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Safety evaluation of a food enzyme with glucan 1,4- α -glucosidase and α -amylase activities from the genetically modified *Aspergillus niger* strain NZYM-BX

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Abstract

The food enzyme with glucan 1,4- α -glucosidase (EC 3.2.1.3) and α -amylase (EC 3.2.1.1) activities is produced with the genetically modified strain of *Aspergillus niger* NZYM-BX by Novozymes A/S. The genetic modifications do not give rise to safety concerns. The food enzyme is free from viable cells of the production organism and its DNA. The food enzyme is intended to be used in starch processing for the production of glucose syrups and distilled alcohol. 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 was not calculated. Genotoxicity tests did not raise a safety concern. The repeated dose 90-day oral toxicity study in rats made with a substitute enzyme was not considered suitable. However, since no exposure was expected from the intended uses, this study was not considered necessary. Similarity of the amino acid sequence of the food enzyme 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 by dietary exposure cannot be excluded, but the likelihood is considered to be low. Based on the data provided, the Panel concluded that this food enzyme does not give rise to safety concerns under the intended conditions of use.

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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 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. 1331/2008² 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 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.

Two applications have been introduced by the companies AB Enzymes and Novozymes A/S for the authorisation of the food enzymes endo 1,4-beta xylanase from a genetically modified strain of *Trichoderma reesei* (strain RF5427), and glucan 1,4- α -glucosidase (principal activity) and alpha amylase (subsidiary activity) from a genetically modified strain of *Aspergillus niger* (strain NZYM-BX) respectively.

Following the requirements of Article 12.1 of Regulation (EC) No 234/2011³ implementing Regulation (EC) No 1331/2008, the Commission has verified that the two applications fall within the scope of the food enzyme Regulation and contain all the elements required under Chapter II of that Regulation.

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.

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 endo 1,4-beta xylanase from a genetically modified strain of *Trichoderma reesei* (strain RF5427), and glucan 1,4- α -glucosidase (principal activity) and alpha amylase (subsidiary activity) from a genetically modified strain of *Aspergillus niger* (strain NZYM-BX) in accordance with Article 17.3 of Regulation (EC) No 1332/2008 on food enzymes.

1.2. Interpretation of the Terms of Reference

The present scientific opinion addresses the European Commission's request to carry out the safety assessment of food enzyme glucan 1,4- α -glucosidase (principal activity) and alpha amylase (subsidiary activity) from a genetically modified strain of *A. niger* (strain NZYM-BX).

2. Data and Methodologies

2.1. Data

The applicant has submitted a dossier in support of the application for authorisation of the food enzyme glucoamylase and alpha-amylase from a genetically modified *A. niger* (strain NZYM-BX). The dossier was submitted on 30 September 2013.

Additional information was requested from the applicant during the assessment process on 2 September 2014 and 22 December 2014 and was consequently provided. Spontaneous additional information was received from the applicant on 4 November 2020 (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) 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 guidances of EFSA Scientific Committees.

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 CEP Panel statement on the exposure assessment of food enzymes (EFSA CEP Panel, 2016).

3. Assessment

The food enzyme under application contains two declared activities:

IUBMB nomenclature	glucan 1,4- α -glucosidase
Systematic name	4- α -D-glucan glucohydrolase
Synonyms	glucoamylase; amyloglucosidase; acid maltase; exo-1,4- α -glucosidase; glucose amylase;
IUBMB No	EC 3.2.1.3
CAS No	9032-08-0
EINECS No	232-877-2

The glucan 1,4- α -glucosidase catalyses the hydrolysis of terminal (1-4)-linked α -D-glucose residues successively from non-reducing ends of amylopectin and amylose with the release of glucose.

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

The α -amylase catalyses 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 dextrans and other malto-oligosaccharides.

The food enzyme is intended to be used in starch processing for the production of syrups and distilled alcohol production.

3.1. Source of the food enzyme

The glucoamylase and the α -amylase are produced with the genetically modified filamentous fungus *A. niger* strain NZYM-BX, which is deposited at the German Collection of Microorganisms and Cell Cultures (DSMZ, Germany), with deposit number [REDACTED].⁴

3.1.1. Characteristics of the parental and recipient microorganisms

The parental strain is [REDACTED]

[REDACTED]

[REDACTED]⁶

The recipient strain [REDACTED]

[REDACTED]

[REDACTED]

3.1.2. Characteristics of the introduced sequences

[REDACTED]

3.1.3. Description of the genetic modification process

The purpose of genetic modification was to enable the production strain to overproduce glucon 1,4- α -glucosidase and α -amylase. [REDACTED]

[REDACTED]

⁴ Technical dossier/GMM dossier/Annex A4.

