Safety evaluation of the food enzyme pullulanase from genetically modified Bacillus subtilis strain NZYM-AK

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Safety evaluation of the food enzyme pullulanase from genetically modified *Bacillus subtilis* strain NZYM-AK

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Abstract

The food enzyme considered in this opinion is a pullulanase (pullulan 6-α-glucanohydrolase; EC 3.2.1.41), produced with the genetically modified *Bacillus subtilis* strain NZYM-AK by Novozymes A/S (Denmark). The pullulanase food enzyme is intended to be used in starch processing for the production of glucose syrups. Since the residual amounts of total organic solids (TOS) in glucose syrups after filtration and purification during starch processing were considered negligible, no dietary exposure was calculated. Genotoxicity tests made with the food enzyme indicated no genotoxic concern. A repeated dose 90-day oral toxicity study in rodents, carried out with a pullulanase produced with a predecessor strain, showed no concern with respect to systemic toxicity. The allergenicity was evaluated by searching for similarity of the amino acid sequence to those of known allergens; no match was found. The Panel considers that there are no indications for allergic reactions. Based on the microbial source, genetic modifications performed, the manufacturing process, the compositional and biochemical data provided, the findings in the toxicological studies and allergenicity assessment, the Panel concludes that this food enzyme does not give rise to safety concerns under the intended conditions of use.

Keywords: food enzyme, pullulanase, pullulan 6-α-glucanohydrolase, EC 3.2.1.41, *Bacillus subtilis*, genetically modified microorganism

Requestor: European Commission

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Note: The full opinion will be published in accordance with Article 12(3) of Regulation (EC) No 1331/2008 once decision on confidentiality will be received from the European Commission. The following information has been provided under the confidentiality framework and has been redacted awaiting the decision of the Commission: monitoring of the production strain; manufacturing process and raw materials used; and genetic modifications.

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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 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, and
- 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 a food enzyme for 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. According to this Regulation, a food enzyme that falls within the scope of Regulation (EC) No 1829/2003[3] on genetically modified food and feed should be authorised in accordance with that Regulation as well as under this Regulation.

An application has been introduced by Novozymes A/S for authorisation of the food enzyme pullulanase obtained by fermentation with the genetically modified *Bacillus subtilis* NZYM-AK.

Following the requirements of Article 12.1 of Commission Regulation (EU) No 234/2011[4] 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

The European Commission requests EFSA to carry out the safety assessment on the food enzyme pullulanase obtained with the genetically modified *Bacillus subtilis* strain NZYM-AK in accordance with Article 17.3 of Regulation (EC) No 1332/2008 on food enzymes.

[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, p. 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, p. 1–6.

[3] Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. OJ L 268, 18.10.2003, p. 1–23.

[4] 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, p. 15–24.

1.2. Information on existing authorisation and evaluations

According to the applicant, the French and Danish food authorities have evaluated and authorised the use of pullulanase from a predecessor production strain in the same strain lineage, in a number of food and beverage manufacturing processes. The Danish authorities specify the conditions of use, including the dosage for specific foods, which were up to a level of 675 pullulanase units NPUN[5]/kg starch for glucose syrup, beer and beverage alcohol.

2. Data and methodologies

2.1. Data

The applicant has submitted a dossier in support of the application for authorisation of the food enzyme pullulanase produced with a genetically modified *B. subtilis* (strain NZYM-AK). The food enzyme is intended for use in starch processing for the production of glucose syrups.[6]

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 Guidances from the EFSA Scientific Committee.

The current guidance on the submission of a dossier for safety evaluation of a food enzyme (EFSA, 2009a) has been followed by the CEF Panel 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:	Pullulanase
Systematic name:	Pullulan 6-α-glucanohydrolase
Synonyms:	α-Dextrin endo-1,6-α-glucosidase, amylopectin 6-glucanohydrolase
IUBMB No:	EC 3.2.1.41
CAS No:	9075-68-7
EINECS No:	232-983-9.

3.1.2. Chemical Parameters

The pullulanase produced with the genetically modified *Bacillus subtilis* strain NZYM-AK is a single polypeptide of 928 amino acids. The molecular mass of 101.5 kDa was deduced from the amino acid sequence. The protein homogeneity of the food enzyme was investigated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The gel showed a single major protein band at about 100 kDa, together with a number of other bands with lower staining intensity.

The food enzyme was tested for other enzyme activities in the food enzyme product, i.e. α-amylase, glucoamylase, lipase and protease, which were below the limits of detection. No other enzymatic side activities have been reported by the applicant.

Data on the chemical parameters of the food enzyme have been provided for three commercial food enzyme batches and one batch to be used for toxicological tests (Table 1). The average total organic solids (TOS) content of the three commercial food enzyme batches was 11.6%; the values ranged from 10.5% to 13.0%.

