

Safety evaluation of the food enzyme subtilisin from the non-genetically modified *Bacillus licheniformis* strain NZYM-CX

EFSA Panel on Food Contact Materials, Enzymes and Processing Aids (CEP) |

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Abstract

The food enzyme subtilisin (EC 3.4.21.62) is produced with the non-genetically modified *Bacillus licheniformis* strain NZYM-CX by Novozymes A/S. The production strain met the requirements for the qualified presumption of safety (QPS) approach. The food enzyme is intended to be used in eight food manufacturing processes: processing of cereals and other grains for the production of brewed products; processing of dairy products for the production of modified milk proteins and flavouring preparations; processing of plant- and fungal-derived products for the production of plant-based analogues of milk and milk products, protein hydrolysates and edible oils from algae; processing of meat and fish products for the production of protein hydrolysates; processing of yeast and yeast products. Since residual amounts of total organic solids (TOS) are removed in the production of edible oils from algae, dietary exposure was calculated only for the remaining seven food manufacturing processes. Exposure was estimated to be up to 2.393 mg TOS/kg body weight (bw) per day in European populations. As the production strain qualified for the QPS approach and no issues of concern arose from the production process of the food enzyme, the Panel considered that toxicological studies were unnecessary. A search for the similarity of the amino acid sequence of the food enzyme to known allergens was performed, and a total of 20 matches were found, 17 to respiratory allergens, two to food allergens (found in muskmelon and pomegranate) and one to a contact allergen. The Panel considered that the risk of allergic reactions upon dietary exposure to this food enzyme cannot be excluded, especially in individuals sensitised to muskmelon or pomegranate, but would not exceed the risk of consuming these foods. 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

Bacillus licheniformis, EC 3.4.21.62, food enzyme, non-genetically modified microorganism, subtilisin

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1 | INTRODUCTION

Article 3 of the Regulation (EC) No 1332/2008¹ 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 1331/2008² 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 1332/2008¹ on food enzymes.

Six applications have been introduced by the companies “Decernis, LLC”, “Keller and Heckman LLP”, the “Association of Manufacturers and Formulators of Enzyme Products (AMFEP)” and “Novozymes A/S” for the authorisation of the food enzymes Cyclomaltodextrin glucanotransferase from *Geobacillus stearothermophilus*, Dextranase from *Chaetomium gracile*, Subtilisin from *Bacillus licheniformis*, Mucorpepsin from *Rhizomucor miehei*, Animal rennet consisting of chymosin and pepsin from the abomasum of *Bos primigenius* (cattle), *Bubalus bubalis* (buffalo), *Capra aegagrus hircus* (goat) and *Ovis aries* (sheep), and Lipase from a genetically modified strain of *Aspergillus niger* (strain NZYM-DB) respectively.

Following the requirements of Article 12.1 of Regulation (EC) No 234/2011³ implementing Regulation (EC) No 1331/2008², the Commission has verified that the six applications fall within the scope of the food enzyme Regulation and contain all the elements required under Chapter II of that Regulation.

1.1.2 | Terms of Reference

The European Commission requests the European Food Safety Authority to carry out the safety assessments on the food enzymes Cyclomaltodextrin glucanotransferase from *Geobacillus stearothermophilus*, Dextranase from *Chaetomium gracile*, Subtilisin from *Bacillus licheniformis*, Mucorpepsin from *Rhizomucor miehei*, Animal rennet consisting of chymosin and pepsin from the abomasum of *Bos primigenius* (cattle), *Bubalus bubalis* (buffalo), *Capra aegagrus hircus* (goat) and *Ovis aries* (sheep), and Lipase from a genetically modified strain of *Aspergillus niger* (strain NZYM-DB) in accordance with Article 17.3 of Regulation (EC) No 1332/2008¹ on food enzymes.

¹Regulation (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.

²Regulation (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.

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

1.2 | 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 subtilisin from *B. licheniformis* submitted by Novozymes A/S.

