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Safety evaluation of the food enzyme endo-1,3(4)-β-glucanase from the genetically modified *Bacillus subtilis* strain DP-Ezm28

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Abstract

The food enzyme endo-1,3(4)-β-glucanase (3(or 4)-β-D-glucan 3(4)-glucanohydrolase; EC 3.2.1.6) is produced with a genetically modified *Bacillus subtilis* strain DP-Ezm28 by Danisco US Inc. The genetic modifications do not give rise to safety concerns. The production strain of the food enzyme contains multiple copies of a known antimicrobial resistance gene. However, based on the absence of viable cells and DNA from the production organism in the food enzyme, this is not considered to be a risk. The food enzyme is intended to be used in distilled alcohol production and brewing processes. Since residual amounts of total organic solids (TOS) are removed by distillation, dietary exposure was only calculated for brewing processes. Based on the maximum use levels recommended for brewing processes and individual data from the EFSA Comprehensive European Food Database, dietary exposure to the food enzyme–total organic solids was estimated to be up to 0.183 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 1,000 mg TOS/kg bw per day, the highest dose tested, which when compared with the estimated dietary exposure, results in a margin of exposure of at least 5464. Similarity of the amino acid sequence to those of known allergens was searched and two matches were found. 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 is considered 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, endo-1,3(4)-β-glucanase, EC 3.2.1.6, Laminaranase, *Bacillus subtilis*, genetically modified microorganism

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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 EU 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 companies 'Chr. Hansen' for the authorisation of the food enzyme Endothiapepsin from a genetically modified strain of *Cryphonectria parasitica* (strain DSM 29549), 'Nagase (Europa) GmbH' for the authorisation of the food enzymes L-Ascorbate oxidase from *Cucurbita pepo* and *Cucurbita moschata*, and Microbial collagenase from a genetically modifies strain of *Streptomyces violaceoruber* (strain pCol); 'Novozymes A/S' for the authorisation of the food enzyme Inulinase from *Aspergillus niger* (strain NZYM-KF), and 'Danisco US Inc.' for the authorisation of the food enzyme Endo-1,3(4)-beta-glucanase from a genetically modified strain of *Bacillus subtilis* (DP-Ezm28).

Following the requirements of Article 12.1 of Commission Regulation (EU) No 234/2011[3] implementing Regulation (EC) No 1331/2008[4], the Commission has verified that the five applications

[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, p. 15–24.

[4] 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, p. 1–6.

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 Endothiapepsin from a genetically modified strain of *Cryphonectria parasitica* (strain DSM 29549), L-Ascorbate oxidase from *Cucurbita pepo* and *Cucurbita moschata*, Microbial collagenase from a genetically modifies strain of *Streptomyces violaceoruber* (strain pCol), Inulinase from *Aspergillus niger* (strain NZYM-KF) and Endo-1,3(4)-beta-glucanase from a genetically modified strain of *Bacillus subtilis* (DP-Ezm28) in accordance with the 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 the food enzyme endo-1,3(4)-β-glucanase from a genetically modified strain of *B. subtilis* (strain DP-Ezm28).

2. Data and methodologies

2.1. Data

The applicant has submitted a dossier in support of the application for authorisation of the food enzyme endo-1,3(4)-β-glucanase from a genetically modified strain of *B. subtilis* (strain DP-Ezm28).

Additional information was requested from the applicant during the assessment process on 18 December 2018, on 11 September 2019, on 27 March 2020 and on 20 October 2020, and was consequently provided (see 'Documentation provided to EFSA').

Following the reception of additional data by EFSA on 30 July 2019, EFSA requested a clarification teleconference on 16 January 2020, after which the applicant provided additional data on 11 March 2020.

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) as well as in the 'Statement on characterisation of microorganisms used for the production of food enzymes' (EFSA CEP Panel, 2019) and following the relevant existing guidance's of EFSA Scientific Committee.

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

3. Assessment

IUBMB nomenclature:	Endo-1,3(4)-β-glucanase
Systematic name:	3-(1→3;1→4)-β-D-glucan 3(4)-glucanohydrolase
Synonyms:	Endo-1,3-β-D-glucanase; β-1,3-glucanase; laminaranase
IUBMB No:	EC 3.2.1.6
CAS No:	62213-14-3.

The endo-1,3(4)-β-glucanase catalyses the hydrolysis of 1,3- or 1,4-β-glycosidic linkages in β-D-glucans resulting in the generation of partially hydrolysed β-D-glucans. It is intended to be used in distilled alcohol production and brewing processes.