The average enzyme activity/TOS ratio of the batches was 64 New Pullulanase Unit Novozymes per mg TOS (NPUN/mg TOS), see Section 3.1.3; the values ranged from 46 to 77 NPUN/mg TOS (Table 1).

[5] New Pullulanase Unit Novozymes (see Section 3.1.2).

[6] European Commission 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.

Table 1: Compositional data of the food enzyme

Parameter	Units	Batches			
		1	2	3	4[(a)]
Pullulanase activity	NPUN/g batch[(b)]	9,140	8,050	5,240	8,070
Protein	%	6.6	5.8	5.3	NA[(c)]
Ash	%	2.2	1.8	2.0	2.3
Water	%	84.8	87.7	86.6	85.8
Total Organic Solids (TOS)[(d)]	%	13.0	10.5	11.4	11.9
Pullulanase activity/mg TOS	NPUN/mg TOS	70	77	46	68

(a): Batch for the Genotoxicity studies.
(b): NPUN/g batch: New Pullulanase Unit Novozymes (see Section 3.1.3).
(c): NA: Not analysed.
(d): TOS calculated as 100% – % water – % ash.

The applicant provided analytical data on potential contaminants in the three commercial batches of the food enzyme and a further batch used for toxicological studies. Results showed that the lead content (< 0.5 mg/kg) was below the specification for lead (≤ 5 mg/kg) as laid down in the general specifications and considerations for enzymes used in food processing (FAO/WHO, 2006). Furthermore, the levels of arsenic, cadmium and mercury were below their respective levels of detection (As: 0.3 mg/kg; Cd, Hg: 0.05 mg/kg). No antimicrobial activity was detected in any of these batches (FAO/WHO, 2006).

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 are not more than 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 pullulanase catalyses the hydrolysis of 1,6-α-D-glucosidic linkages in pullulan and partially hydrolysed amylopectin as well as α- and β-amylase limit dextrins of amylopectin, resulting in the generation of maltotetraose and maltotriose with reducing carbohydrate ends.

The pullulanase activity is quantified in a multistep reaction. In the first step, the enzyme is added to borohydride (BH_4), reduced pullulan (5.3 g/L) in buffer (pH 5.0, 50°C, 9 min), resulting in the hydrolysis of 1,6-α-D-glucosidic linkages and release of maltotriose units. The reaction is stopped by the addition of a buffer containing a glucokinase (pH 9.6). This phosphorylates glucose to the non-reducing D-glucose-6-phosphate, leaving maltotriose as the only source of reducing groups. Then bismuth acetate/*p*-hydroxy-benzoic acid hydrazide reacts with the reducing groups of maltotriose, generating a yellow complex measured at 405 nm. The activity is expressed as NPUN/g. The international unit correspondence is described as follows: 1 NPUN = 0.35 PUN (Pullulanase Unit Novozymes), where 1 PUN is defined as the amount of enzyme, which under the standard conditions releases 1 μmol of glucose per minute.

The effect of temperature and pH on the activity of pullulanase has been characterised. The enzyme is active at temperatures at least up to 60°C (with an optimum of 50°C, at pH 5.5). The pullulanase exhibits activity in the pH range 4.0–7.0 (with an optimum of pH 6.0, at 30°C). The thermostability of the pullulanase was tested after a 30 min pre-incubation (pH 5.5). The enzyme stability decreases rapidly above 60°C, with no enzymatic activity remaining at 70°C. The activity itself was measured under standard assay conditions.

3.1.4. Information of the microbial source material

3.1.4.1. Information relating to the genetically modified microorganism

The pullulanase production strain *Bacillus subtilis* NZYM-AK is deposited in the DSMZ with the deposit number [redacted]

3.1.4.2. Characteristics of the recipient or parental microorganism

The parental microorganism is the bacterium *B. subtilis*, strain A164 [redacted].

B. subtilis species is included in the list of bacterial species considered suitable for a Qualified Presumption of Safety (QPS) approach to safety assessment, with the qualification that the absence of acquired antibiotic resistance genes and toxigenic activity are verified for the specific strain used (EFSA BIOHAZ Panel 2017a,b). The identity of the parental strain has been confirmed. The parental strain has been tested for the absence of cytotoxicity in VERO cells. An intermediate strain has been tested both in CHO-K1 (Pedersen et al., 2002) and in VERO cells. Both proved negative. [redacted] Consequently, the parental strain is presumed to be safe for production purposes.

The recipient strain, *B. subtilis* [redacted], has been developed from the parental strain A164 [redacted]

3.1.4.3. Characteristics of the donor organisms

[redacted]

3.1.4.4. Description of the genetic modification process

The production strain NZYM-AK was developed from the recipient strain [redacted]

[redacted]

3.1.4.5. Safety aspects of the genetic modification

[redacted]

3.1.5. Manufacturing process

The food enzyme is manufactured with food safety procedures based on Hazard Analysis and Critical Control Points (HACCP), in accordance with current Good Manufacturing Practice (GMP) and, when produced in the EU, in accordance with the Food Hygiene Regulation (EC) 852/2004[7].

