Safety evaluation of the food enzyme $\alpha,\alpha$-trehalase glucohydrolase from *Trichoderma reesei* (strain DP-Nzs51)

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Abstract

The food enzyme trehalase ($\alpha,\alpha$-trehalase glucohydrolase, EC 3.2.1.28) is produced with a genetically modified *Trichoderma reesei* DP-Nzs51 by Danisco US Inc. The genetic modifications do not give rise to safety concerns. The food enzyme is free from viable cells of the production organism and recombinant DNA. The trehalase 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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**Keywords:** food enzyme, $\alpha,\alpha$-trehalase glucohydrolase, EC 3.2.1.28, trehalase, *Trichoderma reesei*, genetically modified microorganism

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Safety of the food enzyme α,α-trehalase glucohydrolase from Trichoderma reesei (strain DP-Nzs51)

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1. Introduction

Article 3 of the Regulation (EC) No. 1332/2008 provides definition for 'food enzyme' and 'food enzyme preparation'.

'Food enzyme' means a product obtained from plants, animals or micro-organisms or products thereof including a product obtained by a fermentation process using micro-organisms: (i) containing one or more enzymes capable of catalysing a specific biochemical reaction; and (ii) added to food for a technological purpose at any stage of the manufacturing, processing, preparation, treatment, packaging, transport or storage of foods.

'Food enzyme preparation' means a formulation consisting of one or more food enzymes in which substances such as food additives and/or other food ingredients are incorporated to facilitate their storage, sale, standardisation, dilution or dissolution.

Before January 2009, food enzymes other than those used as food additives were not regulated or were regulated as processing aids under the legislation of the Member States. On 20 January 2009, Regulation (EC) No. 1332/2008 on food enzymes came into force. This Regulation applies to enzymes that are added to food to perform a technological function in the manufacture, processing, preparation, treatment, packaging, transport or storage of such food, including enzymes used as processing aids. Regulation (EC) No. 1331/2008 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:

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

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

The 'Guidance on submission of a dossier on food enzymes for safety evaluation' (EFSA 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 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 company "Danisco US Inc." for the authorisation of the food enzymes Beta-galactosidase from a genetically modified strain of Aspergillus oryzae (DP-Bzg59), Alpha, alpha trehalase from a genetically modified strain of Trichoderma reesei (DP-Nzs51), Alpha-amylase from a genetically modified strain of Bacillus licheniformis (DP-Dzb45), Glucose oxidase from a genetically modified strain of Aspergillus niger (DP-Aze23), and Alpha-amylase from Geobacillus stearothermophilus (DP-Gzb47).

Following the requirements of Article 12.1 of Regulation (EC) No 234/2011 implementing Regulation (EC) No 1331/2008, 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.

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1 Regulation (EC) No. 1332/2008 of the European Parliament and of the Council of 16 December 2008 on Food Enzymes and Amending Council Directive 83/417/EEC, Council Regulation (EC) No. 1493/1999, Directive 2000/13/EC, Council Directive 2001/112/EC and Regulation (EC) No 258/97. OJ L 354, 31.12.2008, pp. 7–15.
2 Regulation (EC) No. 1331/2008 of the European Parliament and of the Council of 16 December 2008 establishing a common authorisation procedure for food additives, food enzymes and food flavourings. OJ L 354, 31.12.2008, pp. 1-6.
3 Commission Regulation (EU) No 234/2011 of 10 March 2011 implementing Regulation (EC) No 1331/2008 of the European Parliament and of the Council establishing a common authorisation procedure for food additives, food enzymes and food flavourings. OJ L 64, 11.3.2011, p. 15–24.
1.1.2. Terms of Reference

The European Commission requests the European Food Safety Authority to carry out the safety assessments on the food enzymes Beta-galactosidase from a genetically modified strain of Aspergillus oryzae (DP-Bzg59), Apha, alpha trehalase from a genetically modified strain of Trichoderma resei (DP-Nzs51), Alpha-amylase from a genetically modified strain of Bacillus licheniformis (DP-Dzb45), Glucose oxidase from a genetically modified strain of Aspergillus niger (DP-Aze23), and Alpha-amylase from Geobacillus stearothermophilus (DP-Gzb47) 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 Alpha, alpha trehalase from a genetically modified strain of Trichoderma resei (DP-Nzs51).