3.1. Source of the food enzyme

The endo-1,3(4)-β-glucanase is produced with a genetically modified strain of *B. subtilis* (DP-Ezm28; [redacted]), which is deposited at the Westerdijk Fungal Biodiversity Institute (CBS, The Netherlands) with deposit number [redacted].[5]

3.1.1. Characteristics of the parental and recipient microorganisms

The parental microorganism [redacted]

The recipient strain [redacted]

The recipient strain [redacted]

Data on the absence of cytotoxicity were provided for the parental strain but not for the production strain.[6]

3.1.2. Characteristics of the introduced sequences[7]

The gene encoding endo-1,3(4)-β-glucanase [redacted]

During the genetic modification, [redacted]

3.1.3. Description of the genetic modification process[8]

[redacted]

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 *B. subtilis* DP-Ezm28 differs from the recipient strain [redacted] in its ability to produce the endo-1,3(4)-β-glucanase enzyme from the inserted multiple copies of the [redacted]

[5] Technical dossier/Additional data/Annex AK.
[6] Technical dossier/Additional information July 2019/Annex AC.
[7] Technical dossier/1st submission/Annex Q; Technical dossier/2nd submission/Annex S_updated; Additional information July 2019.
[8] Technical dossier/1st submission/Annex R.

[redacted]. Other than the [redacted] gene, whole genome sequence (WGS) analysis in preparation, expected to be published by March 2021 in demonstrated that no antimicrobial resistance (AMR) genes were introduced in the production strain by the genetic modification.[9]

The production strain contains multiple copies of [redacted] gene which is considered a hazard. No other issues of concern arising from the genetic modification were identified.

3.2. Production of the food enzyme

The food enzyme is manufactured according to Food Hygiene Regulation (EC) No 852/2004[10], with food safety procedures based on Hazard Analysis and Critical Control Points (HACCP) and in accordance with current Good Manufacturing Practice (GMP).[11]

The production strain is grown as a pure culture using a typical industrial medium in a submerged, fed-batch fermentation system with conventional process controls in place. After completion of the fermentation, the solid biomass is removed from the fermentation broth by microfiltration leaving a supernatant containing the food enzyme. The filtrate containing the enzyme is then further purified and concentrated, including an ultrafiltration step in which enzyme protein is retained while most of the low molecular weight material passes the filtration membrane and is discarded. 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.[12]

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 endo-1,3(4)-β-glucanase is a single polypeptide of [redacted] amino acids, including a signal sequence of [redacted] amino acids, which is cleaved off during the secretion of the enzyme.[13] The molecular mass of the mature protein, derived from the amino acid sequence, was calculated to be [redacted] kDa. The food enzyme was analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis. A consistent protein pattern was observed across all batches. The gels showed the target protein migrating slightly above the [redacted]-kDa reference protein.[14] No other enzyme activities were reported.[15]

The in-house determination of endo-1,3(4)-β-glucanase activity is based on the hydrolysis of barley β-glucan (reaction conditions: pH 5.0, 30°C, 20 min). The enzymatic activity is determined by measuring the release of reducing sugar using 3,5-dinitrosalicylic acid. The enzyme activity is expressed in 'Beta-Glucanase from Brew 2 Units (BBU)'. One unit of β-glucanase is defined as the amount of enzyme required to generate 1 μmol glucose reducing sugar per minute under the assay conditions.[16]

The food enzyme has a temperature optimum between 55 and 65°C (pH 5.0) and a pH optimum between 6 and 7 at 30°C.[17] Thermostability was tested after a pre-incubation of the food enzyme for 10 min at different temperatures (pH 5.5). No enzyme activity was detected above 80°C.[18]

3.3.2. Chemical parameters

Data on the chemical parameters of the food enzyme were provided for three food enzyme batches used for commercialisation and one batch used for toxicological testing.[19] The average total organic

[9] Technical dossier/Additional data November 2020/Annex AJ.
[10] 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, 321 pp.
[11] Technical dossier/2nd submission/Annex J.
[12] Technical dossier/1st submission/Annex L/Additional data November 2020/Annex AL.
[13] Technical dossier/1st submission/Annex H.
[14] Technical dossier/2nd submission/Updated dossier/p. 38.
[15] Technical dossier/2nd submission/Updated dossier/p. 41.
[16] Technical dossier/1st submission/Annex D.
[17] Technical dossier/2nd submission/Updated dossier/p. 42.
[18] Technical dossier/Additional data March 2020/Annex AG.
[19] Technical dossier/2nd submission/Updated dossier/p 37 and Technical dossier/1st submission/Annex F; Additional data March 2020/Annex AD/Annex AE/Annex AF; Additional data October 2020/Annex AH.

solids (TOS) content of the three food enzyme batches used for commercialisation is 9.05% and the average enzyme activity/TOS ratio is 994 BBU/mg TOS.

