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Safety evaluation of the food enzyme 4-phytase from a genetically modified *Trichoderma reesei* (strain DP-Nzt55)

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Abstract

The food enzyme Myo-inositol-hexakisphosphate 4-phosphohydrolase (4-phytase, EC 3.1.3.26) is produced with a genetically modified *Trichoderma reesei* DP-Nzt55 by Danisco US Inc. The production strain contains a known antimicrobial resistance gene. However, based on the absence of viable cells and recombinant DNA of the production strain in the food enzyme, this is not considered to be a risk. The 4-phytase is intended to be used in distilled alcohol production. Since residual amounts of total organic solids are removed by distillation (> 99%), toxicological data were not considered necessary and dietary exposure was not calculated. Similarity of the amino acid sequence to those of known allergens was searched and no matches were found. The Panel considered that, under the intended conditions of use, the risk of allergic sensitisation and elicitation reactions by dietary exposure can be excluded. 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 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 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.

Five applications have been introduced by the companies "Erbsloh Geisenheim AG" for the authorisation of the food enzyme Endo-1,3(4)-beta-glucanase from *Talaromyces versatilis* (strain PF8), "Novozymes A/S" for the authorisation of the food enzyme Lipase from a genetically modified strain of *Aspergillus oryzae* (strain NZYM-PH), and "Danisco US Inc." for the authorisation of the food enzymes 4-phytase from a genetically modified strain of *Trichoderma reesei* (DP-Nzt55), Alpha-amylase from a genetically modified strain of *Bacillus licheniformis* (DP-Dzb54) and Pullulanase from a genetically modified strain of *Bacillus licheniformis* (DP-Dzp39).

Following the requirements of Article 12.1 of Regulation (EC) No 234/2011² implementing Regulation (EC) No 1331/2008³, the Commission has verified that the five 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, p. 7–15.

² 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.

³ 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.

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-1,3(4)-beta-glucanase from *Talaromyces versatilis* (strain PF8), Lipase from a genetically modified strain of *Aspergillus oryzae* (strain NZYM-PH), 4-phytase from a genetically modified strain of *Trichoderma reesei* (DP-Nzt55), Alpha-amylase from a genetically modified strain of *Bacillus licheniformis* (DP-Dzb54) and Pullulanase from a genetically modified strain of *Bacillus licheniformis* (DP-Dzp39) 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 request to carry out the safety assessment of the food enzyme 4-phytase from a genetically modified strain of *T. reesei* (DP-Nzt55).

2. Data and methodologies

2.1. Data

The applicant has submitted a dossier in support of the application for authorisation of the food enzyme 4-phytase produced with the genetically modified *T. reesei* (strain DP-Nzt55).

Additional information was requested from the applicant during the assessment process on 3 October 2018 and was consequently provided (see 'Documentation provided to EFSA').

2.2. Methodologies

The assessment was conducted in line with the principles described in the EFSA Guidance on transparency in the scientific aspects of risk assessment (EFSA, 2009b) 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 guidance of the EFSA Scientific Committee.

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.

3. Assessment

IUBMB nomenclature: 4-phytase
 Systematic name: Myo-inositol-hexakisphosphate 4-phosphohydrolase
 Synonyms: phytase; Phytate 6-phosphatase; Myoinositol-hexakisphosphate 6-phosphohydrolase
 IUBMB No: 3.1.3.26
 CAS No: 9001-89-2

The 4-phytase catalyses the hydrolysis of phytic acid (myo-inositol hexakisphosphate) to 1D-myo-inositol 1,2,3,5,6-pentakisphosphate and phosphate. It is intended to be used in distilled alcohol production processes.

3.1. Source of the food enzyme

The 4-phytase is produced with a genetically modified filamentous fungus *T. reesei* DP-Nzt55, which is deposited at the Westerdijk Fungal Biodiversity Institute culture collection (The Netherlands) with deposition number [REDACTED]⁴

3.1.1. Characteristics of the parental and recipient microorganisms⁵

[REDACTED]⁶

⁴ Technical dossier/Additional information July 2019/Annex AG.

⁵ Technical dossier/Annex V.

⁶ Technical dossier/Annex J.