⁵ Technical dossier/GMM dossier/Annex A3.

⁶ Technical dossier/GMM dossier/Annexes A1 and A2.

⁷ Technical dossier/GMM dossier/Annex C1.

activity is determined by measuring the release of glucose. The glucan 1,4- α -glucosidase activity is quantified relative to an enzyme standard and expressed in Amyloglucosidase Units/g (AGU/g).¹⁷

The glucan 1,4- α -glucosidase has a temperature optimum around 60°C (pH 5.0) and a pH optimum around pH 4.0 (37°C). Thermostability was tested after a pre-incubation of the food enzyme for 30 min at different temperatures (pH 5.0). Glucan 1,4- α -glucosidase activity decreased above 50°C, showing no residual activity above 70°C.¹⁸

3.3.1.2. α -amylase

The in-house determination of α -amylase activity is based on hydrolysis of starch (reaction conditions: pH 2.5, temperature 37°C, reaction time 3 min). The enzymatic activity is determined by measuring the remaining starch, which is dyed with iodine and detected spectrophotometrically at 600 nm. The activity is quantified relative to an internal enzyme standard and expressed in Fungal α -amylase Units/g (FAU(A)/g).¹⁹

Thermostability was tested after a pre-incubation of the food enzyme for 30 min at different temperatures (pH 5.0). α -Amylase activity decreased above 60°C, showing no residual activity at 80°C.²⁰

3.3.2. Chemical parameters

Data on the chemical parameters of the food enzyme were provided for three batches (no. 1-3) used for commercialisation and one batch (no 4) produced for the toxicological tests (Table 1).²¹ The mean total organic solids (TOS) of the three food enzyme batches for commercialisation are 12.1% and the mean enzyme activity/TOS ratios are 1.4 AGU/mg TOS and 0.5 FAU(F)/mg TOS.

Table 1: Compositional data of the food enzyme

Parameters	Unit	1	2	3	4 ^(a)
Glucan 1,4-α-glucosidase activity	AGU/g batch ^(b)	163	164	161	156
α-Amylase activity	FAU(A)/g batch ^(c)	55.5	51.4	55.8	50.9
Protein	%	9.4	8.9	8.4	8.4
Ash	%	0.7	0.7	0.6	0.6
Water	%	86.5	86.9	88.3	88.4
Total organic solids (TOS)^(d)	%	12.8	12.4	11.1	11.0
Glucan 1,4-α-glucosidase activity/mg TOS	AGU/mg TOS	1.27	1.32	1.45	1.42
α-amylase activity/mg TOS	FAU(A)/mg TOS	0.43	0.41	0.50	0.46

(a): Batch used for the genotoxicity studies.

(b): AGU: Amyloglucosidase Units (see Section 3.3.1).

(c): FAU(F): Fungal α -amylase Units (see Section 3.3.1).

(d): TOS calculated as 100% - % water - % ash.

3.3.3. Purity

The lead content in the three commercial batches and in the two batches used for toxicological studies was below 0.5 mg/kg,^{22,23} which complies with the specification for lead (≤ 5 mg/kg), as laid down in the general specifications for enzymes used in food processing (FAO/WHO, 2006). In addition, the levels of arsenic, cadmium and mercury were below the limits of detection of the employed methodologies^{24,25} except for one batch, in which arsenic was found at 0.13 mg/kg. The Panel considered this concentration as not of concern.

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

¹⁷ Technical dossier/pg. 42-43/Annex 3.01.

¹⁸ Technical dossier/pg. 45/Annex 9.01.

¹⁹ Technical dossier/pg. 46-47/Annex 3.02.

²⁰ Technical dossier/pg. 48/Annex 9.02.

²¹ Technical dossier/pg. 37; Spontaneous submission of data November 2020.

²² Technical dossier/pg. 39.

²³ Spontaneous submission of data November 2020.

²⁴ LoDs: Pb = 0.5 mg/kg; As = 0.1 mg/kg; Cd = 0.05 mg/kg; Hg = 0.03 mg/kg.

²⁵ Technical dossier/pg. 39; Spontaneous submission of data November 2020.