Table 1: Compositional data of the food enzyme

Parameter	Unit	Batch			
		1	2	3	4[(a)]
Endo-1,3(4)-β-glucanase	BBU/g batch[(b)]	91,127	125,154	58,565	160,365
Protein	%	6.01	8.00	5.96	10.23
Ash	%	0.55	0.74	0.59	0.81
Water	%	91.03	88.49	91.46	89.03
Total Organic Solids (TOS)[(c)]	%	8.42	10.77	7.95	10.16
Activity/mg TOS	BBU/mg TOS	1,082	1,162	737	1,578

(a): Batch used for the toxicological studies.
(b): BBU: Beta-Glucanase from Brew 2 Units (see Section 3.3.1).
(c): TOS calculated as 100% – % water – % ash.

3.3.3. Purity

The lead content in the three commercial batches and in the batch used for toxicological studies was below 5 mg/kg[20] which complies with the specification for lead (≤ 5 mg/kg) as laid down in the general specifications and considerations for enzymes used in food processing (FAO/WHO, 2006).[21] In addition, the levels of arsenic, cadmium and mercury were below the limits of detection of the employed methodologies.[22] The food enzyme complies with the microbiological criteria (for total coliforms, *Escherichia coli* and *Salmonella*) as laid down in the general specifications and considerations for enzymes used in food processing (FAO/WHO, 2006).[23] No antimicrobial activity was detected in any of the tested batches (FAO/WHO 2006).[24]

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 production strain in the food enzyme was demonstrated in nine independent batches analysed in triplicate. [redacted] No colonies were produced.[25]

The absence of recombinant DNA in the food enzyme was demonstrated by polymerase chain reaction (PCR) analysis of three batches in triplicate. No DNA was detected [redacted][26]

3.4. Toxicological data

A battery of toxicological tests including a bacterial gene mutation assay (Ames test), an *in vitro* mammalian chromosomal aberration test, and a repeated dose 90-day oral toxicity study in rats has been provided. The batch 4 (Table 1) used in these studies has similar chemical purity as the commercial batches, and thus is considered suitable as a test item.

[20] Technical dossier/1st submission/Annex F; Additional data March 2020/Annex AD/Annex AE/Annex AF; Additional data October 2020/Annex AH.
[21] Technical dossier/2nd submission/Updated dossier/p. 39 and Technical dossier/1st submission/Annex F; Additional data March 2020/Annex AF.
[22] LODs: Pb = 5 mg/kg; As = 3 mg/kg; Cd = 0.5 mg/kg and Hg = 0.5 mg/kg.
[23] Technical dossier/1st submission/Annex F; Additional data March 2020/Annex AF.
[24] Technical dossier/1st submission/Annex F, Additional data March 2020/Annex AF.
[25] Technical dossier/1st submission/Annex I; Additional data July 2019/Annex Z.
[26] Technical dossier/1st submission/Annex U; Additional data July 2019/Annex AA.

3.4.1. Genotoxicity

3.4.1.1. Bacterial reverse mutation test

A bacterial reverse mutation assay was performed according to OECD Test Guideline 471 (OECD, 1997a) and following Good Laboratory Practice (GLP) in four strains of *Salmonella* Typhimurium (TA 1535, TA 1537, TA 98, and TA 100) and *E. coli* WP2uvrA, in the presence or absence of metabolic activation (S9-mix) applying the treat and plate method.[27] Two separate experiments in triplicate were carried out using six different concentrations of the food enzyme: 15, 50, 150, 500, 1,500 and 5,000 μg total proteins/plate (corresponding to 15.4, 51.2, 153.5, 511.5, 1,534.6 and 5,115.3 μg TOS/plate).[28] No growth inhibition of any test strain was reported. An increase in revertant counts with *Salmonella* TA98 strain and *E. coli* WP2 *uvr*A was found that was not dose-related in the first experiment, and these increases were within the historical vehicle control ranges and were not reproduced in the second experiment. No increase in revertant colony numbers above the control values was observed in any other *Salmonella* strains at any concentration of the food enzyme.