2. Data and methodologies

2.1. Data

The applicant has submitted a dossier in support of the application for authorisation of the food enzyme \( \alpha,\alpha\)-trehalase glucohydrolase from Trichoderma resei (strain DP-Nzs51).

Additional information was requested from the applicant during the assessment process on 3 October 2018 and 25 June 2019 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, 2009) as well as in the EFSA Guidance on the characterisation of microorganisms used as feed additives or as production organisms (EFSA FEEDAP Panel, 2018) and following the relevant existing guidance's of EFSA Scientific Committees.

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

IUBMB nomenclature: Trehalase
Systematic name: \( \alpha,\alpha\)-trehalase glucohydrolase
Synonyms: \( \alpha,\alpha\)-trehalase, \( \alpha\)-Glucopyranosyl-\( \alpha\)-Glucopyranoside
IUBMB No: EC 3.2.1.28
CAS No: 9025-52-9

The trehalase catalyses the hydrolysis of the \( \alpha\)-\( \alpha\)-glucosidic linkage of \( \alpha,\alpha\)-trehalose, releasing initially equimolar amounts of \( \alpha\)- and \( \beta\)-D-glucose. It is intended to be used in distilled alcohol production processes.

3.1. Source of the food enzyme

The trehalase is produced with a genetically modified filamentous fungus Trichoderma resei DP-Nzs51 which is deposited at the Westerdijk Fungal Biodiversity Institute culture collection (The Netherlands) with deposition number 433.

3.1.1. Characteristics of the parental and recipient microorganisms

The parental strain 4

4 Technical dossier/Additional information June 2019/Annex AC.
T. reesei is considered to be non-pathogenic. T. reesei produces antimicrobial peptides called peptaibols (Degenkolb et al., 2012). Demonstration of the lack of antimicrobial activity (Section 3.3.3), confirmed that peptaibols are not present in the food enzyme in amounts that would raise a safety concern.

The recipient strain

3.1.2. Characteristics of introduced sequences

3.1.3. Description of the genetic modification process

3.1.4. Safety aspects of the genetic modification

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

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

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5 Technical dossier/1st submission/Annex S.
6 Technical dossier/2nd submission/Annex Z.
7 Technical dossier/Additional information June 2019/Annex AG.
3.2. Production of the food enzyme

The food enzyme is manufactured according to the Food Hygiene Regulation (EC) No. 852/2004, 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 or 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.

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 trehalase is a single polypeptide chain of amino acids. The food enzyme was analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and a consistent protein pattern was observed. The gels showed a major protein band corresponding to an apparent molecular mass of about 107 kDa. No other enzymatic side activities were reported.

The determination of trehalase activity is based on hydrolysis of the O-α-glucosidic linkage of α,α-trehalose, releasing equimolar amounts of α- and β-D-glucose (reaction conditions: pH 4.5, 50°C, 10 min). The enzymatic activity is determined by measuring the release of the reducing sugar glucose by the action of trehalase on the disaccharide trehalose. The rate of reducing sugar release is measured by the reaction with 3,5-dinitrosalicylic acid (DNS). The enzyme activity is expressed in THU/g. One THU is defined as the amount of enzyme required to generate 1 μmole of glucose reducing sugar equivalents per minute, under the conditions of this assay.

The food enzyme has a temperature optimum around 55°C (pH 4.5) and a pH optimum around pH 3–5 (50°C). Thermostability was tested after a pre-incubation of the food enzyme for 30 min at different temperatures. Under the conditions (pH 4.5) of the applied temperature stability assay, trehalase activity decreased above 50°C showing no residual activity above 65°C.

