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Safety evaluation of the food enzyme α-amylase from a genetically modified *Aspergillus niger* (strain NZYM-SB)

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Abstract

The food enzyme is an α-amylase (4-α-D-glucan glucanohydrolase; EC 3.2.1.1), produced with the genetically modified *Aspergillus niger* strain NZYM-SB by Novozymes A/S. The food enzyme does not contain the production organism or its DNA; therefore, there is no safety concern for the environment. The α-amylase is intended for use in starch processing, beverage alcohol (distilling) processes and baking processes. Residual amounts of total organic solids (TOS) are removed by distillation and by the purification steps applied during the production of glucose syrups (by > 99%). Consequently, dietary exposure was not calculated for these two uses. Based on the maximum use levels recommended for the baking processes and individual consumption data from the EFSA Comprehensive European Food Consumption Database, dietary exposure to the food enzyme–TOS was estimated to be up to 3.075 mg TOS/kg body weight per day in European populations. The food enzyme did not induce gene mutations in bacteria or micronuclei in human lymphocytes. Subchronic toxicity was assessed by means of a repeated-dose 90-day oral toxicity study in rodents. A no observed adverse effect level (NOAEL) was derived that, compared with the dietary exposure, resulted in a sufficiently high margin of exposure (MOE). Similarity of the amino acid sequence to those of known allergens was searched and two matches were found. The Panel considered that the risk of allergic sensitisation and elicitation reactions by dietary exposure cannot be excluded, but the likelihood is considered low. Based on the genetic modifications, the manufacturing process, the compositional and biochemical data, the findings in the toxicological and genotoxicity studies, as well as the estimated dietary exposure, the Panel concluded that this food enzyme does not give rise to safety concerns under the intended conditions of use.

Keywords: food enzyme, α-amylase, 4-α-D-glucan glucanohydrolase, EC 3.2.1.1, *Aspergillus niger*, NZYM-SB, 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 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. 1331/2008[2] established the European Union (EU) procedures for the safety assessment and the authorisation procedure of food additives, food enzymes and food flavourings. The use of a food enzyme shall be authorised only if it is demonstrated that:

- it does not pose a safety concern to the health of the consumer at the level of use proposed;
- there is a reasonable technological need;
- its use does not mislead the consumer.

All food enzymes currently on the EU market and intended to remain on that market, as well as all new food enzymes, shall be subjected to a safety evaluation by the European Food Safety Authority (EFSA) and approval via an EU Community list.

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

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

1.1.1. Background as provided by the European Commission

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

Two applications have been put forward by the company 'Novozymes A/S' for the authorisation of the food enzymes serine protease (with trypsin specificity) from a genetically modified strain of *Fusarium venenatum* (strain NZYM-FG) and α-amylase from a genetically modified strain of *Aspergillus niger* (strain NZYM-SB).

Following the requirements of Article 12.1 of Commission Regulation (EU) No. 234/2011[3] 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 items required under Chapter II of that Regulation.

1.1.2. Terms of Reference

The European Commission (EC) requests the European Food Safety Authority (EFSA) to carry out the safety assessments on the food enzymes serine protease (with trypsin specificity) from a genetically modified strain of *Fusarium venenatum* (strain NZYM-FG) and α-amylase from a genetically

[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, 11.03.2011, pp. 15–24.

modified strain of *Aspergillus niger* (strain NZYM-SB) 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 the food enzyme α-amylase from a genetically modified (GM) strain of *A. niger* (strain NZYM-SB).

1.3. Information on existing authorisations and evaluations

The applicant reports that Danish and French authorities have not yet evaluated and authorised the use of α-amylase from a GM strain of *A. niger* (strain NZYM-SB).

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 GM *A. niger* (strain NZYM-SB). The food enzyme is intended to be used in starch processing for the production of glucose syrups, distilled alcohol production and baking processes.

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, 2009) and following the relevant existing Guidances from the EFSA Scientific Committee.

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

3. Assessment

3.1. Technical data

3.1.1. Identity of the food enzyme

IUBMB nomenclature: α-Amylase
Systematic name: 4-α-D-Glucan glucanohydrolase
Synonyms: Endo-amylase, 1,4-α-D-glucan glucanohydrolase
IUBMB No.: EC 3.2.1.1
CAS No.: 9000-90-2
EINECS No.: 232-565-6.

3.1.2. Chemical parameters

The α-amylase produced with the GM *A. niger* (strain NZYM-SB) consists of a single-polypeptide chain of 580 amino acids. The molecular mass, derived from the amino acid sequence, was calculated to be 63.2 kDa. The protein pattern of the food enzyme was investigated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The gels presented for the four food enzyme batches are comparable, showing multiple protein bands including a band in the 63 kDa region.

