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Safety evaluation of the food enzyme α-amylase from the genetically modified *Bacillus subtilis* strain AR-651

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Abstract

The food enzyme α-amylase (4-α-D-glucan glucanohydrolase; EC 3.2.1.1) is produced with the genetically modified *Bacillus subtilis* strain AR-651 by AB Enzymes. The genetic modifications do not give rise to safety concerns. The food enzyme is considered free from viable cells of the production organism and its DNA. It is intended to be used in baking processes. Dietary exposure to the food enzyme–total organic solids (TOS) was estimated to be up to 1.19 mg TOS/kg body weight (bw) per day in European populations. The production strain carries known antimicrobial resistance genes and consequently, it does not fully fulfil the requirements for the qualified presumption of safety (QPS) approach to safety assessment. However, considering the absence of viable cells and DNA from the production organism in the food enzyme, this is not considered to be a risk. As no other concerns arising from the microbial source and its subsequent genetic modification or 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 three matches with respiratory allergens were found. The Panel considered that, under the intended conditions of use, the risk of allergic sensitisation and elicitation reactions by dietary exposure cannot be excluded, but the likelihood for this to occur is considered to be low. Based on the data provided, the Panel concludes that this food enzyme does not give rise to safety concerns, under the intended conditions of use.

Keywords: α-amylase, 4-α-D-glucan glucanohydrolase, EC 3.2.1.1, glycogenase, *Bacillus subtilis*, genetically modified microorganism

Requestor: European Commission

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Legal notice: Confidential information has been redacted according to Commission Decision of 16.12.2022 on the confidentiality of certain information contained in the application submitted by AB Enzymes GmbH for the authorisation of the food enzyme α-amylase from the genetically modified Bacillus subtilis strain AR-651 under Regulation (EC) No 1332/2008 of 16 December 2008 on food enzymes (EFSA-Q-2021-00307, EC 3.2.1.1, 2021/22).

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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 "AB Enzymes GmbH" for the authorisation of the food enzyme Alpha-amylase from a genetically modified strain of *Bacillus subtilis* (strain AR-651).

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 subtilis* (strain AR-651), 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. subtilis* (strain AR-651).

Additional information was requested from the applicant during the assessment process on 27 January 2022 and subsequently 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 Committee.

The Scientific Guidance for the submission of dossiers on food enzymes (EFSA CEP Panel, 2021a) 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
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 baking processes.

3.1. Source of the food enzyme

The α-amylase is produced with the genetically modified bacterium *B. subtilis* strain AR-651 ([redacted]), which is deposited at the Westerdijk Fungal Biodiversity Institute culture collection (the Netherlands), with the deposit number [redacted][4] The production strain was identified as *B. subtilis* [redacted][6]

The species *B. subtilis* 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 production strain *B. subtilis* AR-651 was found not to be cytotoxic to VERO cells.[7] [redacted] and other antibiotics.[8]

3.1.1. Characteristics of the parental microorganism

[redacted]

[4] Technical dossier/Volume II/Annex 3.
[5] Technical dossier/Volume II/Annex 2.
[6] Technical dossier/additional information April 2022/Annex 1.
[7] Technical dossier/Volume I/Annex 13.
[8] Technical dossier/Volume II/Annex 4 and additional information April 2022/Annex 2.

3.1.2. Characteristics of introduced sequences

[redacted]

3.1.3. Description of the genetic modification

[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.

[redacted] No antimicrobial resistance genes were introduced during the genetic modification.

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

3.2. Production of the food enzyme

The food enzyme is manufactured according to the Food Hygiene Regulation (EC) No 852/2004[9], with food safety procedures based on hazard analysis and critical control points, and in accordance with current good manufacturing practice.[10]

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. 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.[11] 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.

[9] 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.
[10] Technical dossier/Volume I/pg. 19/Annex 8.
[11] Technical dossier/Volume I/pg. 19-27/Annex 10.
[12] Technical dossier/Volume I/pg. 19-20/Annexes: 9, 11, 12.

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.[13] The molecular mass of the mature protein, calculated from the amino acid sequence, is [redacted] kDa.[14] 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 major protein band corresponding to an apparent molecular mass of about [redacted] kDa, consistent with the expected mass of the enzyme. The food enzyme was tested for protease activity and none was detected.[15] No other enzymatic activities were reported.

