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Safety evaluation of the food enzyme α-amylase from the genetically modified *Bacillus licheniformis* strain NZYM-AY

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Abstract

The food enzyme α-amylase (4-α-D-glucan glucanhydrolase; EC 3.2.1.1) is produced with the genetically modified *Bacillus licheniformis* strain NZYM-AY by Novozymes A/S. 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 starch processing for the production of glucose syrup and other starch hydrolysates, and distilled alcohol production. Since residual amounts of total organic solids are removed by distillation and by the purification steps applied during the production of glucose syrups, dietary exposure estimation was considered unnecessary. The production strain of the food enzyme fulfils the requirements for the qualified presumption of safety (QPS) approach to safety assessment. As no other concerns arising from the manufacturing process have been identified, the Panel considers that toxicological tests are not needed for the assessment of this food enzyme. 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 (other than distilled alcohol production) 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 did not give rise to safety concerns under the intended conditions of use.

Keywords: α-Amylase, 4-α-D-glucan glucanhydrolase, EC 3.2.1.1, glycogenase, *Bacillus licheniformis*, 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 European Union market and intended to remain on that market, as well as all new food enzymes, shall be subjected to a safety evaluation by the European Food Safety Authority (EFSA) and approval via an EU Community list.

The 'Guidance on submission of a dossier on food enzymes for safety evaluation' (EFSA, 2009a) lays down the administrative, technical and toxicological data required.

1.1. Background and Terms of Reference as provided by the requestor

1.1.1. Background as provided by the European Commission

Only food enzymes included in the European Union (EU) Community list may be placed on the market as such and used in foods, in accordance with the specifications and conditions of use provided for in Article 7(2) of Regulation (EC) No 1332/2008 on food enzymes.

An application has been introduced by the applicant "Novozymes A/S" for the authorisation of the food enzyme Alpha-amylase from a genetically modified strain of *Bacillus licheniformis* (strain NZYM-AY).

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

In accordance with Article 29(1)(a) of Regulation (EC) No 178/2002, the European Commission requests the European Food Safety Authority to carry out the safety assessment on the following food enzyme: Alpha-amylase from a genetically modified strain of *Bacillus licheniformis* (strain NZYM-AY), in accordance with Regulation (EC) No 1331/2008 establishing a common authorisation procedure for food additives, food enzymes and food flavourings.

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

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

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

2. Data and methodologies

2.1. Data

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

Additional information was requested from the applicant during the assessment process on 25 October 2021 and was consequently provided (see 'Documentation provided to EFSA').

2.2. Methodologies

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

The 'Scientific Guidance for the submission of dossiers on food enzymes' (EFSA CEP Panel, 2021) has been followed for the evaluation of the application.

3. Assessment

IUBMB nomenclature	α-amylase
Systematic name	4-α-D-glucan glucanohydrolase
Synonyms	Glycogenase, endoamylase, 1,4-α-D-glucan glucanohydrolase, Taka-amylase A
IUBMB No	EC 3.2.1.1
CAS No	9000-90-2
EINECS No	232–565-6

α-Amylases catalyse the hydrolysis of 1,4-α-glucosidic linkages in starch (amylose and amylopectin), glycogen and related polysaccharides and oligosaccharides, resulting in the generation of soluble dextrins and other gluco-oligosaccharides. The food enzyme is intended to be used in starch processing for the production of glucose syrup and other starch hydrolysates, and distilled alcohol production.

3.1. Source of the food enzyme

The α-amylase is produced with the genetically modified bacterium *B. licheniformis* strain NZYM-AY, which is deposited at the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ, Germany), with deposit number [redacted][4] The production strain was identified as *B. licheniformis* [redacted][5]

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) genes and toxigenic activity are verified for the specific strain used (EFSA, 2007; EFSA BIOHAZ Panel, 2020). The absence of cytotoxic activity was confirmed on the production strain using a VERO cell assay and lactate dehydrogenase release as a measure of cellular function.[6] [redacted] did not identify [redacted] antimicrobial resistance genes [redacted][7]

3.1.1. Characteristics of the parental microorganism

The parental strain is *B. licheniformis* Ca63 (DSM 9552). The strain shows no cytotoxic activity in Chinese hamster ovary cells (Pedersen et al., 2002).

3.1.2. Characteristics of introduced sequences

[redacted]

[4] Technical dossier/Annex A2.
[5] Technical dossier/Annex A1.
[6] Technical dossier/Annex A4.
[7] Technical dossier/Additional information April 2022/Annex 5.

[redacted]

3.1.3. Description of the genetic modification process

[redacted][8][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.