Therefore, the Panel concluded that the food enzyme did not induce gene mutations under the conditions employed.

3.4.1.2. *In vitro* mammalian chromosomal aberration test

The *in vitro* chromosomal aberration test was carried out according to the OECD Test Guideline 473 (OECD, 1997b) and following GLP.[29] Human peripheral blood lymphocytes were exposed in duplicate to the food enzyme in a short treatment (4 h + 16 h recovery) in the presence and absence of S9-mix and in a continuous treatment for 20 h without S9-mix. Based on the results of a range finding test the concentration levels for the chromosome aberration assay were set at 1,000, 2,500 and 5,000 μg total protein/mL (corresponding to 1,023.1, 2,557.6 and 5,115.3 μg TOS/mL) for short-term treatment and at 100, 150 and 250 μg total protein/mL (corresponding to 102.3, 153.5 and 255.8 μg TOS/mL)[30] for the 20-h continuous treatment. A reduction of the mitotic index of 51%, 46% and 56% was detected at the highest concentrations tested at 4-h treatment with and without S9-mix and at continuous 20-h treatment, respectively. No statistically significant increase in the incidence of cells with chromosomal aberrations or in the incidence of polyploid cells was observed after treatment with the test article at any concentration tested.

The Panel concluded that the food enzyme did not induce chromosomal aberrations in mammalian cells under the conditions employed.

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

A repeated dose 90-day oral toxicity study was performed according to OECD test guideline 408 (OECD, 1998), and following GLP.[31] Groups of 10 male and 10 female Crl:CD(SD) rats received the food enzyme orally via gavage for 90 days at dose levels of 100, 300 and 1,000 mg TOS/kg body weight (bw) per day, and referred to as low-, mid- and high-dose groups, respectively. A control group received water alone.

No mortality was observed.

In locomotor activity testing in week 13 statistically significantly higher values than in controls were recorded for basic movements and rearing counts (time intervals 20–30 min, 0–60 min), fine movements and total distance (a time interval 0–60 min) in low-dose females and for basic movements in mid-dose females (time interval 0–10 min). Despite these findings being statistically significant, they were considered to be incidental and not treatment related as they were without dose dependency and were limited to one sex.

Statistically significantly lower feed intake was recorded during several periods in all female groups and the overall feed intake (days 1–91) was statistically significantly lower in mid- and high-dose females than in the controls. As the lower feed intake was observed only in one sex and did not result in statistically significant differences in body weight between the control and treated females, this finding was considered by the Panel as not toxicologically significant.

[27] Technical dossier/Additional data March 2020/Annex AD.
[28] TOS = 10.16%, total proteins = 10.23%, specific gravity = 1.03 g/mL; Additional data March 2020/Annex AD.
[29] Technical dossier/Additional data March 2020/Annex AE.
[30] TOS = 10.16%, total proteins = 10.23%, specific gravity = 1.03 g/mL; Additional data March 2020/Annex AE.
[31] Technical dossier/Additional data March 2020/Annex AF.

Among haematological parameters, the only statistically significant differences to controls were lower values for mean corpuscular volume in week 6 and at termination and mean corpuscular haemoglobin at termination in low-dose females. As no significant changes were observed for the measured parameters (red blood cell count, haemoglobin concentration, haematocrit), the small changes in these calculated parameters were considered by the Panel to be not of toxicological significance.

Clinical chemistry analyses revealed several statistically significant differences to controls. Plasma concentration of sodium was higher in low-dose females in week 6 and in low- and mid-dose males at termination. Urea nitrogen concentration was lower in mid-dose males in week 6 and in low-dose females at termination. Creatinine concentration was lower in high-dose males. As the differences lacked dose–response relationship (for sodium and urea nitrogen), were generally of small magnitude or confined to one sex they were considered by the Panel to be not of toxicological significance.

The relative heart weight of low-dose males was statistically significantly lower than in the controls. In the high-dose males, the absolute relative to body weight or relative to brain weight testes weights were lower than in the controls. These findings were not accompanied with macro- or microscopic changes. The Panel considered these changes as not toxicologically relevant.

No other significant effects were observed.

The Panel identified a no observed adverse effect level (NOAEL) of 1,000 mg TOS/kg bw per day, the highest dose tested.