[REDACTED]

3.1.2. Characteristics of the inserted sequences

[REDACTED]

3.1.3. Description of the genetic modification process

[REDACTED]

3.1.4. Safety aspects of the genetic modifications

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

[REDACTED]

The presence of an antimicrobial resistance gene [REDACTED] in the production strain is a possible safety concern [REDACTED] which is further discussed in this opinion.

⁷ Technical dossier/Additional information June 2019/Annex AL.

⁸ Technical dossier/Annex AA.

3.2. Production of the food enzyme

The food enzyme is manufactured according to the Food Hygiene Regulation (EC) No. 852/2004⁹, with food safety procedures based on hazard analysis and critical control points, and in accordance with current Good Manufacturing Practice.

The production strain is grown as a pure culture using a typical industrial medium in a submerged, batch/fed-batch fermentation system with conventional process controls in place. After completion of the fermentation, the solid biomass is removed from the fermentation broth by filtration leaving a supernatant containing the food enzyme. The filtrate containing the enzyme is then further purified and concentrated, including an ultrafiltration step in which enzyme protein is retained while most of the low molecular weight material passes the filtration membrane and is discarded. The applicant provided information on the identity of the substances used to control the fermentation and in the subsequent downstream processing of the food enzyme.¹⁰

The Panel considered that sufficient information has been provided on the manufacturing process and the quality assurance system implemented by the applicant to exclude issues of concern.

3.3. Characteristics of the food enzyme

3.3.1. Properties of the food enzyme

The 4-phytase is a single polypeptide chain of ■ amino acids.¹¹ The food enzyme was analysed by SDS-PAGE analysis. A consistent protein pattern was observed across all batches. The gels showed a single major protein band migrating between the marker proteins of 36.5 and the 55.4 kDa. No other enzymatic side activities were reported.

The in-house determination of 4-phytase activity is based on the ability to catalyse the hydrolysis of phytic acid to 1D-myo-inositol 1,2,3,5,6-pentakisphosphate and phosphate. The released inorganic phosphate forms a yellow complex with an acidic molybdate-vanadate reagent. The absorbance of the yellow complex is then measured at a wavelength of 415 nm and the inorganic phosphate released is quantified relative to a phosphate standard curve¹² and expressed in phytase activity Units (FTU).

The food enzyme has a temperature optimum between 75 and 85°C (pH 5.5) and a pH optimum between pH 3.5 and 4.5 (37°C). Thermostability was tested after a pre-incubation of the food enzyme for 10 min at different temperatures. Under the conditions (pH 5.5) of the applied temperature stability assay, 4-phytase activity decreased above 85°C showing no residual activity above 95°C.

3.3.2. Chemical parameters

Data on the chemical parameters of the food enzyme have been provided for three batches used for commercialisation (Table 1). The average total organic solids (TOS) of the three food enzyme batches for commercialisation was 31.5%. The average enzyme activity/TOS ratio of the three food enzyme batches for commercialisation is 361.0 FTU/mg TOS.

Table 1: Compositional data of the food enzyme¹³

Parameter	Units	Batches		
		1	2	3
4-Phytase activity	FTU/g batch ^(a)	115,335	113,127	111,628
Protein	%	30.9	28.1	29.6
Ash	%	< 0.04	0.23	0.13
Water	%	66.9	69.5	69.0

⁹ Regulation (EC) No. 852/2004 of the European Parliament and of the Council of 29 April 2004 on the hygiene of food additives. OJ L 226, 25.6.2004, pp. 3–21.

¹⁰ Technical dossier/Additional information June 2019/Annex AE.

¹¹ Technical dossier/Annex H.

¹² Technical dossier/Annex D.

¹³ Technical dossier/Additional information June 2019/Annex AC.

Parameter	Units	Batches		
		1	2	3
TOS ^(b)	%	33.1	30.3	30.9
4-phytase-activity/mg TOS	FTU/mg TOS	348.4	373.4	361.3

(a): FTU: phytase-activity Unit/g (see Section 3.1.3).

(b): TOS calculated as 100% – % water – % ash.

3.3.3. Purity

The lead content in the three commercial batches was below 5 mg/kg,¹⁴ which complies with 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).¹³

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. No antimicrobial activity was detected in any of these batches (FAO/WHO, 2006).¹³

The presence of mycotoxins (T-2 toxin, total aflatoxin, ochratoxin, zearalenone, fumonisin and sterigmatocystin) was examined in the three food enzyme batches, and were below the limits of detection (LoDs) of the applied analytical methods.¹⁵

Strains of *T. reesei*, in common with most filamentous fungi, have the capacity to produce a range of secondary metabolites (Frisvad et al., 2017). The applicant did not provide information on other secondary metabolites produced under the conditions of fermentation which might contribute to the food enzyme TOS.