The in-house determination of α-amylase activity is based on hydrolysis of starch (reaction conditions: pH 5.0, 30°C, 20 min). The released reducing sugars react with *p*-hydroxybenzoic acid hydrazide (PAHBAH). Colour intensity is measured spectrophotometrically at 412 nm. The enzyme activity is expressed in AZ/g. One AZ unit is the amount of enzyme that cleaves glycosidic bonds equivalent to the release of 0.075 mmol of reducing groups under the condition of the assay.[16]

The food enzyme has a temperature optimum around [redacted] and a pH optimum around pH [redacted]. Thermostability was tested after a pre-incubation of the food enzyme for 10 min at 90°C (pH 5.0). No activity was detected after 2 min at 90°C.[17]

3.3.2. Chemical parameters

Data on the chemical parameters of the food enzyme were provided for three batches (Table 1).[18] The mean total organic solids (TOS) of the three food enzyme batches is 6.6% and the mean enzyme activity/TOS ratio is 1.8 AZ/mg TOS.

Table 1: Composition of the food enzyme

Parameters	Unit	Batches		
		1	2	3
α-Amylase activity	AZ/g batch[(a)]	103	129	101
Protein	%	2.4	2.7	2.2
Ash	%	2.2	0.3	1.8
Water	%	92.8	92.5	90.7
Total organic solids (TOS)[(b)]	%	5.0	7.2	7.5
Activity/mg TOS	AZ/mg TOS	2.1	1.8	1.4

(a): AZ: See Section 3.3.1.
(b): TOS calculated as 100% – % water – % ash.

3.3.3. Purity

The lead content in the three batches was below 5 mg/kg which complies with the specification for lead as laid down in the general specifications for enzymes used in food processing (FAO/WHO, 2006).[19] In addition, the concentrations of arsenic, cadmium and mercury were below the limits of detection (LoDs) of the employed methods.[19,20]

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).[19] No antimicrobial activity was detected in any of the tested batches (FAO/WHO, 2006).[19]

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

[13] Technical dossier/Volume I/pg. 10/Annex 2.
[14] Technical dossier/Additional information April 2022.
[15] Technical dossier/Volume I/pg. 15/Annex 3.
[16] Technical dossier/Volume I/Annex 5.
[17] Technical dossier/Volume I/Annex 6.
[18] Technical dossier/Volume I/pg. 12/Annexes: 3, 4, 5.
[19] Technical dossier/Volume I/pg. 12-13/Annexes: 3, 4.
[20] LoDs: Pb, Cd, Hg = 0.025 mg/kg each; As = 0.25 mg/kg.

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][21] [redacted][22]

The absence of recombinant DNA in the food enzyme was demonstrated [redacted][23]

3.4. Toxicological data

Although all other requirements for the QPS have been met, the production strain carries several acquired antimicrobial resistance genes and therefore cannot be considered as suitable for the QPS approach. However, no risk is expected from the presence of these antimicrobial resistance genes in the production strain, as the enzyme has been shown not to contain viable cells and DNA (Section 3.3.4). As no other concerns arising from the microbial source and its subsequent genetic modification or from the manufacturing process have been identified, the Panel considers that no toxicological studies other than assessment of allergenicity are needed for the assessment of this food enzyme.

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. subtilis* strain AR-651 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, three matches were found. The matching allergens were two α-amylases from *Aspergillus oryzae* and one α-amylase from *Periplaneta americana* (American cockroach), all known as respiratory allergens.[24]

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 allergen associated with baker's asthma. However, several studies have shown that adults with respiratory allergy caused by an enzyme (as described for α-amylase from *A. oryzae*) can ingest the 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).

According to the information provided, [redacted] a known source of allergens, is present in the media fed to the microorganisms. 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 these materials employed as protein sources are 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, but the likelihood of such reactions to occur is considered to be low.

[21] Technical dosier/Additional information April 2022/Annex 3.
[22] Technical dosier/Additional information April 2022/Annex 4.
[23] Technical dosier/Volume II/Annex 12.
[24] Technical dosier/Volume I/pg. 35-36/Annex 2.