3.4.3. Allergenicity

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

The potential allergenicity of endo-1,3(4)-β-glucanase from the genetically modified *B. subtilis* strain DP-Ezm28 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 window of 80 amino acids as criterion, two matches were found with the *Aspergillus fumigatus* allergens Asp f 9 and Asp f 16.[32]

No information is available on oral sensitisation and elicitation reactions of this endo-1,3(4)-β-glucanase.

The matching *A. fumigatus* allergens for endo-1,3(4)-β-glucanase are well known allergens that can trigger respiratory sensitisation and asthma (Chaudhary and Marr, 2011). However, several studies have shown that adults with occupational asthma to a food enzyme (like α-amylase from *A. oryzae*) may be able to ingest the corresponding enzyme without acquiring clinical symptoms of food allergy (Cullinan et al., 1997; Poulsen, 2004; Armentia et al., 2009). Considering the wide use of α-amylase as a food enzyme, only a low number of case reports has been described in the literature focused on allergic reactions upon oral exposure to α-amylase in individuals respiratory sensitised to α-amylase (Losada et al., 1992; Quirce et al., 1992; Baur and Czuppon, 1995; Kanny and Moneret-Vautrin, 1995; Moreno-Ancillo et al., 2004).

According to the information provided, substances or products that may cause allergies or intolerances (Regulation EU 1169/2011)[33] are used as raw materials ([redacted]) in media fed to the microorganisms. In addition, [redacted], known allergens are also present in the media fed to the microorganisms. However, during the fermentation process, these products will be degraded and utilised by the microorganisms for cell growth, cell maintenance and production of enzyme protein. In addition, the microbial biomass and fermentation solids are removed. Taking into account the fermentation process and downstream processing, the Panel considered that potentially allergenic residues of these materials employed as protein sources are not expected to be present in the food enzyme.

[32] Technical dossier/1st submission/Annex P.

[33] 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.

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 as in the case of distilled alcohol production. The food enzyme remains in brewed beverages.

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 can be excluded for distilled alcohol production. The risk cannot be excluded for brewing processes, 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 two food manufacturing processes at the recommended use levels summarised in Table 2.[34]

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

Food manufacturing process[(a)]	Raw material	Recommended dosage of the food enzyme
Brewing processes	Cereals	Up to 40 mg TOS/kg cereals
Distilled alcohol production	Cereals	Up to 40 mg TOS/kg cereals

(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.

In distilled alcohol production, the food enzyme is added in the pre-treatment, pre-saccharification or fermentation step. It is used to break down cell wall polysaccharides in the cereal grain to increase the yield of fermentable sugars, and to reduce wort/mash viscosity.

Concerning distilled alcohol production, technical information and experimental data provided on the removal of food enzyme TOS was considered by the Panel as sufficient to exclude this process from the exposure assessment (Annex B in EFSA CEF Panel, 2016).

In brewing processes, the food enzyme is added during the mashing step. It is used to hydrolyse starch and related polysaccharides to delay the staling process. The food enzyme hydrolyses the substrates (β-glucans) and facilitates filtration. It also degrades the cell walls, releasing starch and protein and, therefore, increasing the brewing yield.

The food enzyme remains in brewed beverages. Based on data provided on thermostability (see Section 3.3.1), it is expected that the endo-1,3(4)-β-glucanase is inactivated during brewing processes.

3.5.2. Dietary exposure estimation

As residual amounts of TOS are removed by distillation, foods/ingredients derived through this process, i.e. distilled alcohol, were excluded from the estimation.

For brewing processes, chronic exposure was calculated using the methodology described in the CEF Panel statement on the exposure assessment of food enzymes (EFSA CEF Panel, 2016). The assessment involved selection of relevant food categories from the EFSA Comprehensive European Food Consumption Database and application of process and technical conversion factors (Annex B in EFSA CEF Panel, 2016).

Chronic exposure was calculated by combining the maximum recommended use level provided by the applicant (see Section 3.5.1/Table 2) with the relevant FoodEx categories (Annex B in EFSA CEF Panel, 2016), based on individual consumption data. Exposure from individual FoodEx categories was subsequently summed up, averaged over the total survey period and normalised for bodyweight. 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).

[34] Technical dossier/2nd submission/Updated dossier/p. 64.

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 35 different dietary surveys (covering infants, toddlers, children, adolescents, adults and the elderly), carried out in 22 European countries (Appendix B).