The application was submitted initially as a joint dossier⁴ and identified as EFSA-Q-2015-00232. During the risk assessment phase, it was found that the technical dossier is too generic to be evaluated. During an ad-hoc meeting between EFSA, the European Commission and the Association of Manufacturers and Formulators of Enzyme Products (AMFEP),⁵ it was agreed that joint dossiers will be split into individual data packages.

The current opinion addresses one data package originating from the joint dossier EFSA-Q-2015-00232. This data package, identified as EFSA-Q-2022-00545, concerns the food enzyme subtilisin that is produced with a strain of *B. licheniformis* (NZYM-CX) and submitted by Novozymes A/S.

2 | DATA AND METHODOLOGIES

2.1 | Data

The applicant has submitted a dossier in support of the application for authorisation of the food enzyme subtilisin from a non-genetically modified *B. licheniformis* (strain NZYM-CX).

Additional information was requested from the applicant during the assessment process on 13 February 2023 and received on 10 May 2023 (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 the EFSA Scientific Committee.

A data package originated from a joint dossier should fulfil the data requirements in the 'Submission of a Dossier on Food Enzymes for Safety Evaluation' (EFSA CEF Panel, 2009a). During the evaluation, the Panel applied, whenever possible, the updated current 'Scientific Guidance for the submission of dossiers on Food Enzymes' (EFSA CEP Panel, 2021) and the guidance on the 'Food manufacturing processes and technical data used in the exposure assessment of food enzymes' (EFSA CEP Panel, 2023).

3 | ASSESSMENT

IUBMB nomenclature	Subtilisin
Systematic name	–
Synonyms	Alcalase; bacillopeptidase; colistinase; <i>Bacillus subtilis</i> alkaline proteinase; protease S
IUBMB No	EC 3.4.21.62
CAS No	9014-01-1
EINECS No	232-752-2

Subtilisins catalyse the hydrolysis of peptide bonds of proteins with a broad specificity, releasing peptides and amino acids.

The enzyme under assessment is intended to be used in eight food manufacturing processes: processing of cereals and other grains for the production of brewed products; processing of dairy products for the production of modified milk proteins and flavouring preparations; processing of plant- and fungal-derived products for the production of plant-based analogues of milk and milk products, protein hydrolysates and edible oils from algae; processing of meat and fish products for the production of protein hydrolysates and processing of yeast and yeast products.

⁴Commission Implementing Regulation (EU) No 562/2012 of 27 June 2012 amending Commission Regulation (EU) No 234/2011 with regard to specific data required for risk assessment of food enzymes Text with EEA relevance. OJ L 168, 28.6.2012, pp. 21–23.

⁵The full detail is available online <https://www.efsa.europa.eu/en/events/event/ad-hoc-meeting-industry-association-amfep-joint-dossiers-food-enzymes>

3.1 | Source of the food enzyme

The enzyme is produced with the non-genetically modified bacterium *B. licheniformis* strain NZYM-CX, which is deposited at the [REDACTED] with the deposit number [REDACTED].⁶ The production strain was identified as *B. licheniformis* by Whole Genome Sequencing (WGS) analysis [REDACTED].^{7,8} *B. licheniformis* NZYM-CX was obtained by [REDACTED].⁹

The species *B. licheniformis* is included in the list of organisms for which the qualified presumption of safety (QPS) may be applied, provided that the absence of acquired antimicrobial resistance (AMR) and toxigenic activity are verified for the specific strain used (EFSA, 2007; EFSA BIOHAZ Panel, 2020). A cytotoxicity test made with culture supernatants indicated that the production strain *B. licheniformis* strain NZYM-CX did not induce cell damage to Vero cells using the lactate dehydrogenase assay.¹⁰ The WGS analysis of the production strain was interrogated for the presence of antimicrobial resistance genes using two databases with thresholds of > 80% identity and > 70% coverage. No genes of concern were identified.¹¹ Therefore, the production strain was considered to qualify for the QPS approach.