The food enzyme was tested for other enzyme activities. Lipase and protease activities were below the detection limits of the employed methods, but glucoamylase activity was detected. However, the Panel did not consider this of being of safety concern.[4]

[4] LODs: glucoamylase = 0.825 AGU/g; lipase = 0.02 KLU/g; protease = 196 HUT/g.

Data on the chemical parameters of the food enzyme have been provided for four food enzyme batches, three batches used for commercialisation and one batch used for the toxicological tests (Table 1). The average total organic solids (TOS) content of the three commercial enzyme batches was 10.4% (w/w); the values ranged from 7.5 to 13.9% (Table 1).

The enzyme activity/mg TOS ratio of the three food enzyme batches ranged from 0.46 to 0.71 Fungal α-Amylase Units (FAU(F))/mg TOS (Table 1). The average value of 0.58 FAU(F)/mg TOS was used for subsequent calculations.

Table 1: Compositional data of the food enzyme

Parameter	Unit	Batches			
		1	2	3	4[(a)]
α-Amylase activity	FAU(F)/g batch[(b)]	63.6	55.9	53.3	53.3
Protein	%	9.3	7.9	7.6	7.8
Ash	%	0.4	0.9	0.8	0.7
Water	%	85.7	89.4	91.7	87.6
Total organic solids (TOS)[(c)]	%	13.9	9.7	7.5	11.7
α-Amylase activity/mg TOS	FAU(F)/mg TOS	0.46	0.58	0.71	0.46

(a): Batch used for the toxicological tests.
(b): FAU(F): fungal alpha-amylase units (relative to an internal standard 'F') (see Section 3.1.3).
(c): TOS calculated as 100% – % water – % ash.

The lead content in the three commercial batches (not more than 5 mg/kg), complies with the specification for lead as laid down in the general specifications and considerations 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.[5]

No antimicrobial activity was detected in any of these batches (FAO/WHO, 2006).

Mycotoxins ochratoxin A and fumonisins are of major concern in species of *A. niger* (Blumenthal, 2004; Frisvad et al., 2007, 2011; EFSA, 2017). The presence of the mycotoxins ochratoxin A and fumonisin B2 was examined in the four food enzyme batches and they were below the limits of detection of the applied analytical methods.[6]

The food enzyme complies with the microbiological criteria as laid down in the general specifications and considerations for enzymes used in food processing (FAO/WHO, 2006), which stipulate that *Escherichia coli* and *Salmonella* species are absent in 25 g of sample and total coliforms should not exceed 30 colony-forming units (CFU) per gram.

The applicant has provided information on the identities of the antifoam agents used. Taking into account the nature and properties of the antifoam agents, the manufacturing process and the quality assurance system implemented by the applicant, the Panel considers their use as of no safety concern.

The Panel considered the compositional data provided for the food enzyme as sufficient.

3.1.3. Properties of the food enzyme

The α-amylase catalyses the hydrolysis of 1,4-α-glycosidic linkages in starch (amylose and amylopectin), resulting in the production of soluble dextrins and other malto-oligosaccharides.

The analytical principle to determine α-amylase activity is based on hydrolysis of the substrate 4,6-ethylidene(G_7)-*p*-nitrophenyl(G_1)-α,D-maltoheptaoside, which is cleaved to glucose and *p*-nitrophenol (reaction conditions: pH 7.15, temperature 37°C, reaction time 5 min). The enzymatic activity is determined by measuring the formation of *p*-nitrophenol spectrophotometrically at 405 nm. The α-amylase activity is measured relative to an internal enzyme standard and expressed in fungal α-amylase units/g (FAU(F)/g).

The α-amylase enzyme has been characterised under different temperature and pH conditions. The α-amylase is active at temperatures up to 80°C (with an optimum of 46–62°C at pH 5.5) and within a pH range of 2–10 (with an optimum of about pH 3 at 30°C).

The thermostability of the α-amylase was tested over the range of 30–90°C after a pre-incubation at the different temperatures at pH 5.5 for 30 min. The activity itself was measured at 30°C and pH

[5] LODs: Pb = 0.5 mg/kg; As = 0.1 mg/kg; Cd = 0.05 mg/kg; Hg = 0.03 mg/kg.
[6] LODs: ochratoxin A and fumonisin B2 = 0.0003 mg/kg.

5.5 using an insoluble blue-dyed cross-linked starch as substrate. The enzyme shows about 100% activity up to approximately 40°C, less than 50% residual activity above 70°C and no activity remains at 80°C after 30 min.

3.1.4. Information of the microbial source material

3.1.4.1. Information on the genetically modified microorganism

The technical dossier contains detailed information on the recipient microorganism, the donor organism and the genetic modification process.

