.
[35] Technical dossier/Additional data August 2021/Annex AR.

study authors, to the presence of hydrogen peroxide, which is formed from the catalytic activity of glucose oxidase on glucose. The Panel considered this explanation plausible.

3.4.1.5. Conclusions on genotoxicity

Two batches of the food enzyme were tested using a bacterial gene mutation assay up to over 8,000 μg TOS/plate in the presence and absence of metabolic activation with negative results. An *in vitro* study with a mammalian chromosomal aberration test in human peripheral blood lymphocytes, at the limit concentration of food enzyme, reported severe toxicity, even in the presence of catalase and an increase of structural chromosomal aberrations at the highest concentrations analysed. Further studies conducted with different enzyme solutions reported severe toxicity and led to the conclusion that the food enzyme is incompatible with *in vitro* testing in either the chromosomal aberration or the micronucleus assay.

An *in vivo* mammalian erythrocyte micronucleus test carried out at the maximum feasible dose of food enzyme reported negative results. An *in vivo* Comet assay carried out up to the limit dose reported negative results in liver and a slight increase of DNA strand breaks in duodenum only at the highest dose, which was considered to be due to the production of the hydrogen peroxide.

Considering the results of the Ames tests, the *in vivo* micronucleus test and the *in vivo* Comet assay, the Panel concluded that there is no concern for genotoxicity.

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

The repeated dose 90-day oral toxicity study was performed in accordance with OECD Test Guideline 408 (OECD, 1998) and following GLP.[36] Groups of 10 male and 10 female Sprague-Dawley (Ntac:SD) rats received by gavage the food enzyme (batch 4, Table 1) in three doses corresponding to 3.24, 6.47 and 19.55 mg TOS/kg bw per day. Controls received the vehicle (water).

One high-dose male died on day 29 due to misdosing, confirmed by necropsy findings.

A statistically significant lower feed intake as compared to controls was recorded in week 10 in mid-dose males. This finding was isolated and was considered by the Panel not related to the treatment.

Haematological investigation revealed a statistically significantly higher red blood cell count (RBC), a statistically significantly lower mean cell haemoglobin (MCH) and mean cell volume (MCV) in high-dose males. A statistically significantly higher percentage and absolute number of eosinophils (EOS) were also observed in low-dose males.

Clinical chemistry investigation revealed that the cholesterol concentration was statistically significantly decreased in low-dose females.

Urinalysis revealed a statistically significant lower concentration of potassium and chloride, and the presence of bacteria in low-dose females.

All the changes in haematology, blood chemistry parameters and urinalysis were considered by the Panel as not toxicologically relevant, because the differences were small (RBC, MCH, MCV) or without an apparent dose dependency (EOS, cholesterol, potassium, chloride, presence of bacteria in urine) and only observed in one sex.

No other statistically significant differences to controls were observed.

The Panel identified the no observed adverse effect level (NOAEL) of 19.55 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 glucose oxidase produced with the genetically modified *Aspergillus niger* strain DP-Aze23 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 [redacted]

[36] Technical dossier/Annex R.

[redacted] can induce immunoglobulin E (IgE)- and T-cell-mediated allergic reaction in atopic eczema patients characterised by an impaired skin barrier. Considering that oral allergic reactions are mediated by IgE, elicitation reactions upon dietary exposure to this food enzyme cannot be excluded, but as the yeast that expresses this allergen is a ubiquitous component of the skin microflora, the Panel considers that the likelihood that this will pose a risk is low.

No information is available on oral or respiratory sensitisation and elicitation reactions to the glucose oxidase under evaluation.[37]

According to the information provided,[15] a known source of allergens ([redacted]) is present in the medium fed to the microorganism. However, during the fermentation process, this product will be degraded and utilised by the microorganism 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.

Quantifying the risk for allergenicity is not possible in view of the individual susceptibility to food allergens. Allergenicity can be ruled out only if the proteins are fully removed. However, the food enzyme remains in the egg, cereal-based and baked products.

The Panel considered that, under the intended conditions of use, the risk of allergic sensitisation and elicitation reactions upon dietary exposure to this food enzyme cannot be excluded, but the likelihood of such reactions to occur is 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 processes. Intended uses and the recommended use levels are summarised in Table 2.

Table 2: Intended uses and recommended use levels of the food enzyme as provided by the applicant[(b)]

Food manufacturing process[(a)]	Raw material (RM)	Recommended dosage of the food enzyme (mg TOS/kg RM)
Baking processes	Flour	0.85–3.4
Cereal-based processes	Flour	0.85–3.4
Egg processing	Whole egg, egg white, egg yolk	2.32–7.72

(a): The description provided by the applicant has been harmonised by EFSA according to the 'EC working document describing the food processes in which food enzymes are intended to be used' – not yet published at the time of adoption of this opinion.

(b): Technical dossier/pp. 61-64 and Additional data August 2021/Annex 1.