3.2 | Production of the food enzyme

The food enzyme is manufactured according to the Food Hygiene Regulation (EC) No 852/2004¹², with food safety procedures based on Hazard Analysis and Critical Control Points and in accordance with current Good Manufacturing Practice.¹³

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 filtration. The filtrate containing the enzyme is 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.¹⁴ The applicant provided information on the identity of the substances used to control fermentation and in the subsequent downstream processing of the food enzyme.¹⁵

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

Subtilisin is a single polypeptide chain of [REDACTED] amino acids.¹⁶ The molecular mass of the mature protein, calculated from the amino acid sequence, is [REDACTED] kDa.¹⁶ The food enzyme was analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. A consistent protein pattern was observed across all batches. The gel showed a single major protein band corresponding to an apparent molecular mass of about [REDACTED] kDa, consistent with the expected mass of the enzyme.¹⁷ The food enzyme was also tested for α -amylase, glucoamylase and lipase activities;¹⁸ only α -amylase and lipase activities were detected. No other enzymatic activities were reported.

The in-house determination of subtilisin activity is based on hydrolysis of N-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide (reaction conditions: pH 9.0, 37°C, 63 s). The enzymatic activity is determined by measuring the release of *p*-nitroanilide spectrophotometrically at 405 nm, quantified relative to an internal enzyme standard and expressed in Anson Units (Alcalase)/g (AU-A/g).¹⁹

The food enzyme has a temperature optimum around 70°C (pH 7.0) and a pH optimum around pH 9.0 (30°C). Thermostability was tested after a pre-incubation of the food enzyme for 30 min at different temperatures (pH 7.0). Enzyme activity decreased above 70°C, showing no residual activity above 80°C pre-incubation.²⁰

⁶Technical dossier/Annex 8.

⁷Technical Dossier/Annex 6.

⁸Technical Dossier/Annex 7.

⁹Technical Dossier/p. 44.

¹⁰Technical Dossier/Annex 9.

¹¹Technical Dossier/Annexes: 10.01, 10.02, 10–03.

¹²Regulation (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.

¹³Technical Dossier/p. 48/ Annex 11.

¹⁴Technical Dossier/pp. 48–54.

¹⁵Technical Dossier/Annex 12.

¹⁶Technical Dossier/p. 36/Annex 1.

¹⁷Technical Dossier/p. 38.

¹⁸Technical Dossier/pp. 42–43/Annexes: 3.02, 3.03, 3.04.

¹⁹Technical Dossier/pp. 40–41/Annex 3.01.

²⁰Technical Dossier/pp. 41–42/Annex 5.

3.3.2 | Chemical parameters

Data on the chemical parameters of the food enzyme were provided for three batches intended for commercialisation (Table 1).²¹ The mean total organic solids (TOS) of the three food enzyme batches was 15.1% and the mean enzyme activity/TOS ratio was 0.036 AU-A/mg TOS.

TABLE 1 Composition of the food enzyme

Parameters	Unit	Batches		
		1	2	3
Subtilisin activity	AU-A/g ^a	5.41	6.05	4.88
Protein	%	11.2	11.9	9.2
Ash	%	0.9	0.8	1.1
Water	%	83.4	84.1	84.5
Total organic solids (TOS) ^b	%	15.7	15.1	14.4
Activity/TOS ratio	AU-A/mg TOS	0.035	0.040	0.034

^aAU-A/g: Anson Unit (Alcalase)/g (see Section 3.3.1).

^bTOS calculated as 100% – % water – % ash.

3.3.3 | Purity

The lead content in the three commercial batches was below 0.5 mg/kg,²² which complies with the specification for lead as laid down in the general specifications for enzymes used in food processing (FAO/WHO, 2006). In addition, arsenic, mercury and cadmium contents were below the limits of detection of the employed methods.^{22,23}

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 the tested batches.²⁵

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

3.4 | Toxicological data

As the production strain qualifies for the QPS approach to safety assessment and no issue of concern arising from the production process of the food enzyme was identified (see Sections 3.1, 3.2 and 3.3), the Panel considered that no toxicological studies other than the assessment of allergenicity were necessary (EFSA CEP Panel, 2021).