The α-amylase production organism *A. niger* NZYM-SB is deposited at the [redacted] with deposit number [redacted]

3.1.4.2. Characteristics of the parental and recipient microorganisms

Some strains of *A. niger* have a long history of use in the production of food enzymes (Schuster et al., 2002; van Dijck et al., 2003; Park et al., 2017).

The parental strain is *A. niger* [redacted] The first intermediate strain [redacted] derived from the parental strain by [redacted] was found not to produce [redacted] Strain [redacted] was identified as *A. niger* by [redacted]

Recipient strain *A. niger* [redacted] was derived from [redacted] by [redacted]

3.1.4.3. Characteristics of introduced sequences

[redacted]

3.1.4.4. Description of the genetic modification process

[redacted]

3.1.4.5. Safety aspects of the genetic modification

The recipient strain *A. niger* differs from the parental strain

The production strain *A. niger* NZYM-SB differs from the recipient strain

The absence of antibiotic resistance genes used during the genetic modification was confirmed by

Phenotypic stability of the *A. niger* NZYM-SB strain was confirmed by

The genetic stability of the production strain *A. niger* NZYM-SB was demonstrated by

3.1.5. Manufacturing process

The food enzyme is manufactured according to the Food Hygiene Regulation (EC) No. 852/2004[7], with food safety procedures based on Hazard Analysis and Critical Control Points (HACCP), and in accordance with current Good Manufacturing Practice (GMP).

The food enzyme is produced by a pure culture in a contained, fermentation system with conventional process controls in place. The identity and purity of the culture are checked at each transfer step from frozen vials until the end of fermentation.

The food enzyme produced is recovered from the fermentation broth after biomass separation using filtration. Further purification and concentration involve a series of filtration steps, including and After stabilisation with the food enzyme preparation is commercialised in a liquid or in a solid form.

The absence of the production strain in the food enzyme was demonstrated

No DNA was detected

The Panel considered the information provided on the raw materials and the manufacturing process as sufficient.

3.1.6. Safety for the environment

The production strain and its DNA were not detected in the final product. The Panel concluded that there is no safety concern for the environment.

3.1.7. Case of need and intended conditions of use

The original uses proposed by the applicant were starch processing for glucose syrup production, distilled alcohol production and baking and cereal-based processes. In the course of the evaluation process, the applicant informed EFSA about withdrawal of the intended use in cereal-based processes.

The resulting intended uses and the recommended use levels are summarised in Table 2.

[7] 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.

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

Food manufacturing process[(a)]	Raw material	Recommended dosage of the food enzyme
Starch processing for glucose syrups production	Starch dry matter	Up to 200 FAU(F)/kg of starch dry matter, corresponding to 345 mg TOS/kg starch dry matter
Distilled alcohol production	Starch dry matter	Up to 200 FAU(F)/kg of starch dry matter, corresponding to 345 mg TOS/kg starch dry matter
Baking processes	Flour	Up to 160 FAU(F)/kg of flour, corresponding to 274 mg TOS/kg flour

(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 adoption of this opinion.

In starch processing for glucose syrups production, the α-amylase is typically added during the saccharification step when it degrades gelatinised starch into dextrins. The α-amylase can also be used for raw starch hydrolysis where the starch is not completely gelatinised. In this case, the enzyme can be added to the feed tank, where process conditions are adapted to the temperature and pH profile of the enzyme.

In distilled alcohol production, the α-amylase is typically applied during the presaccharification together with other saccharification enzymes (e.g. glucoamylase) to degrade the dextrins to fermentable sugars. In plants using the simultaneous saccharification and fermentation (SSF) process, liquefied mash is pumped into the fermenter, where the α-amylase and other saccharification enzymes are added together with the yeast at the beginning of fermenter fill.

In baking processes, the food enzyme is added to the raw materials during the preparation of the dough. The α-amylase hydrolyses starch from granules that have been damaged during the milling and therefore releasing fermentable sugars and dextrins. These are further degraded by other enzymes. By hydrolysing damaged starch granules, α-amylase liberates water for optimal gluten development and improved gas retention.

3.1.8. Reaction and fate in food

The α-amylase catalyses the hydrolysis of 1,4-α-glycosidic linkages in starch (amylose and amylopectin) resulting in the generation of dextrins and other malto-oligosaccharides.

Experimental data have been provided on the removal (> 99%) of protein in the course of distilled alcohol production and starch processing for the production of glucose syrups (Documentation provided to EFSA No. 5). The Panel considered the evidence as sufficient to conclude that residual amounts of TOS (including substances other than proteins) are removed by distillation. In addition, when taking into account the purification steps applied to the production of glucose syrups i.e. filtration, ion exchange chromatography, treatment with active carbon, the Panel also considers that the amount of TOS in the final glucose syrup is removed to a similar degree.

The data and the information provided indicate that the α-amylase is inactivated during the baking processes under the intended conditions of use.