3.5. Dietary exposure

3.5.1. Intended use of the food enzyme

The food enzyme is intended to be used in baking process at a maximum recommended use level of 100 mg TOS/kg flour.[25]

In baking processes, the food enzyme is added to flour during the preparation of dough. The α-amylase hydrolyses starch and releases dextrins. This reaction reduces viscosity of the dough, which consequently facilitates the handling of the dough and results in more uniform products with better properties (increased firmness, reduced oil absorption and less stockiness). The food enzyme–TOS remains in the dough.

Based on data provided on thermostability (see Section 3.3.1), it is expected that the α-amylase is inactivated during baking.

3.5.2. Dietary exposure estimation

Chronic exposure to the food enzyme–TOS was calculated by combining the maximum recommended use level with individual consumption data (EFSA CEP Panel, 2021a). The estimation involved selection of relevant food categories and application of technical conversion factors (EFSA CEP Panel, 2021b). Exposure from all FoodEx categories was subsequently summed up, averaged over the total survey period (days) and normalised for body weight. This was done for all individuals across all surveys, resulting in distributions of individual average exposure. Based on these distributions, the mean and 95th percentile exposures were calculated per survey for the total population and per age class. Surveys with only one day per subject were excluded and high-level exposure/intake was calculated for only those population groups in which the sample size was sufficiently large to allow calculation of the 95th percentile (EFSA, 2011).

Table 2 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 41 dietary surveys (covering infants, toddlers, children, adolescents, adults and the elderly), carried out in 22 European countries (Appendix B). The highest dietary exposure to the food enzyme–TOS was estimated to be 1.19 mg TOS/kg body weight (bw) per day in infants. The estimation made by the applicant using the FEIM-baking calculator (version 2018)[26] matches the results of EFSA's own calculation.

Table 2: 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)	0.019–0.278 (11)	0.215–0.599 (15)	0.240–0.578 (19)	0.131–0.355 (21)	0.098–0.218 (22)	0.098–0.219 (22)
Min–max 95th percentile (number of surveys)	0.109–1.190 (9)	0.530–1.018 (13)	0.471–1.085 (19)	0.293–0.750 (20)	0.216–0.452 (22)	0.196–0.374 (21)

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 3.

[25] Technical dossier/pg. 30.
[26] Technical dossier/Annex 11.

Table 3: 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	+/–

TOS: total organic solids.
+: Uncertainty with potential to cause overestimation of exposure.
–: Uncertainty with potential to cause underestimation of exposure.

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

3.6. Margin of exposure

Since toxicological tests are considered unnecessary by the Panel, the margin of exposure was not calculated.

4. Conclusions

Based on the data provided, the Panel concludes that the food enzyme α-amylase produced with the genetically modified *B. subtilis* strain AR-651 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 from a genetically modified strain of *Bacillus subtilis*. October 2021. Submitted by AB Enzymes.
2) Additional information. April 2022. Submitted by AB Enzymes.

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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
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://efsa.onlinelibrary.wiley.com/doi/10.2903/j.efsa.2023.7468#support-information-section).

The file contains two sheets, corresponding to two tables.

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

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

Appendix B – Population groups considered for the exposure assessment

Population	Age range	Countries with food consumption surveys covering more than one day
Infants	From 12 weeks on up to and including 11 months of age	Bulgaria, Cyprus, Denmark, Estonia, Finland, France, Germany, Italy, Latvia, Portugal, Slovenia
Toddlers	From 12 months up to and including 35 months of age	Belgium, Bulgaria, Cyprus, Denmark, Estonia, Finland, France, Germany, Hungary, Italy, Latvia, Netherlands, Portugal, Slovenia, Spain
Children	From 36 months up to and including 9 years of age	Austria, Belgium, Bulgaria, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Italy, Latvia, Netherlands, Portugal, Spain, Sweden
Adolescents	From 10 years up to and including 17 years of age	Austria, Belgium, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Italy, Latvia, Netherlands, Portugal, Romania, Slovenia, Spain, Sweden
Adults	From 18 years up to and including 64 years of age	Austria, Belgium, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Netherlands, Portugal, Romania, Slovenia, Spain, Sweden
The elderly[(a)]	From 65 years of age and older	Austria, Belgium, Cyprus, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Netherlands, Portugal, Romania, Slovenia, Spain, Sweden

(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).