3.4.1 | Allergenicity

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

The potential allergenicity of the subtilisin produced with the non-genetically modified *B. licheniformis* strain NZYM-CX 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, 17 matches with proteases annotated as respiratory allergens, two with proteases classified as food allergens and one protease known to be a contact allergen were found. The matching food allergens were Cuc m 1, a subtilisin-like protease from *Cucumis melo* (Muskmelon) and Pun g 14, a chitinase III from *Punica granatum* (Pomegranate); the matching contact allergen was Tri r 2 from *Trichophyton rubrum* (Athlete's foot fungus),²⁶ and a match was also found with an alkaline protease from the same organism.

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

No allergic reactions to oral ingestion of the contact allergen alkaline protease from *Trichophyton rubrum*, a fungus residing on the skin, were expected. Several studies have shown that adults respiratorily sensitised to a food enzyme may

²¹Technical Dossier/p. 37/Annexes: 2.01, 2.02, 2.03, 3.01, 4.

²²Technical Dossier/p. 38/Annex 2.04.

²³LoDs: Pb = 0.5 mg/kg; As = 0.3 mg/kg; Hg, Cd = 0.05 mg/kg each.

²⁴Technical Dossier/pp. 39–40/Annexes: 2.06, 2.07, 2.08, 2.09.

²⁵Technical Dossier/p. 39/Annex 2.05.

²⁶Technical Dossier/pp. 63–68/Annexes 13.01, 13.02.

be able to ingest the corresponding allergen without acquiring clinical symptoms of food allergy (Armentia et al., 2009; Cullinan et al., 1997; Poulsen, 2004).

Allergic reaction cannot be excluded in individuals allergic to muskmelon and pomegranate. [REDACTED], a product that may cause allergies or intolerances (listed in the Regulation (EU) No 1169/2011²⁷), is used as raw material. However, during the fermentation process, this product 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 no potentially allergenic residues from this source are present in the food enzyme.

The Panel considered that, under the intended conditions of use, the risk of allergic reactions upon dietary exposure to this food enzyme cannot be excluded especially in individuals sensitised to muskmelon or pomegranate, but it would not exceed the risk of consuming these foods.

3.5 | Dietary exposure

3.5.1 | Intended use of the food enzyme

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

TABLE 2 Intended uses and recommended use levels of the food enzyme as provided by the applicant²⁸

Food manufacturing process ^a	Raw material (RM)	Recommended use level (mg TOS/kg RM) ^b
Processing of cereals and other grains		
• Production of brewed products	Cereals (malted or unmalted)	15–100
Processing of dairy products		
• Production of modified milk proteins	Liquid whey	2.7–6.7
	Whey proteins	333–833
• Production of flavouring preparations from dairy products	Cheese, cream, butter, etc.	15–100
Processing of plant- and fungal-derived products		
• Production of plant-based analogues of milk and milk products	Cereals, nuts, seeds, etc	17–83
• Production of protein hydrolysates from plants and fungi	Plant and microbial proteins	83–667
• Production of edible oils from algae	Algal cells	17–85
Processing of meat and fish products		
• Production of protein hydrolysates from meat and fish proteins	Meat and fish proteins	83–667
Processing of yeast and yeast products	Yeast	17–85

Abbreviation: TOS, total organic solids.

^aThe 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, 2023).

^bThe numbers in bold were used for calculation.

In the production of brewed products, the food enzyme is added to malted or unmalted grains during the mashing step.²⁹ The subtilisin hydrolyses proteins in the cereals to release free amino nitrogen for the optimal growth of the brewer's yeast during fermentation.³⁰ The food enzyme–TOS remains in the beers.

In the production of modified milk proteins, the food enzyme is added to whey or whey proteins³¹ to achieve the desired degree of hydrolysis. The hydrolysis by subtilisin can also enhance the flavour of the resulting milk protein products (e.g. whey protein hydrolysates, whey concentrates and whey protein isolates), which are subsequently used as ingredients in a variety of foods, including infant formula, follow-on formula and foods for special medical purposes. The food enzyme–TOS remains in the final foods.

²⁷Regulation (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.

²⁸Technical dossier/Additional information May 2023/Appendix A.

²⁹Technical dossier/p. 77.

³⁰Technical dossier/p. 56.