3.2. Dietary exposure

As residual amounts of TOS are removed by distillation and by the purification steps applied during the production of glucose syrups (by > 99%), a dietary exposure to the food enzyme resulting from these intended uses was not calculated.

For baking processes, dietary exposure estimates were calculated using the methodology described in the CEF Panel statement on the exposure assessment of food enzymes (EFSA CEF Panel, 2016). The assessment of the food processes covered in this opinion involved the selection of relevant food groups and application of process and technical conversion factors (Appendix B). These input data were subject to a stakeholder consultation through open calls[8] and adjusted in accordance with feedback received.

[8] http://www.efsa.europa.eu/en/data/call/161110

3.2.1. EFSA Comprehensive European Food Consumption Database

Since 2010, the EFSA Comprehensive European Food Consumption Database (hereafter the EFSA Comprehensive Database[9]) has been populated with detailed national data on food consumption. Competent authorities in European countries provide EFSA with data on the level of food consumption by individual consumers, as taken from the most recent national dietary survey in their country (EFSA, 2011a).

The food consumption data gathered by EFSA were collected using different methodologies, and thus, direct country-to-country comparisons should be made with caution. Depending on the food category and the level of detail used in exposure calculations, uncertainties might be introduced owing to subjects underreporting and/or misreporting of consumption amounts. Nevertheless, the EFSA Comprehensive Database is the best available source of food consumption data across Europe.

Food consumption data from the population groups, infants, toddlers, children, adolescents, adults and the elderly, were used for the exposure assessment. For the present assessment, food consumption data were available from 33 different dietary surveys carried out in 19 European countries (Appendix A).

Consumption records were codified according to the standardised food classification and description system (FoodEx) (EFSA, 2011b).

3.2.2. Exposure assessment methodology

Chronic exposure was calculated based on individual consumption data, averaged over the total survey period, excluding surveys with only 1 day per subject. 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, 2011a).

The exposure per FoodEx category (Appendix B) was subsequently added to derive an individual total exposure per day. Finally, these exposure estimates were averaged over the number of survey days and normalised for individual body weight (bw), resulting in an individual average exposure/day per kg bw for the survey period. This was carried out for all individuals in the survey and per age class, resulting in distributions of individual average exposure per survey and age class. Based on these distributions, the mean and 95th percentile exposures were calculated per survey for the total population and per age class.

3.2.3. Exposure to food enzyme–TOS according to the intended use proposed by the applicant

Exposure to the food enzyme–TOS was based on intended use and the recommended maximum use levels of the food enzyme–TOS provided by the applicant (Section 3.1.7). Foods/ingredients derived through distilled alcohol production and starch processing were excluded from the analysis as the Panel considered the presence of residual amounts of TOS in distilled alcohol and glucose syrups as negligible (see Section 3.1.8). Therefore, dietary exposure to food enzyme–TOS was calculated from foods produced involving a baking process only.

Relevant food groups and/or individual foods were selected from the Comprehensive Database and were assumed to always contain the food enzyme–TOS at the maximum recommended use level. This will result in an overestimation of exposure to food enzyme–TOS.

To facilitate matching of the reported use levels for baking processes with foods identified in the Comprehensive Database, the selected foods were disaggregated to ingredient level as appropriate and converted into the corresponding raw material i.e. flour and malted barley/starch, via the application of conversion factors (Appendix B). For example, consumption of 100 g of bread was converted into an intake of 70 g flour (recipe fraction of 0.7) and then multiplied by 274 mg TOS/kg flour (Table 2) to arrive at an exposure of 19.18 mg TOS/100 g bread.

Exposure to the food enzyme–TOS was calculated by multiplying values reported for each food category by their respective consumption amount per kilogram of body weight (kg bw) separately for each individual in the database. Table 3 provides an overview of the derived exposure estimates. Average and 95th percentile exposure to the food enzyme–TOS per age class, country and survey are reported in Appendix C – Table 1. The contribution of the food enzyme–TOS from each FoodEx category to the total dietary exposure is indicated in Appendix C – Table 2.

[9] http://www.efsa.europa.eu/en/food-consumption/comprehensive-database

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

Population group	Estimated exposure (mg/kg bw 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)	0.191–0.836 (6)	0.717–1.731 (10)	0.752–1.635 (18)	0.449–1.090 (17)	0.325–0.662 (17)	0.312–0.575 (14)
Min–max 95th percentile (number of surveys)	1.114–2.355 (5)	1.627–2.919 (7)	1.407–3.075 (18)	0.803–2.153 (17)	0.631–1.302 (17)	0.590–1.035 (14)

bw: body weight.

3.2.4. Uncertainty analysis

In accordance with the guidance provided in the EFSA Opinion of the Scientific Committee related to uncertainties in dietary exposure assessment (EFSA, 2007), the following sources of uncertainties have been considered and are summarised in Table 4.