The food enzyme is produced by a pure culture in a contained, submerged, fed-batch fermentation system with conventional process controls in place. The identity and the purity of the culture are checked at each transfer step and at regular and critical steps until the end of fermentation.

The downstream processing includes recovery, purification, concentration and stabilisation. The food enzyme produced is recovered from the fermentation broth after biomass separation via press filtration. The liquor is then filtered to remove the remaining microorganisms, and concentrated by ultrafiltration, which removes the low-molecular-weight material.

Subsequently, the food enzyme concentrate is formulated and commercialised as a liquid or a solid preparation. To this end, the concentrated food enzyme solution is stabilised by the addition of preservatives [redacted], and finally subjected to polish- and germ-filtration.

The absence of the production strain in the product was demonstrated [redacted].

No recombinant DNA was detected in three independent batches in triplicate [redacted]

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

3.1.6. Safety for the environment

Neither the production strain nor its recombinant DNA was detected in the final product. Accordingly, no environmental risk assessment is required (EFSA GMO Panel, 2011).

[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, p. 3–21.

3.1.7. Reaction and fate in food

The pullulanase catalyses the hydrolysis of 1,6-α-D-glucosidic linkages in pullulan and partially hydrolysed amylopectin as well as α- and β-amylase limit dextrins of amylopectin, resulting in the generation of maltotetraose and maltotriose with reducing carbohydrate ends. These products are naturally present in starch-containing foods.

The information and data provided indicate that the pullulanase is removed during processing under the intended conditions of use (starch processing: filtration, carbon treatment and ion exchange).

The food enzyme was tested for the presence of other enzyme activities, i.e. α-amylase, glucoamylase, lipase and protease, which were below the limits of detection. No other side activities have been reported by the applicant.

3.1.8. Case of need and proposed conditions of use

As proposed by the applicant, the food enzyme pullulanase is intended for use in starch processing for the production of glucose syrups containing maltose, at an intended use level of up to 78.1 mg TOS/kg starch.

In starch processing, the pullulanase is added after the liquefaction during the saccharification step in order to convert liquefied starch into maltose-rich glucose syrups.

According to the applicant, the food enzyme is used at the minimum amount necessary to achieve the desired reaction according to GMP. The use level applied by a food manufacture in practice depends on the particular process.

3.2. Dietary exposure

For this application, the intended use of this pullulanase is to hydrolyse starch in order to produce maltose-containing glucose syrups. Experimental data on the significant removal (> 99%) of protein in the course of this process have been provided (Documentation provided to EFSA n. 4). The Panel considered this evidence as sufficient to conclude that the presence of residual amounts of TOS after the purification steps applied during the production of glucose syrups, i.e. filtration, ion exchange chromatography, carbon treatment and crystallisation, is negligible. Consequently, no exposure was calculated.

3.3. Toxicological assessment

3.3.1. Genotoxicity

Test item used for the genotoxicity studies is described in Table 1 (batch 4).

3.3.1.1. Bacterial reverse mutation test

To investigate the potential of pullulanase to induce gene mutations, a bacterial reverse mutation assay (an Ames test) was performed according to OECD Test Guideline 471 (OECD, 1997) and following Good Laboratory Practice (GLP). Four strains of *Salmonella* Typhimurium (TA1535, TA100, TA1537 and TA98) as well as *E. coli* WP2uvrA were tested in the presence and absence of metabolic activation (S9-mix). The direct 'plate incorporation assay' was applied using six different concentrations of the food enzyme test substance (156, 313, 625, 1,250, 2,500 and, 5,000 μg/plate, corresponding to approximately 18.6, 37.3, 74.4, 148.8 and 595 μg TOS/plate), appropriate positive controls, and sterile deionised water as a negative control. Three experiments were performed. Upon treatment with food enzyme, there was no increase in revertant colony numbers in the first experiment, whereas significant increases were evident at the highest dose in tests with *S.* Typhimurium strain TA1535 (in the presence and absence of S9-mix), *S.* Typhimurium strain TA98 and *E. coli* WP2uvrA (in the absence of S9-mix) in the second experiment. To clarify the outcome of this experiment, these test series were repeated in a third experiment and all results were clearly negative. Since the results obtained in the second experiment were not reproducible, the Panel concluded that the food enzyme did not induce gene mutations under the conditions employed for this study.