³¹Technical dossier/Additional information May 2023/pp. 7–8.

In the production of flavouring preparations from dairy products, the food enzyme is added to dairy products (e.g. cheese, cream or butter) to modify the sensory properties.³² The food enzyme–TOS remains in these enzyme-modified dairy ingredients (EMDI), which are subsequently used as an ingredient in the formulation of a variety of foods, such as processed cheese, cheese sauce, cheese powder, salad dressing and snack foods. The food enzyme–TOS remains in those EMDIs.

In the production of plant-based analogues of milk and milk products, the food enzyme is added to the slurry of plant materials (e.g. oat flour) during the saccharification step.³³ The food enzyme can also be added to plant-based beverages prior to fermentation to produce fermented plant-based analogues.³⁴ The subtilisin is used to increase yield and to enhance flavours. The food enzyme–TOS remains in the final foods.

In the production of hydrolysed proteins from different sources, the food enzyme is added to the protein isolated from plant, fungal or animal sources during the hydrolysis step.³⁵ The subtilisin is used alone or together with other peptidases to achieve the desired degree of hydrolysis, to increase the yield and to enhance flavours.³⁶ The food enzyme–TOS remains in these protein hydrolysates.

In the production of edible oils from algae, the food enzyme is added to the algae concentrate during the cell lysis step³⁷ to hydrolyse proteins of the algae cell wall.³⁸ The food enzyme–TOS is removed in the final processed foods by repeated washing during refinement (EFSA CEP Panel, 2023).

In the processing of yeast and yeast products, the food enzyme is added during the autolysis step.³⁹ The subtilisin hydrolyses insoluble proteins, optimising the extraction process and improving the sensory properties of the yeast extract,⁴⁰ which are used (in paste or powder form) as an ingredient in a wide range of foods. The food enzyme under application is not used to treat the yeast cell wall.⁴¹ The food enzyme–TOS remains in yeast extracts.

Based on the data provided on thermostability (see Section 3.3.1) and the downstream processing step applied in the food manufacturing processes, it is expected that the enzyme is inactivated or removed in all the food manufacturing processes listed in Table 2.

3.5.2 | Dietary exposure estimation

In accordance with the guidance document (EFSA CEP Panel, 2021), a dietary exposure was calculated only for food manufacturing processes where the food enzyme–TOS remains in the final foods: processing of cereals and other grains for the production of brewed products; processing of dairy products for the production of modified milk proteins and flavouring preparations; processing of plant- and fungal-derived products for the production of plant-based analogues of milk and milk products and protein hydrolysates; processing of meat and fish products for the production of protein hydrolysates and processing of yeast and yeast products.

Chronic exposure to the food enzyme–TOS was calculated by combining the maximum recommended use level with individual consumption data (EFSA CEP Panel, 2021). The estimation involved selection of relevant food categories and application of technical conversion factors (EFSA CEP Panel, 2023). Exposure from all FoodEx categories was subsequently summed up, averaged over the total survey period (days) and normalised for body weight (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 about 2.393 mg TOS/kg bw per day in infants at the 95th percentile.

³²Technical dossier/p. 79.

³³Technical dossier/p. 81.

³⁴Technical dossier/p. 82.

³⁵Technical dossier/p. 84.

³⁶Technical dossier/p. 83.

³⁷Technical dossier/p. 88.

³⁸Technical dossier/p. 87.

³⁹Technical dossier/p. 89.

⁴⁰Technical dossier/p. 89.

⁴¹Technical dossier/Additional information May 2023/p. 1.

TABLE 3 Summary of the 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)	0.080–1.012 (12)	0.095–0.668 (15)	0.068–0.158 (19)	0.014–0.091 (21)	0.022–0.137 (22)	0.008–0.093 (23)
Min–max 95th percentile (number of surveys)	0.195–2.393 (11)	0.317–1.765 (14)	0.187–0.438 (19)	0.044–0.342 (20)	0.083–0.563 (22)	0.037–0.278 (22)

Abbreviation: TOS, total organic solids.