Table 4: Qualitative evaluation of the influence of uncertainties on the dietary exposure estimate

Sources of uncertainties	Direction of impact
	Exposure to food enzyme–TOS
Model input data	
Consumption data: different methodologies/representativeness/ underreporting/misreporting/no portion size standard	+/–
Use of data from food consumption survey 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 likely to contain the food enzyme	+/–
Use of technical factors in the exposure model	+/–

+: 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 a considerable overestimation of the exposure.

3.3. Toxicological assessment

A battery of toxicological tests that includes bacterial gene mutation assay (Ames test), *in vitro* mammalian cell micronucleus test and a repeated-dose 90-day oral toxicity study in rats has been provided. The batch used for toxicological testing (Table 1, batch 4) was considered as suitable by the Panel.

3.3.1. Genotoxicity

3.3.1.1. Bacterial reverse mutation test

To investigate the potential of α-amylase to induce gene mutations, 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), in four strains of Salmonella Typhimurium (TA98, TA100, TA1535 and TA1537) and *E. coli* WP2uvrA pKM101 in the presence and absence of metabolic activation applying the `treat and plate´ assay. Two experiments were carried out in triplicate plating using six different concentrations of the food enzyme (156, 313, 625, 1,250, 2,500, 5,000 μg dry matter/plate, corresponding to approximately 147, 295, 590, 1,179, 2,359, 4,718 μg TOS/plate), appropriate positive controls and sterile deionised water as a negative control. All positive control chemicals showed a significant increase of induced revertant colony numbers, confirming the sensitivity of the tests and the efficacy of the S9-mix. Negative and positive control values were within the historical control ranges. No toxicity was observed at any concentration level of the test substance. Growth stimulation measured as increased viable count was observed in some tested conditions after treatment with the food enzyme; the increase did not result in noticeable increase in the corresponding number of revertant colonies. On treatment with the food enzyme, the numbers of the revertant colonies were comparable to the values observed in the vehicle control groups in any tester strain both in the presence and absence of metabolic activation. The Panel concluded that the food enzyme α-amylase did not induce gene mutations in the bacterial reverse mutation assay under the test conditions employed for this study.

3.3.1.2. *In vitro* mammalian cell micronucleus test

The *in vitro* mammalian cell micronucleus test was carried out according to OECD Test Guideline 487 (OECD, 2010) and following GLP. Whole blood cultures were treated with purified water (negative control), the food enzyme or appropriate positive controls both in the presence and absence of metabolic activation. Based on the results obtained in a cytotoxicity-range finding test, the cells were treated with 3,000, 4,000 and 5,000 μg food enzyme/mL (corresponding to 351, 468, 585 μg TOS/mL), applying a short-term treatment (3 h followed by 21 h of recovery) in the presence and absence of S9-mix, and a continuous treatment (24 + 24 h of recovery) in the absence of S9-mix. No significant changes in osmolarity and pH were detected. Two thousand cells were scored per experimental point. The positive controls induced statistically significant increases in micronucleus frequency and the system was considered sensitive and valid. Negative controls were within the historical vehicle control ranges. No significant increase of cytotoxicity was observed at the highest concentration tested (4, 0 and 0% after short-term treatment with or without S9-mix, and continuous treatment, respectively). No statistically significant increase in the frequency of micronuclei was observed in the treated cultures compared to the negative controls in all the tested conditions. The Panel concluded that the food enzyme α-amylase did not induce micronuclei under the test conditions employed for this study.

The Panel concludes on the basis of the *in vitro* studies that there is no concern for genotoxicity for the food enzyme tested.

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

The repeated-dose 90-day oral toxicity study in rodents was performed in accordance with OECD Test Guideline 408 (OECD, 1998) and following GLP. Groups of 10 male and 10 female Sprague–Dawley (Crl:CD(SD)) rats received via oral gavage the food enzyme (batch 4) at dose levels of 122, 400 or 1,220 mg TOS/kg bw per day for 90 days. Controls received the vehicle (water).

One high-dose female was sacrificed for welfare reasons, necropsy showed some findings that were not treatment related.

Group mean high-beam scores (rearing activity) for high-dose males were statistically significantly decreased at 24 min. There was no corresponding decrease in low-beam scores (cage floor activity), which were, in contrast, higher than those of controls for all treated males (18 min). Motor activity scores for all treated females showed a statistically significant decrease in group mean high- and low-beam scores at 48 min. These effects were incidental and not considered to be of safety concern.

Statistically significant increases in haematocrit (4.85%), haemoglobin concentration (4.3%) and eosinophil counts (50%) were observed in high-dose females. These changes were considered as not toxicologically relevant and within historical control ranges.