Strains of *Aspergillus*, in common with most filamentous fungi, have the capacity to produce a range of secondary metabolites (Frisvad et al., 2017). The presence of ochratoxin A and fumonisin B2 was examined in four food enzyme batches and not detected at the LODs of the applied analytical methods.^{25,26}

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

3.3.4. Viable cells and DNA of the production strain

The absence of the production strain in the food enzyme was demonstrated [REDACTED]

[REDACTED]²⁷

The absence of recombinant DNA in the food enzyme [REDACTED]

[REDACTED]²⁸

3.4. Toxicological data

The food enzyme is intended to be used in starch processing for the production of syrups and distilled alcohol production. In the course of these processes, the food enzyme is removed by the applied purification steps (see Section 3.5) and, consequently, no toxicological studies other than assessment of allergenicity are needed for the assessment of this food enzyme.

The applicant provided a bacterial gene mutation assay (Ames test), and an *in vitro* micronucleus test performed with the food enzyme under assessment (batch 4, Table 1). Batch 4 has a similar protein pattern and chemical purity as the batches used for commercialisation, and thus is considered suitable as a test item. For systemic toxicity, the applicant provided data on a substitute food enzyme glucan 1,4- α -glucosidase produced with the *A. niger* strain BO-1, which is an early ancestor in the strain lineage leading to the production strain NZYM-BX. The genotoxicity tests are reported as supporting evidence, but the repeated dose 90-day oral toxicity study was not considered. This was because a round of chemical mutagenesis was applied in the last step of the development of the production strain NZYM-BX, and, therefore, the test item cannot be considered suitable for the assessment of the food enzyme.

3.4.1. Genotoxicity

3.4.1.1. Bacterial reverse mutation test

A bacterial reverse mutation assay (Ames test) was performed according to Organisation for Economic Co-operation and Development (OECD) Test Guideline 471 (OECD, 1997) and following Good Laboratory Practice (GLP).²⁹ Four strains of *Salmonella* Typhimurium (TA98, TA100, TA1535 and TA1537) and *Es. coli* WP2uvrA were used in the presence or absence of metabolic activation, applying the direct plate incorporation method. Two separate experiments were carried out using six concentrations of the food enzyme (156, 313, 625, 1,250, 2,500 and 5,000 μ g/plate, corresponding to 148, 296, 593, 1185, 2370 and 4740 μ g TOS/plate).³⁰ No cytotoxicity was observed at any concentration level of the test. Upon treatment with the food enzyme, there was no significant increase in revertant colony numbers above the control values in any strain with or without S9-mix.

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

3.4.1.2. *In vitro* micronucleus assay

The *in vitro* micronucleus test was carried out according to OECD Draft Guideline 487 (OECD, 2010) and following GLP.³¹ The experiment was performed in duplicate cultures of human peripheral whole blood lymphocytes. Cells were exposed to the test substance for 3 hours in the presence or absence of

²⁶ LoD: ochratoxin A = 0.003 mg/kg; fumonisin B2 = 0.003 mg/kg.

²⁷ Technical dossier/GMM dossier/Annex E1.

²⁸ Technical dossier/GMM dossier/Annex E2.

²⁹ Technical dossier/Annex 7.01.

³⁰ Technical dossier/Supplementary information to Ames study no. 20088014.

³¹ Technical dossier/Annex 7.02.

S9-mix and harvested 24 hours after the beginning of treatment. Additionally, a continuous 24-hour treatment without S9-mix was included. The food enzyme was tested at 2,450, 3,500 and 5,000 $\mu\text{g/mL}$, corresponding to 269.5, 385 and 550 $\mu\text{g TOS/mL}$. After treatments, no cytotoxicity was observed neither in the presence nor in the absence of S9-mix. In the short-term and continuous treatments in the absence of S9-mix, the frequency of binucleated cells with micronuclei (MNBN) was comparable to the negative controls at all concentrations tested. In the short-term treatment in the presence of S9-mix, a statistically significant increase in MNBN was observed for all concentrations analysed. However, the values of MNBN in the treated cultures were within the 95th percentile of the historical control range and the increase was not concentration-related, therefore, these effects were considered not to be biologically relevant.

The Panel concluded that, under the test conditions employed in this study, the food enzyme did not induce an increase in the frequency of MNBNs in cultured human peripheral blood lymphocytes.

3.4.2. 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 glucan 1,4- α -glucosidase and the α -amylase produced with the genetically modified *A. niger* strain NZYM-BX 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, two matches were found. The matching allergens were Sch c 1, a glucoamylase produced by *Schizophyllum commune*, and Asp o 21, an α -amylase produced by *Aspergillus oryzae*, known as occupational respiratory allergens.³²

No information is available on oral and respiratory sensitisation or elicitation reactions of these glucan 1,4- α -glucosidase and α -amylase activities.