3.5.3 | Uncertainty analysis

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

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

Sources of uncertainties	Direction of impact
Model input data	
Consumption data: different methodologies/representativeness/underreporting/misreporting/no portion size standard	+/–
Use of data from food consumption surveys of a few days to estimate long-term (chronic) exposure for high percentiles (95th percentile)	+
Possible national differences in categorisation and classification of food	+/–
Model assumptions and factors	
Selection of broad FoodEx categories for the exposure assessment	+
The higher use level given for the process ‘production of modified milk proteins’ was used in the calculation	+
In the absence of analytical data to demonstrate the removal of the food enzyme–TOS in infant formulae, follow-on formulae and foods for special medical purposes, ^a these highly regulated formulae were included in the calculation	+/–
The calculation for yeast processing included not only food groups relevant for yeast extracts but also those relevant for yeast cell walls	+
Exposure to food enzyme–TOS was always calculated based on the recommended maximum use level	+
Use of recipe fractions in disaggregation FoodEx categories	+/–
Use of technical factors in the exposure model	+/–
Exclusion of one process from the exposure assessment: – production of edible oils from algae	–

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

^aTechnical dossier/Additional information May 2023/p. 6.

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

The exclusion of one food manufacturing process from the exposure assessment was based on > 99% of TOS removal. This was not expected to have an impact on the overall estimate derived.

3.6 | Margin of exposure

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

4 | CONCLUSION

Based on the data provided, the outcome of the QPS assessment of the production strain and the absence of concerns arising from the production process, the Panel concluded that the subtilisin produced with the non-genetically modified *B. licheniformis* strain NZYM-CX does not give rise to safety concerns under the intended conditions of use.

5 | DOCUMENTATION AS PROVIDED TO EFSA

Subtilisin produced by *Bacillus licheniformis* (strain NZYM-CX). September 2022. Submitted by Novozymes A/S. Additional information. May 2023. Submitted by Novozymes A/S.

ABBREVIATIONS

bw	body weight
CAS	Chemical Abstracts Service
CEF	EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
CEP	EFSA Panel on Food Contact Materials, Enzymes and Processing Aids
EC	European Commission
EINECS	European Inventory of Existing Commercial Chemical Substances
EU	European Union
FAO	Food and Agricultural Organization of the United Nations
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
QPS	qualified presumption of safety
TOS	total organic solids
WGS	whole genome sequencing
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

European Commission

QUESTION NUMBER

EFSA-Q-2022-00545

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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.

How to cite this article: EFSA CEP Panel (EFSA Panel on Food Contact Materials, Enzymes and Processing Aids), Lambr  , C., Barat Baviera, J. M., Bolognesi, C., Cocconcelli, P. S., Crebelli, R., Gott, D. M., Grob, K., Lampi, E., Mengelers, M., Mortensen, A., Rivi  re, G., Steffensen, I.-L., Tlustos, C., Van Loveren, H., Vernis, L., Zorn, H., Roos, Y., Aguilera, J., ... Chesson, A. (2023). Safety evaluation of the food enzyme subtilisin from the non-genetically modified *Bacillus licheniformis* strain NZYM-CX. *EFSA Journal*, 21(11), e8406. <https://doi.org/10.2903/j.efsa.2023.8406>

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*, Serbia*, Slovenia, Spain
Children	From 36 months up to and including 9 years of age	Austria, Belgium, Bulgaria, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Italy, Latvia, the Netherlands, Portugal, Republic of North Macedonia*, Serbia*, Spain, Sweden
Adolescents	From 10 years up to and including 17 years of age	Austria, Belgium, Bosnia and Herzegovina*, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Italy, Latvia, Montenegro*, the Netherlands, Portugal, Romania, Serbia*, Slovenia, Spain, Sweden
Adults	From 18 years up to and including 64 years of age	Austria, Belgium, Bosnia and Herzegovina*, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Montenegro*, the Netherlands, Portugal, Romania, Serbia*, Slovenia, Spain, Sweden
The elderly^a	From 65 years of age and older	Austria, Belgium, Cyprus, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Montenegro*, the Netherlands, Portugal, Romania, Serbia*, Slovenia, Spain, Sweden

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

^aThe 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).