No other significant effects were observed.

Overall, the Panel derived a No observed adverse effect level (NOAEL) at the high-dose level of 1,220 mg TOS/kg bw per day for both males and females.

A comparison of the NOAEL (1,220 mg TOS/kg bw per day) from the 90-day study with the derived exposure estimates in six human population groups of 0.191–1.731 mg TOS/kg bw per day at the mean and 0.590–3.075 mg TOS/kg bw per day at the 95th percentile, resulted in a margin of exposure (MOE) of at least 397, indicating that there is no toxicological concern.

3.4. 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 GM *A. niger* (strain NZYM-SB) 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 are Asp o 21, an α-amylase produced by *Aspergillus oryzae* and Sch c 1, a glucoamylase produced by *Schizophyllum commune*.

Both glucoamylase from *S. commune* (Toyotome et al., 2014) and α-amylase from *A. oryzae* (Brisman and Belin, 1991; Quirce et al., 1992, 2002; Sander et al., 1998; Brisman, 2002) are known as occupational respiratory allergens associated with baker's asthma. However, several studies have shown that adults with occupational asthma to a food 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). Taking into account the wide use of α-amylase as food enzyme, only a low number of case reports has been described in 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.

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

The Panel noted that an allergic reaction upon oral ingestion of this α-amylase produced with the GM *A. niger* strain NZYM-SB, in individuals respiratory sensitised to α-amylase or to glucoamylase 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 ([redacted]) or intolerances (Regulation EU 1169/2011[10]) are used as raw materials in the growth medium of the production organism. However, during the fermentation, these products will be degraded and utilised by the fungus for cell growth, cell maintenance and production of enzyme protein. In addition, the fungal biomass and fermentation solids will be removed. Taking into account the fermentation process and downstream processing, the Panel considered that potentially allergenic residues of these foods employed as protein 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 (e.g. in distilled alcohol production). In the starch processing for the production of glucose syrups, although experimental data showed a significant removal (> 99%) of protein, traces amount of protein, estimated to be about 0.5 mg/kg, could be present in glucose syrup. Products such as candy and ice creams can contain about 50% and 40% glucose syrup, respectively, and therefore, proteins could be present in a quantity sufficient to elicit an allergic reaction.

The Panel considers that under the intended condition 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.

[10] 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.

Conclusions

Based on the genetic modifications performed, the data provided on the manufacturing process, the compositional and biochemical data provided, the dietary exposure assessment as well as the findings in the toxicological studies, the Panel concludes that the food enzyme α-amylase produced with the GM *A. niger* (strain NZYM-SB) by Novozymes A/S does not give rise to safety concerns under the intended conditions of use.

Regarding the allergenicity assessment, the risk of allergic sensitisation and elicitation reactions upon dietary exposure to this food enzyme cannot be excluded, but the likelihood of such reaction can be considered to be low.

Documentation provided to EFSA

1) Dossier 'Application for authorisation of α-amylase produced by a genetically modified strain of *Aspergillus niger* (strain NZYM-SB)'. 15 May 2014. Submitted by Novozymes A/S.
2) Summary report on toxicological data for α-amylase produced by a genetically modified strain of *Aspergillus niger* (strain NZYM-SB). Delivered by FoBiG GmbH (Freiburg, Germany) on 3 December 2014.
3) Additional information received by 5 October 2017. Submitted by Novozymes A/S.
4) Additional information received by 22 February 2018. Submitted by Novozymes A/S.
5) AMFEP (Association of Manufacturers and Formulators of Enzyme Products), 2017. `Food enzyme removal during the production of cereal based distilled alcoholic beverages' and 'Food enzyme carry-over in glucose syrups'. 22 February 2017. Unpublished document.

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Abbreviations

AMFEP	Association of Manufacturers and Formulators of Enzyme Products
bp	a base pair
bw	body weight
CAS	Chemical Abstracts Service
CEF	EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
CFU	Colony-forming units
[redacted]	[redacted]
EINECS	European Inventory of Existing Commercial Chemical Substances
FAO	Food and Agricultural Organization of the United Nations
FAU(F)	Fungal α-amylase units (F standard)
FoodEx	A standardised food classification and description system
FOA	5-Fluoro-orotic acid
GLP	Good laboratory practice
GM	Genetically modified
GMO	Genetically modified organisms
GMP	Good manufacturing practice
HACCP	Hazard Analysis and Critical Control Points
IUBMB	International Union of Biochemistry and Molecular Biology
[redacted]	[redacted]
kDa	kiloDalton
MOE	Margin of exposure
NOAEL	No observed adverse effect level
OECD	Organisation for Economic Cooperation and Development
PCR	Polymerase chain reaction
S9	Metabolic activation
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SSF	Simultaneous Saccharification and Fermentation
TOS	Total organic solids
[redacted]	[redacted]
WHO	World Health Organisation
w/w	weight for weight