[redacted]

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

The identity of the production strain is established as *B. licheniformis*, a species that qualifies for QPS status. As it was shown to be free of AMR genes, not to be cytotoxic and as the genetic modifications applied did not raise safety concerns, the production strain is considered to meet the requirements of the QPS approach for safety assessment.

3.2. Production of the food enzyme

The food enzyme is manufactured according to the Food Hygiene Regulation (EC) No 852/2004[11], with food safety procedures based on hazard analysis and critical control points, and in accordance with current good manufacturing practice.[12]

[8] Technical dossier/Annexes C1-12.
[9] Technical dossier/Additional data April 2022/Annex A6.5.
[10] Technical dossier/Additional data April 2022/Annexes A6.
[11] Regulation (EC) No 852/2004 of the European Parliament and of the Council of 29 April 2004 on the hygiene of food additives. OJ L 226, 25.6.2004, pp. 3–21.
[12] Technical dossier/p. 44.

The production strain is grown as a pure culture using a typical industrial medium in a submerged, [redacted] fermentation system with conventional process controls in place. After completion of the fermentation, the solid biomass is removed from the fermentation broth by filtration, 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 mass material passes the filtration membrane and 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 α-amylase is a single polypeptide chain of [redacted] amino acids.[15] The molecular mass of the mature protein, calculated from the amino acid sequence, is [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, consistent with the expected mass of the enzyme.[16] The food enzyme was tested for glucan 1,4-α-glucosidase, triacylglycerol lipase, peroxidase and protease activities, and only protease activity was detected. No other enzyme activities were reported.[17]

The in-house determination of α-amylase activity is based on hydrolysis of 4,6-ethylidene(G_7)-*p*-nitrophenyl(G_1)-α,D-maltoheptaoside (ethylidene-G_7PNP) by a coupled reaction which results in the release of *p*-nitrophenol (reaction conditions: pH 7, 37°C, 5 min). The enzymatic activity is quantified by measuring the release of *p*-nitrophenol spectrophotometrically at 405 nm. The α-amylase activity is quantified relative to an internal enzyme standard and expressed in Kilo Novozymes Unit (Avantec High strength)/g (KNU(AH)/g).[18]

The food enzyme has a temperature optimum around 70°C (pH 4.5) and a pH optimum around pH 6.0 (95°C).[19] Thermostability was tested after a pre-incubation of the food enzyme for 30 min at different temperatures (pH 4.0). α-Amylase activity was stable up to 70°C and then decreased sharply with no residual activity at 110°C.[20]

3.3.2. Chemical parameters

Data on the chemical parameters of the food enzyme were provided for three batches used for commercialisation (Table 1). The mean total organic solids (TOS) of the three food enzyme batches for commercialisation is 6.5% and the mean enzyme activity/TOS ratio is 42.6 KNU/(AH)/mg TOS.

[13] Technical dossier/p. 44–50.
[14] Technical dossier/Annex 6.
[15] Technical dossier/p. 30 and Annex 1.
[16] Technical dossier/p. 32.
[17] Technical dossier/p. 38 and Annex 3.02–3.05.
[18] Technical dossier/p. 34–35 and Annex 3.01.
[19] Technical dossier/p. 36 and Annex 8.
[20] Technical dossier/Annex 8.

Table 1: Composition of the food enzyme[(c)]

Parameters	Unit	Batches		
		1	2	3
α-amylase activity	KNU/AH)/g batch[(a)]	2,490	3,410	2,340
Protein	%	4.6	5.9	4.2
Ash	%	0.8	0.8	0.8
Water	%	93.1	91.1	94.0
Total organic solids (TOS)[(b)]	%	6.1	8.1	5.2
Activity/mg TOS	KNU(AH)/mg TOS	40.8	42.1	45.0

(a): KNU(AH): Kilo Novozymes Unit (Avantec High Strength) (see Section 3.3.1).
(b): TOS calculated as 100% – % water – % ash.
(c): Technical dossier/p. 31 and Annexes 2.01–2.03 and 9.

3.3.3. Purity

The lead content in the three commercial batches was below 5 mg/kg[21] which complies with the specification for lead as laid down in the general specifications for enzymes used in food processing (FAO/WHO, 2006).[22] In addition, the levels of arsenic, cadmium and mercury were below the limits of detection (LoDs) of the employed methods.[22,23]

The food enzyme complies with the microbiological criteria (for total coliforms, *Escherichia coli* and *Salmonella*)[24] as laid down in the general specifications for enzymes used in food processing (FAO/WHO, 2006).[23] No antimicrobial activity was detected in any of the tested batches (FAO/WHO, 2006).[25]

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

3.3.4. Viable cells and DNA of the production strain

The absence of viable cells of the production strain in the food enzyme was demonstrated [redacted][26]

The absence of recombinant DNA in the food enzyme was demonstrated [redacted][27]

3.4. Toxicological data

As the production strain qualifies for the QPS approach of safety assessment and as no issue of concern arising from the production process of the food enzyme were identified (see Sections 3.1, 3.2 and 3.3), the Panel considers that no toxicological studies other than an assessment of allergenicity are necessary.