Both glucoamylase from *S. commune* (Toyotome et al., 2014) and α -amylase from *A. oryzae* (Brisman and Belin, 1991; Sander et al., 1998; Quirce et al., 2002; Brisman, 2002) are 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 have been described in the literature that 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). Such information has not been reported for glucoamylase. The Panel noted that an allergic reaction upon oral ingestion of glucan 1,4- α -glucosidase and α -amylase, produced with the genetically modified *A. niger* strain NZYM-BX, in individuals respiratory sensitised to α -amylase cannot be ruled out, but the likelihood of such a reaction to occur is considered to be low.

According to the information provided, substances or products that may cause allergies or intolerances are used as raw materials (██████████) 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 fungal biomass and fermentation solids are removed. Taking into account the fermentation process and downstream processing, the Panel considered that potentially allergenic residues of these foods employed as nitrogen sources are not expected to be present.

Quantifying the risk for allergenicity is not possible in view of the individual susceptibility to food allergens. Allergenicity can be ruled out only if the proteins are fully removed as in the case of distilled alcohol production. In the starch processing for the production of glucose syrups, experimental data showed a significant removal (> 99%) of protein. However, traces of protein could be present in glucose syrup.

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 starch processing, but the likelihood of such reactions to occur is considered to be low.

³² Technical dossier/ Annex 8.

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.

In starch processing, the food enzyme is typically added during the saccharification step where it degrades gelatinised starch into dextrins. The food enzyme can also be used for raw starch hydrolysis where the starch is not completely gelatinised to degrade the dextrins to fermentable sugars.

Table 2: Intended uses and recommended use levels of the food enzyme as provided by the applicant³³

Food manufacturing process ^(a)	Raw material	Recommended dosage of the food enzyme
Starch processing for the production of glucose syrups	starch	Up to 593 mg TOS/kg starch
Distilled alcohol production	cereals	Up to 474 mg TOS/kg cereals

TOS: total organic solids.

(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 typically applied during the pre-saccharification together with other saccharifying enzymes to degrade the dextrins to fermentable sugars. In plants using the simultaneous saccharification and fermentation process, liquefied mash is pumped into the fermenter, where the food enzyme and other saccharifying enzymes are added together with yeast at the beginning of fermenter fill.

3.5.2. Dietary exposure estimation

The technical information and experimental data provided on the removal of food enzyme TOS during distilled alcohol production and starch processing for the production of glucose syrups was considered by the Panel as sufficient to exclude these processes from the exposure assessment (Annex B in EFSA CEF Panel, 2016). Consequently, a dietary exposure was not calculated.

4. Conclusions

Based on the data provided and the removal of TOS during starch processing and distilled alcohol production, the Panel concluded that the food enzyme with glucoamylase and α -amylase activities produced with the genetically modified *A. niger* strain NZYM-BX 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

Glucoamylase and alpha-amylase produced by a genetically modified strain of *Aspergillus niger* (strain NZYM-BX). November 2013. Submitted by 1.1 Novozymes A/S.

Additional information. September 2014. Submitted by Novozymes A/S

Additional information. December 2014. Submitted by Novozymes A/S

Spontaneous additional information. November 2020. Submitted by Novozymes A/S

Summary report on GMM part related to Glucoamylase and alpha-amylase produced from *Aspergillus niger* by Novozymes A/S. January 2015. Delivered by Technical University of Denmark (Lyngby, Denmark).

Summary report on technical part related to Glucoamylase and alpha-amylase produced from *Aspergillus niger* by Novozymes A/S. February 2014. Delivered by Technical University of Denmark (Lyngby, Denmark).

³³ Spontaneous information November 2020.

Summary report on toxicological data related to Glucoamylase and alpha-amylase produced from *Aspergillus niger* by Novozymes A/S. January 2014. Delivered by FoBiG (Freiburg im Breisgau, Germany).

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Abbreviations

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
GLP	Good Laboratory Practice
GMM	genetically modified microorganism
GMO	genetically modified organism
IUBMB	International Union of Biochemistry and Molecular Biology
LoD	limit of detection
MNBN	binucleated cells with micronuclei
OECD	Organisation for Economic Cooperation and Development
PCR	polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TOS	total organic solids
WHO	World Health Organization