Appendix A – Population groups considered for the exposure assessment

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

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

Appendix B – FoodEx categories used to derive exposure estimates for the food enzyme–TOS and the respective conversion factors

FoodEx code	FoodEx category	Conversion factor from FoodEx food group to raw material[a]	Recipe fraction[b]	mg TOS/kg flour
A.01	Grains and grain-based products (unspecified)	0.8	1	274
A.01.03	Grain milling products (unspecified)	1	1	274
A.01.03.001	Wheat milling products (unspecified)	1	1	274
A.01.03.001.001	Wheat flour, brown	1	1	274
A.01.03.001.002	Wheat flour, Durum	1	1	274
A.01.03.001.003	Wheat flour, white	1	1	274
A.01.03.001.004	Wheat flour, wholemeal	1	1	274
A.01.03.001.005	Graham flour	1	1	274
A.01.03.001.006	Wheat flour, gluten free	1	1	274
A.01.03.001.014	Wheat starch	1.2	1	274
A.01.03.002	Rye milling products (unspecified)	1	1	274
A.01.03.002.001	Rye flour, gluten free	1	1	274
A.01.03.002.002	Rye flour, light	1	1	274
A.01.03.002.003	Rye flour, medium	1	1	274
A.01.03.002.004	Rye flour, wholemeal	1	1	274
A.01.03.003	Buckwheat milling products (unspecified)	1	1	274
A.01.03.003.001	Buckwheat flour	1	1	274
A.01.03.004	Corn milling products (unspecified)	1	1	274
A.01.03.004.001	Corn flour	1	1	274
A.01.03.004.003	Corn starch	1.3	1	274
A.01.03.005	Oat milling products (unspecified)	1	1	274
A.01.03.005.002	Oat flour	1	1	274
A.01.03.005.004	Oat starch	1.2	1	274
A.01.03.006	Rice milling products (unspecified)	1	1	274
A.01.03.006.001	Rice flour	1	1	274
A.01.03.006.002	Rice flour white	1	1	274
A.01.03.006.003	Rice flour, instant	1	1	274
A.01.03.006.004	Rice starch	1.2	1	274
A.01.03.007	Spelt milling products	1	1	274
A.01.03.008	Other milling products (unspecified)	1	1	274
A.01.03.008.001	Amaranth flour	1	1	274
A.01.03.008.002	Barley flour	1	1	274
A.01.03.008.003	Chapatti flour	1	1	274
A.01.03.008.004	Flour mix, wheat/rye/barley/oats	1	1	274
A.01.03.008.005	Millet flour	1	1	274
A.01.03.008.007	Sorghum flour	1	1	274
A.01.04	Bread and rolls (unspecified)	1	0.7	274
A.01.04.001	Wheat bread and rolls	1	0.7	274
A.01.04.002	Rye bread and rolls	1	0.7	274
A.01.04.003	Mixed wheat and rye bread and rolls	1	0.7	274
A.01.04.004	Multigrain bread and rolls	1	0.7	274
A.01.04.005	Unleavened bread, crisp bread and rusk (unspecified)	1	0.8	274