3.3.1.2. *In vitro* micronucleus assay

The *in vitro* micronucleus assay was carried out according to the OECD Test Guideline 487 (OECD, 2007) and following GLP. Human peripheral blood lymphocytes were exposed to the food enzyme for a short treatment (3 h + 21 h recovery) in the presence and absence of S9-mix, and for a continuous

treatment (24 h with no recovery) without S9-mix using a concentration range from 1,187 to 5,000 μg/mL (corresponding to 141–595 μg TOS/mL). No biologically relevant increase in the frequency of micronuclei was observed. The Panel concluded that the food enzyme pullulanase did not induce micronuclei under the test conditions employed.

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

For the repeated dose 90-day oral toxicity, a study on a pullulanase preparation isolated from a strain developed from the intermediate strain [redacted] has been submitted. This strain differs from the final production strain by [redacted] The applicant states that this predecessor strain encodes the same pullulanase enzyme as is produced by NZYM-AK. The Panel considered this, in this case, as and adequate substitution.

A repeated dose 90-day oral toxicity study in rodents was performed according to the OECD Test Guideline 408 (OECD, 1998) and following GLP. Groups of 10 male and 10 female SPF Sprague–Dawley rats received daily gavage of the food enzyme test substance for 90 days at dose levels of 0 (water), 10, 30 and 100% in a volume of 10 mL/kg body weight (bw) corresponding to 129, 387 and 1,285 mg TOS/kg bw per day.

No treatment-related deaths or effects on clinical signs, changes in body weight, body weight gain, food consumption, in ophthalmoscopic examinations, in parameters of haematology and clinical chemistry, organ weights, and macroscopic or microscopic pathology were observed. Some changes in urinary parameters were observed, but these were not considered of biological relevance.

The Panel concluded that the no observed adverse effect level (NOAEL) was the highest dose level tested, which corresponds to 1,285 mg TOS/kg bw per day.

3.4. Allergenicity

The potential allergenicity of the pullulanase produced with the genetically modified *B. subtilis* strain NZYM-AK was assessed by comparing its amino acid sequence with those of known allergens according to the scientific opinion on the assessment of allergenicity of genetically modified (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, no match was found.

Pullulanase from *B. subtilis* strain NZYM-AK is not described as a potential allergen and no food allergic reactions to this pullulanase have been reported, so there is no evidence for potential allergenicity of this food enzyme.

According to the information provided, substances or products that may cause allergies or intolerances (Regulation EU 1169/2011)[8] are used as raw materials [redacted] in the media fed to the microorganisms. However, the proteins will be digested during the fermentation process and consumed by the microorganisms for cell growth, cell maintenance and production of enzyme protein. In addition, the microbial biomass and fermentation solids will be removed. Therefore, potentially allergenic residues of these foods employed as protein sources are not expected to be present.

Taken together, the Panel considers that there are no indications for allergic reactions, and therefore, this pullulanase produced with the genetically modified *B. subtilis* (strain NZYM-AK) is not of safety concern.

Conclusions

Based on the genetic modifications performed, the manufacturing process, the compositional and biochemical data provided, the findings in the toxicological studies and allergenicity assessment, the Panel concludes that this food enzyme does not give rise to safety concerns under the intended conditions of use.

[8] 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. OJ L 304,22.11.2011, p. 18–63.

Documentation provided to EFSA

1) Dossier Application for authorisation of Pullulanase from a genetically modified strain of *B. subtilis* (NZYM-AK), March 2015. Submitted by Novozymes A/S (Denmark).
2) Summary reports on technical, toxicological and genetic modifications data were delivered by Hylobates Consulting/BiCT (Rome, Italy) on 5 August 2016, FoBiG GmbH (Freiburg, Germany) on 15 August 2016 and by the Technical University of Denmark (Søborg, Denmark) on 17 November 2016, respectively.
3) Additional information was received from Novozymes A/S on January 2017.
4) Additional information on "Food enzyme removal during the production of cereal based distilled alcoholic beverages" and "Food enzyme carry/over in glucose syrups". February 2017. Provided by the Association of Manufacturers and Formulators of Enzyme Products.

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Abbreviations

bw	body weight
CAS	Chemical Abstracts Service
CASO	Casein Soy Bean Digest
CEF	EFSA Panel on Food Contact Material, Enzymes, Flavourings and Processing Aids
CFU	colony forming units
CHO	Chinese hamster ovaria
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany
EC	Enzyme Commission
EINECS	European Inventory of Existing Commercial Chemical Substances
FAO	Food and Agricultural Organization
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
JECFA	Joint FAO/WHO Expert Committee on Food Additives
mRNA	messenger ribonucleic acid
NOAEL	no observed adverse effect level
NPUN	New Pullulanase Unit Novozymes
OECD	Organisation for Economic Cooperation and Development
PCR	polymerase chain reaction
QPS	Qualified Presumption of Safety
SDS-PAGE	sodium dodecyl sulfate-poly acrylamide gel electrophoresis
TOS	total organic solids
TSA	Tryptic Soy Agar
WHO	World Health Organization