In both baking and cereal-based processes,[38] the food enzyme is added to the flour during the preparation of dough. The glucose oxidase catalyses the oxidation of β-D-glucose to D-glucono-1,5-lactone (glucono δ-lactone), thereby reducing molecular oxygen to hydrogen peroxide. The latter is considered to be the active agent responsible for the intended function of glucose oxidase in dough preparation. Hydrogen peroxide reinforces the gluten network via oxidation of cysteine residues and the resulting formation of disulfide bonds (Bonet et al., 2006). In addition, hydrogen peroxide has been shown to induce the formation of dityrosine cross links through oxidative coupling of tyrosine residues in gluten proteins (Tilley et al., 2001; Takasaki et al., 2005). Concerning the reactions of hydrogen peroxide in the dough, there is no expectation of oxidation products other than those typically formed during the dough making and baking processes.

In egg processing,[39] the oxidation of glucose by the glucose oxidase avoids Maillard type browning reactions and the development of off-flavours in the production of dried egg. This treatment takes place before the pasteurisation step.

[37] Technical dossier/Additional data August 2021/Annex AS.
[38] Technical dossier/pp. 60-62.
[39] Technical dossier/p. 63.

The food enzyme remains in the dough, whole egg, egg white or egg yolk. Based on data provided on thermostability (see Section 3.3.1), it is expected that the glucose oxidase is inactivated during these food manufacturing processes.

3.5.2. Dietary exposure estimation

The chronic exposure to the food enzyme-TOS was calculated by combining the maximum recommended use level provided by the applicant (see Table 2) with the relevant FoodEx categories (EFSA CEP Panel, 2021b). Exposure from individual FoodEx categories was subsequently summed up, averaged over the total survey period 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 average 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 average 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 surveys (covering infants, toddlers, children, adolescents, adults and the elderly), carried out in 23 European countries (Appendix B). The highest dietary exposure to the food enzyme–TOS was estimated to be 0.05 mg TOS/kg bw per day in infants, toddlers and other children.

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**
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)	0.002–0.013 (12)	0.013–0.027 (17)	0.015–0.027 (20)	0.007–0.016 (22)	0.005–0.010 (23)	0.004–0.009 (23)
Min–max 95th percentile (number of surveys)	0.012–0.046 (10)	0.025–0.052 (15)	0.025–0.047 (20)	0.013–0.030 (21)	0.010–0.019 (23)	0.009–0.016 (22)

3.5.3. Uncertainty analysis

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

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	
FoodEx categories included in the exposure assessment were assumed to always contain the food enzyme–TOS	+

Sources of uncertainties	Direction of impact
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	+/–

+: 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 baking processes, cereal-based processes and egg processing, 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

A comparison of the NOAEL (19.55 mg TOS/kg bw per day) from the 90-day rat study with the derived exposure estimates of 0.002–0.027 mg TOS/kg bw per day at the mean and from 0.009–0.052 mg TOS/kg bw per day at the 95th percentile, resulted in margin of exposure (MoE) of at least 376.

4. Conclusions

Based on the data provided and the derived margin of exposure, the Panel concluded that the food enzyme glucose oxidase produced with the genetically modified *Aspergillus niger* strain DP-Aze23 does not give rise to safety concerns under the intended conditions of use.

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

Documentation provided to EFSA

1) Dossier "Application for authorisation of glucose oxidase from a genetically modified strain of *Aspergillus niger* (DP-Aze23) in accordance with Regulation (EC) No 1331/2008", February 2016. Submitted by Danisco US, Inc.
2) Additional information. August 2021. Submitted by Danisco US, Inc.

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Abbreviations

AMFEP	Association of manufacturers and formulators of enzyme product
CAS	Chemical Abstracts Service
CBS	Westerdijk Fungal Biodiversity Institute
CEF	EFSA Panel on Food Contact Material, Enzymes, Flavourings and Processing Aids
CEP	EFSA Panel on Food Contact Materials, Enzymes and Processing Aids
FAO	Food and Agriculture Organization of the United Nations
GLP	good laboratory practice
GMO	genetically modified organisms
GOU	glucose oxidase units
IUBMB	International Union of Biochemistry and Molecular Biology
MoE	margin of exposure
NOAEL	no observed adverse effect level
PCR	polymerase chain reaction

SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TOS	total organic solids
WGS	whole genome sequencing
WHO	World Health Organization

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

Information provided in this appendix is shown in an Excel file (downloadable https://efsa.onlinelibrary.wiley.com/doi/10.2903/j.efsa.2022.7181#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 one 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, United Kingdom
Toddlers	From 12 months up to and including 35 months of age	Belgium, Bulgaria, Cyprus, Denmark, Estonia, Finland, France, Germany, Hungary, Italy, Latvia, Netherlands, Portugal, Slovenia, Spain, United Kingdom
Children	From 36 months up to and including 9 years of age	Austria, Belgium, Bulgaria, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Italy, Latvia, Netherlands, Portugal, Spain, Sweden, United Kingdom
Adolescents	From 10 years up to and including 17 years of age	Austria, Belgium, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Italy, Latvia, Netherlands, Portugal, Romania, Slovenia, Spain, Sweden, United Kingdom
Adults	From 18 years up to and including 64 years of age	Austria, Belgium, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Netherlands, Portugal, Romania, Slovenia, Spain, Sweden, United Kingdom
The elderly[a]	From 65 years of age and older	Austria, Belgium, Cyprus, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Netherlands, Portugal, Romania, Slovenia, Spain, Sweden, 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).