FoodEx code	FoodEx category	Conversion factor from FoodEx food group to raw material[a]	Recipe fraction[b]	mg TOS/kg flour
A.01.04.005.001	Crisp bread, rye wholemeal	1	0.9	274
A.01.04.005.002	Crisp bread, rye, light	1	0.9	274
A.01.04.005.003	Crisp bread, wheat, wholemeal	1	0.9	274
A.01.04.005.004	Crisp bread, wheat, light	1	0.9	274
A.01.04.005.005	Rusk, light	1	0.9	274
A.01.04.005.006	Rusk, wholemeal	1	0.9	274
A.01.04.005.007	Pita bread	1	0.7	274
A.01.04.005.008	Matzo	1	0.9	274
A.01.04.005.009	Tortilla	1	0.7	274
A.01.04.006	Other bread	1	0.7	274
A.01.04.007	Bread products	1	0.7	274
A.01.07	Fine bakery wares (unspecified)	1	0.5	274
A.01.07.001	Pastries and cakes (unspecified)	1	0.5	274
A.01.07.001.001	Beignets	1	0.15	274
A.01.07.001.002	Buns	1	0.7	274
A.01.07.001.003	Cake from batter	1	0.25	274
A.01.07.001.004	Cheese cream cake	1	0.24	274
A.01.07.001.005	Cheese cream sponge cake	1	0.24	274
A.01.07.001.006	Chocolate cake	1	0.24	274
A.01.07.001.007	Chocolate cake with fruits	1	0.24	274
A.01.07.001.008	Cream cake	1	0.24	274
A.01.07.001.009	Cream cheese cake	1	0.24	274
A.01.07.001.010	Cream custard cake	1	0.24	274
A.01.07.001.011	Cream custard sponge cake	1	0.24	274
A.01.07.001.012	Croissant	1	0.5	274
A.01.07.001.013	Croissant, filled with chocolate	1	0.5	274
A.01.07.001.014	Croissant, filled with cream	1	0.5	274
A.01.07.001.015	Croissant, filled with jam	1	0.5	274
A.01.07.001.016	Croquembouche	1	0.15	274
A.01.07.001.017	Doughnuts	1	0.24	274
A.01.07.001.018	Clair	1	0.15	274
A.01.07.001.019	Flan	1	0.5	274
A.01.07.001.020	Fruit cake	1	0.6	274
A.01.07.001.021	Fruit pie	1	0.15	274
A.01.07.001.022	Cheese pie	1	0.15	274
A.01.07.001.023	Fruit tart	1	0.15	274
A.01.07.001.024	Gingerbread	1	0.6	274
A.01.07.001.025	Gougère	1	0.15	274
A.01.07.001.026	Kringles	1	0.25	274
A.01.07.001.027	Nut cream cake	1	0.24	274
A.01.07.001.028	Pancakes	1	0.25	274
A.01.07.001.029	Profiterole	1	0.15	274
A.01.07.001.030	Pyramid cake	1	0.25	274
A.01.07.001.031	Rhubarb flan	1	0.15	274
A.01.07.001.032	Scone	1	0.5	274
A.01.07.001.033	Sponge dough	1	0.25	274
A.01.07.001.034	Sponge cake	1	0.25	274

FoodEx code	FoodEx category	Conversion factor from FoodEx food group to raw material[a]	Recipe fraction[b]	mg TOS/kg flour
A.01.07.001.035	Sponge cake roll	1	0.25	274
A.01.07.001.036	Muffins	1	0.25	274
A.01.07.001.037	Waffles	1	0.25	274
A.01.07.001.038	Apple strudel	1	0.15	274
A.01.07.001.039	Cream-cheese strudel	1	0.24	274
A.01.07.001.040	Cheese pastry goods from puff pastry	1	0.15	274
A.01.07.001.041	Croissant from puff pastry	1	0.6	274
A.01.07.001.042	Brioche	1	0.5	274
A.01.07.001.044	Lebkè	1	0.6	274
A.01.07.001.045	Dumpling	1	0.5	274
A.01.07.001.046	Cake marbled, with chocolate	1	0.5	274
A.01.07.001.047	Marzipan pie	1	0.25	274
A.01.07.001.048	Baklava	1	0.15	274
A.01.07.002	Biscuits (cookies)	1	0.9	274
A.01.07.002.001	Biscuits, sweet, plain	1	0.9	274
A.01.07.002.002	Biscuits, chocolate filling	1	0.81	274
A.01.07.002.003	Biscuits, cream filling	1	0.81	274
A.01.07.002.004	Biscuits, fruit filling	1	0.81	274
A.01.07.002.005	Biscuits, vanilla filling	1	0.81	274
A.01.07.002.006	Butter biscuits	1	0.81	274
A.01.07.002.007	Biscuit, iced	1	0.81	274
A.01.07.002.008	Speculaas	1	0.9	274
A.01.07.002.009	Biscuits, sweet, wheat wholemeal	1	0.9	274
A.01.07.002.010	Biscuits, oat meal	1	0.9	274
A.01.07.002.011	Biscuits, spelt meal	1	0.9	274
A.01.07.002.012	Biscuits, salty	1	0.9	274
A.01.07.002.013	Biscuits, salty, with cheese	1	0.81	274
A.01.07.002.014	Sticks, salty	1	0.81	274
A.17.03.003	Biscuits, rusks and cookies for children	1	0.9	274
A.18.04.001	Find bakery products for diabetics	1	0.5	274
A.19.01.001	Sandwich and sandwich-like meal	1	0.32	274
A.19.01.002	Pizza and pizza-like pies	1	0.3	274

FoodEx: A standardised food classification and description system; TOS: total organic solids.
(a): Available online: http://www.fao.org/fileadmin/templates/ess/documents/methodology/tcf.pdf (FAO, 2000)
(b): Derived from publically available recipe information, and/or food label information (such as Mintel Global New Products Database: http://www.mintel.com/global-new-products-database).

Appendix C – Dietary exposure estimates to the food enzyme–TOS in details

Information provided in this appendix is shown in an excel file (downloadable http://onlinelibrary.wiley.com/wol1/doi/10.2903/j.efsa.2018.5320/suppinfo). The file contains two sheets, corresponding to two tables.

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

Table 2: The contribution of the food enzyme–TOS from each FoodEx category to the total dietary exposure