3.4.1. 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 the α-amylase produced with the genetically modified B. licheniformis strain NZYM-AY 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,

[21] Technical dossier/p. 32 and Annexes 2.04 and 9.
[22] Technical dossier/p. 11.
[23] LoDs: Pb = 0.5 mg/kg; As = 0.3 mg/kg; Cd = 0.05 mg/kg; Hg = 0.05 mg/kg.
[24] Technical dossier/p. 34 and Annexes 2.08–2.10 and 9.
[25] Technical dossier/p. 32 and Annexes 2.06 and 9.
[26] Technical dossier/Annex D1.
[27] Technical dossier/Additional information April 2022/Annex D2 version2.

one match was found. The matching allergen was Asp o 21, an α-amylase produced by *Aspergillus oryzae* known as an occupational respiratory allergen.[28]

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

α-Amylase from *A. oryzae* (Brisman and Belin, 1991; Sander et al., 1998; Quirce et al., 2002; Brisman, 2002) is known as occupational respiratory allergens associated with baker's asthma. However, several studies have shown that adults with occupational asthma caused by an enzyme (as described for α-amylase from *A. oryzae*) can ingest respiratory allergens 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).

A product that may cause allergies or intolerances (listed in the Regulation (EU) No 1169/2011[29]) is used as raw material ([redacted]).[30] 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 potentially allergenic residues of this protein source is not expected to be present in the food enzyme.

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 (except for distilled alcohol production), 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 processes at the recommended use levels summarised in Table 2.

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

Food manufacturing process(a)	Raw material	Recommended use level(b)
Starch processing for the production of glucose syrup and other starch hydrolysates	Starch	1.88–4.69 mg TOS/kg starch
Distilled alcohol production	Starch	1.88–4.69 mg TOS/kg starch

TOS; total organic solids.
(a): The description has been harmonised 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): Based on the mean enzyme activity/TOS ratio of 42.6 KNU(AH)/mg TOS.
(c): Technical dossier/p. 54.

In starch processing for the production of glucose syrups and other starch hydrolysates, the food enzyme is added during the mixing, during the secondary liquefaction and during the saccharification steps.[31] It degrades gelatinised starch into maltodextrins and dextrins, thus, lowering the viscosity. The food enzyme–TOS is removed from the final glucose syrups by treatment with activated charcoal or similar, and with ion-exchange resins. The same consideration is extended to the production of other starch hydrolysates (EFSA CEP Panel, 2021).

[28] Technical dossier/2nd submission/p. 15, 56–59/ Annex 7.01–7.02.
[29] 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.
[30] Technical dossier/2nd submission/Annex 6.
[31] Technical dossier/p. 70.

In distilled alcohol production, the food enzyme is added during the slurry mixing step, in the liquefaction step and, if needed, also in the pre-saccharification step.[32] It is intended to convert partially liquefied starch into long-chain dextrins, providing suitable substrate for saccharification. The food enzyme–TOS is not carried over with the distilled alcohol (EFSA CEP Panel, 2021).

3.5.2. Dietary exposure estimation

The Panel accepted the evidence provided as sufficient to conclude that the residual amounts of food enzyme–TOS in the final distilled alcohol and glucose syrups is negligible. Consequently, a dietary exposure was not calculated.

4. Conclusions

Based on the data provided and removal of TOS during the intended food production processes, the Panel concludes that the food enzyme α-amylase produced with the genetically modified *B. licheniformis* strain NZYM-AY 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.

5. Documentation as provided to EFSA

1) Alpha-amylase produced by a genetically modified strain of *Bacillus licheniformis* (strain NZYM-AY). August 2021. Submitted by Novozymes A/S.
2) Additional information. April 2022. Submitted by Novozymes A/S.

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[32] Technical dossier/p. 72.

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Abbreviations

AMR	antimicrobial resistance
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
EINECS	European Inventory of Existing Commercial Chemical Substances
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
PCR	polymerase chain reaction
QPS	qualified presumption of safety
SDS–PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
TOS	total organic solids
WHO	World Health Organization
WGS	whole genome sequence