3.3.4. Viable cells and DNA of the production strain

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

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The absence of recombinant DNA in the food enzyme was demonstrated

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3.4. Toxicological data

The food enzyme is intended to be used in distilled alcohol production. In the course of this process, the food enzyme is removed ($> 99\%$) and, consequently, the Panel did not consider the toxicological data provided to be necessary.

3.5. 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 this 4-phytase produced with the genetically modified *T. reesei* strain DP-NZS51 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 window of 80 amino acids as the criterion, no match was found.

No information is available on oral sensitisation or elicitation reactions of this 4-phytase.

¹⁴ LoD: 0.05 mg/Kg.

¹⁵ LoDs: T2 toxin: 10 µg/Kg; aflatoxin: 5 µg/Kg; ochratoxin: 2 µg/Kg; zearalenone: 25 µg/Kg; fumonisin: 100 µg/Kg; sterigmatocystin: 100 µg/Kg.

¹⁶ Technical dossier/Annex F and Additional information July 2019/Annex AH.

¹⁷ Technical dossier/Additional information July 2019/Annex AI.

In a few studies phytase is described as the cause for occupational respiratory allergy (Doekes et al., 1999; O'Connor et al., 2001; Baur et al., 2002; Caballero et al., 2007). However, several studies have shown that adults with occupational asthma to a food enzyme can ingest respiratory allergens without acquiring clinical symptoms of food allergy (Cullinan et al., 1997; Brisman, 2002; Poulsen, 2004; Armentia et al., 2009). Such information is not available for phytases.

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 removed, as is the case for distilled alcohol production.

The Panel considered that, under the intended conditions of use, the risk of allergic sensitisation and elicitation reactions can be excluded.

3.6. Dietary exposure

The food enzyme is intended to be used in distilled alcohol production processes at a recommended use level up to 13.4 mg TOS/kg cereals.

In distilled alcohol production, the food enzyme is typically applied during the pre-treatments or the fermentation step to degrade phytic acid originating from cereals.

Experimental data have been provided on the removal (> 99%) of protein in the course of distilled alcohol production (Documentation provided to EFSA No 4). The Panel considered the evidence as sufficient to conclude that residual amounts of TOS (including substances other than proteins) are removed by distillation.

As residual amounts of TOS are removed by distillation, a dietary exposure was not calculated.

Conclusions

Based on the data provided and, in particular, considering the removal of TOS during distilled alcohol production, the Panel concluded that the food enzyme 4-phytase produced with the genetically modified *T. reesei* strain DP-Nzt55 does not give rise to safety concerns under the intended conditions of use.

The production strain of the food enzyme contains multiple copies of a known antimicrobial resistance gene. However, based on the absence of viable cells and DNA from the production organism in the food enzyme, this is not considered to be a risk.

Documentation provided to EFSA

- 1) Dossier "Application for authorisation of 4-phytase from a genetically modified strain of *Trichoderma reesei* (DP-Nzt55)". February 2015. Submitted by Danisco Us Inc.
- 2) Additional information received from by DuPont on 11 June 2019.
- 3) Spontaneous additional information received from by DuPont on 2 July 2019.
- 4) Additional information on 'Food enzyme removal during the production of cereal based distilled alcoholic beverages'. February 2017. Provided by the Association of Manufacturers and Formulators of Enzyme Products.

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Abbreviations

CAS	Chemical Abstracts Service
CEF	EFSA Panel on Food Contact Material, Enzymes, Flavourings and Processing Aids
CFU	colony forming units
EC	Enzyme Commission
FAO	Food and Agricultural Organization
FOA	5-fluoroorotic Acid
GM	genetically modified
GMO	genetically modified organism
IUBMB	International Union of Biochemistry and Molecular Biology
LoD	limit of detection
PCR	polymerase chain reaction
rRNA	ribosomal ribonucleic acid
SDS–PAGE	sodium dodecyl sulfate–poly acrylamide gel electrophoresis
TOS	Total Organic Solids
WGS	whole genome sequence
WHO	World Health Organization