3.3.2. Chemical parameters

Data on the chemical parameters of the food enzyme were provided for three food enzyme batches used for commercialisation (Table 1). The mean total organic solids (TOS) of the three food enzyme batches for commercialisation was 30.9%. The mean enzyme activity/TOS ratio of the three food enzyme batches for commercialisation is 53.4.

| Parameter          | Units          | Batch 1 | Batch 2 | Batch 3 |
|--------------------|----------------|---------|---------|---------|
| Trehalase activity | THU/g batch(3) | 22,233  | 15,748  | 11,272  |
| Protein            | %              | 23.91   | 25.61   | 25.66   |
| Ash                | %              | 0.32    | 0.07    | < 0.04  |
| Water              | %              | 69.06   | 68.95   | 68.89   |

8 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.
9 Technical dossier/1st submission/Annex H.
10 Technical dossier/1st submission/Annex D.
11 Technical dossier/Annex I.
12 Technical dossier/Additional information June 2019/Annex AA.
3.3.3. Purity

The lead content in the three commercial batches was below 5 mg/kg 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 unit (CFU) per gram. No antimicrobial activity was detected in any of these batches (FAO/WHO, 2006).12

The presence of T-2 toxin was examined in the three food enzyme preparation batches, and was below the limit of detection (LoD) of the applied method.14

Strains of *T. reesei*, in common with most filamentous fungi have the capacity to produce a range of secondary metabolites (Frisvad et al., 2018). The applicant did not provide information on 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 demonstrated15

The absence of recombinant DNA in the food enzyme was demonstrated16

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, toxicological data are not considered 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 α,α-trehalase produced with the genetically modified *T. reesei* strain DP-NzsS1 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 plants and microorganisms and derived food and feed of the Scientific Panel on Genetically Modified Organisms (EFSA GMO Panel, 2017). Using higher than 35% identity in a window of 80 amino acids as the criterion, no match was found.17

No information is available on oral or respiratory sensitisation and elicitation reactions to this α,α-trehalase. 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.

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13 Technical dossier/Additional information June 2019/Annex AA. LoD: 0.05 mg/kg.
14 LoD: 10 µg/kg (Technical dossier/2nd submission/Annex G).
15 Technical dossier/Additional information June 2019/Annex AA and AD.
16 Technical dossier/Additional information June 2019/Annex AH.
17 Technical dossier/1st submission/Annex R.
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 of up to 1.2 mg TOS/kg cereals.

In distilled alcohol production, the food enzyme is typically applied during the fermentation step to degrade trehalose originating from the yeast to glucose.

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.

4. Conclusions

Based on the data provided and, in particular, considering the removal of TOS during the intended food production process, the Panel concluded that the food enzyme α,α-trehalase glucohydrolase produced with the genetically modified T. reesei strain DP-Nzs51 does not give rise to safety concerns under the intended conditions of use.

The EFSA Panel on Food Contact Materials, Enzymes and Processing Aids (CEP Panel) considers the food enzyme free from viable cells of the production organism and recombinant DNA.

Documentation provided to EFSA

1) Application for authorisation of alpha,alpha-trehalase from a genetically modified strain of Trichoderma reesei DP-Nzs51 in accordance with Regulation (EC) No 1331/2008. February 2016. Submitted by Danisco US Inc.
2) Additional information. 5 June 2019. Submitted by Du Pont.
3) Additional information. 27 June 2019. Submitted by Du Pont
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**

| Abbreviation | Description |
|--------------|-------------|
| CAS          | Chemical Abstracts Service |
| CEF          | EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids |
| CEP          | EFSA Panel on Food Contact Materials, Enzymes and Processing Aids |
| CFU          | colony forming unit |
| DNS          | 3,5-dinitrosalicylic acid |
| FAO          | Food and Agricultural Organization of the United Nations |
| FOA          | 5-fluoroorotic acid |
| GMO          | EFSA Panel on Genetically Modified Organisms |
| ITS          | internal transcribed spacer |
| IUBMB        | International Union of Biochemistry and Molecular Biology |
| JECFA        | Joint FAO/WHO Expert Committee on Food Additives |
| LoD          | limit of detection |
| PCR          | polymerase chain reaction |
| SDS-PAGE     | sodium dodecyl sulfate-polyacrylamide gel electrophoresis |
| TOS          | total organic solids |
| WGS          | whole genome sequence |
| WHO          | World Health Organization |