Table 3: Summary of estimated dietary exposure to food enzyme–TOS in six population groups

Population group	Estimated exposure (mg TOS/kg body weight per day)					
	Infants	**Toddlers**	**Children**	**Adolescents**	**Adults**	**The elderly**
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 (10)	0 (14)	0–0.001 (19)	0–0.008 (18)	0.003–0.041 (19)	0.001–0.020 (18)
Min–max 95th percentile (number of surveys)	0 (8)	0 (12)	0 (19)	0–0.048 (17)	0.023–0.183 (19)	0.005–0.084 (18)

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, 2007), 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	+
Exposure to food enzyme–TOS was always calculated based on the recommended maximum use level	+
Selection of broad FoodEx categories for the exposure assessment	+
Use of recipe fractions in disaggregation FoodEx categories	+/–
Use of technical factors in the exposure model	+/–
Exclusion of other processes from the exposure estimate: – distilled alcohol production	–

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

The exclusion of one food manufacturing process (distilled alcohol production) from the exposure assessment was based on > 99% of TOS removal during this process and is not expected to have an impact on the overall estimate derived.

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

A comparison of the NOAEL (1,000 mg TOS/kg bw per day) from the 90-day study with the derived exposure estimates of 0–0.041 mg/kg bw per day at the mean and from 0 to 0.183 mg TOS/kg bw per day at the 95th percentile, resulted in margin of exposure (MOE) of at least 5464.

4. Conclusions

Based on the data provided, removal of TOS during the distilled alcohol production process and the derived margin of exposure for brewing processes, the Panel concluded that the food enzyme endo-1,3(4)-β-glucanase produced with the genetically modified *B. subtilis* strain DP-Ezm28 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 Endo-1,3(4)-beta-glucanase from a genetically modified strain of *Bacillus subtilis* DP-Ezm28', March 2015. Submitted by Danisco US Inc.
2) Summary report on GMM part for endo-1,3(4)-beta-glucanase produced by *Bacillus subtilis* strain DP-Ezm28, EFSA-Q-2015-00828'. Delivered by DTU.
3) Additional information. July 2019. Submitted by DuPont Nutrition and Biosciences.
4) Additional information. March 2020. Submitted by DuPont Nutrition and Biosciences.
5) Additional information. October 2020. Submitted by DuPont Nutrition and Biosciences.
6) Additional information. November 2020. Submitted by DuPont Nutrition and Biosciences.

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Abbreviations

AMR	antimicrobial resistance
BBU	beta-glucanase from Brew 2 Units
bp	base pair
bw	body weight
CAS	Chemical Abstracts Service
CFU	colony forming units
EC	Enzyme Commission
EINECS	European Inventory of Existing Commercial Chemical Substances
FAO	Food and Agricultural Organization
GLP	Good Laboratory Practice
GM	genetically modified
GMP	Good Manufacturing Practice
HACCP	Hazard Analysis and Critical Control Points
IUBMB	International Union of Biochemistry and Molecular Biology
JECFA	Joint FAO/WHO Expert Committee on Food Additives
MOE	Margin of Exposure
NOAEL	no observed adverse effect level
OECD	Organisation for Economic Cooperation and Development
PCR	polymerase chain reaction
SDS–PAGE	Sodium Dodecyl Sulfate–poly acrylamide gel electrophoresis
TOS	Total Organic Solids
WGS	whole genome sequence
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://doi.org/10.2903/j.efsa.2021.6431).

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: The contribution of FoodEx categories to the food enzyme–TOS dietary exposure.

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, Denmark, Estonia, Finland, France, Germany, Italy, Latvia, Portugal, United Kingdom
Toddlers	From 12 months up to and including 35 months of age	Belgium, Bulgaria, Denmark, Estonia, Finland, France, Germany, Italy, Latvia, Netherlands, Portugal, Spain, United Kingdom
Children[(a)]	From 36 months up to and including 9 years of age	Austria, Belgium, Bulgaria, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Italy, Latvia, Netherlands, Portugal, Spain, Sweden, United Kingdom
Adolescents	From 10 years up to and including 17 years of age	Austria, Belgium, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Italy, Latvia, Netherlands, Portugal, Spain, Sweden, United Kingdom
Adults	From 18 years up to and including 64 years of age	Austria, Belgium, Croatia, Czech Republic, Denmark, Estonia, Finland, France, Germany, Hungary, Ireland, Italy, Latvia, Netherlands, Portugal, Romania, Spain, Sweden, United Kingdom
The elderly[(a)]	From 65 years of age and older	Austria, Belgium, Denmark, Estonia, Finland, France, Germany, Hungary, Ireland, Italy, Latvia, Netherlands, Portugal, Romania, Spain, Sweden, United Kingdom

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