Safety evaluation of the food enzyme glucan 1,4-\(\alpha\)-glucosidase from *Trichoderma reesei* (strain DP-Nzh63)

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Abstract

The food enzyme 4-\(\alpha\)-D-glucan glucohydrolase (EC 3.2.1.3) is produced with a genetically modified *Trichoderma reesei* DP-Nzh63 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 glucan 1,4-\(\alpha\)-glucosidase 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, Glucan 1,4-\(\alpha\)-glucosidase, glucoamylase, EC 3.2.1.3, *Trichoderma reesei*, DP-Nzh63, genetically modified microorganism

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Table of contents

Abstract ................................................................................................................................................... 1
1. Introduction ................................................................................................................................... 4
1.1. Background and Terms of Reference as provided by the requestor ..................................................... 4
1.1.1. Background as provided by the European Commission ...................................................................... 4
1.1.2. Terms of Reference ........................................................................................................................ 4
1.2. Interpretation of the Terms of Reference .......................................................................................... 5
2. Data and methodologies ................................................................................................................. 5
2.1. Data .............................................................................................................................................. 5
2.2. Methodologies ................................................................................................................................5
3. Assessment .................................................................................................................................... 5
3.1. Source of the food enzyme ............................................................................................................. 5
3.1.1. Characteristics of the parental and recipient microorganisms ............................................................. 5
3.1.2. Characteristics of the inserted sequences ......................................................................................... 6
3.1.3. Description of the genetic modification process ............................................................................. 6
3.2. Production of the food enzyme ........................................................................................................ 7
3.3. Characteristics of the food enzyme .................................................................................................. 7
3.3.1. Properties of the food enzyme ......................................................................................................... 7
3.3.2. Chemical parameters ...................................................................................................................... 7
3.3.3. Purity ............................................................................................................................................ 8
3.3.4. Viable cells and DNA of the production strain ................................................................................... 8
3.4. Toxicological data ........................................................................................................................... 8
3.5. Allergenicity ................................................................................................................................... 8
3.6. Dietary exposure ............................................................................................................................. 9
Conclusions .............................................................................................................................................. 9
Documentation provided to EFSA .............................................................................................................. 9
References ............................................................................................................................................... 9
Abbreviations ........................................................................................................................................... 10
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 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 company “Danisco US Inc.” for the authorisation of the food enzymes Glucan 1,4-alpha-glucosidase from a genetically modified strain of *Trichoderma reesei* (DP-Nzh63), Subtilisin from a genetically modified strain of *Bacillus subtilis* (DP-Ezx62), Subtilisin from a genetically modified strain of *Bacillus subtilis* (DP-Ezx42), Alpha-amylase from *Aspergillus oryzae* (DP-Bzb41), and Glucan 1,4-alpha-glucosidase from a genetically modified strain of *Trichoderma reesei* (DP-Nzh38).

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.

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 Glucan 1,4-alpha-glucosidase from a genetically modified strain of *Trichoderma reesei* DP-Nzh63.
Trichoderma reesei (strain DP-Nzh63), Subtilisin from a genetically modified strain of Bacillus subtilis (strain DP-Ezx62), Subtilisin from a genetically modified strain of Bacillus subtilis (strain DP-Ezx42), Alpha-amylose from Aspergillus oryzae (strain DP-Bzb41), and Glucan 1,4-alpha-glucosidase from a genetically modified strain of Trichoderma reesei (strain DP-Nzh38) 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 food enzyme glucan 1,4-α-glucosidase from a genetically modified strain of T. reesei (DP-Nzh63).

2. Data and methodologies

2.1. Data

The applicant has submitted a dossier in support of the application for authorisation of the food enzyme glucan 1,4-α-glucosidase produced with the genetically modified T. reesei (strain DP-Nzh63).

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’s of EFSA Scientific Committees.

The current Guidance on the submission of a dossier on food enzymes for safety evaluation (EFSA, 2009a) has been followed for the evaluation of the application.

3. Assessment

IUBMB nomenclature: Glucan 1,4-α-glucosidase
Systematic name: 4-α-D-glucan glucohydrolase
Synonyms: Glucoamylase; amyloglucosidase; γ-amylase; lysosomal α-glucosidase; acid maltase; exo-1,4-α-glucosidase; glucose amylase; γ-1,4-glucanglucohydrolase; 1,4-α-D-glucan glucohydrolase

The glucan 1,4-α-glucosidase catalyses the hydrolysis of terminal (1-4)-linked α-D-glucose residues from non-reducing ends of polysaccharides, releasing glucose. It is intended to be used in distilled alcohol production processes.

3.1 Source of the food enzyme

The glucan 1,4-α-glucosidase is produced with a genetically modified filamentous fungus T. reesei DP-Nzh63, which is deposited at the Westerdijk Fungal Biodiversity Institute culture collection (The Netherlands) with deposition number .

3.1.1. Characteristics of the parental and recipient microorganisms

3 Technical dossier/Additional information July 2019/Annex AF.
4 Technical dossier/Annex R.
3.1.2. Characteristics of the inserted sequences

3.1.3. Description of the genetic modification process

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.
Therefore, the enzyme glucan 1,4-α-glucosidase produced by *T. reesei* DP-Nzh63, does not raise safety concern regarding the genetic modification of the production strain.

### 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/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 glucan 1,4-α-glucosidase is a single polypeptide chain of amino acids, including a amino acid signal sequence. The food enzyme was analysed by sodium dodecyl sulfate-poly acrylamide gel electrophoresis (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 55 and 66 kDa, consistent with the declared mass of 63 KDa. No other enzymatic side activities were reported.

Glucan 1,4-α-glucosidase activity is quantified by its ability to catalyse the hydrolysis of *p*-nitrophenyl-α-D-glucopyranoside to glucose and *p*-nitrophenol. At high pH, the nitrophenol forms a yellow colour which is measured spectrophotometrically at 400 nm and quantified against a curve constructed with an enzyme standard (reaction conditions: pH = 4.3, T = 30°C, incubation time 30 min).

The food enzyme has a temperature optimum around 62°C (pH 5.0) and a pH optimum at 5.0 (55°C). Thermostability was tested after a pre-incubation of the food enzyme for 120 min at different temperatures. Under the conditions (pH 5.0) of the applied temperature stability assay, glucan 1,4-α-glucosidase activity decreased above 60°C showing no residual activity above 70°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 was 33.90%. The average enzyme activity/TOS ratio is 4.92 GAU/mg TOS.
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 (\(\leq 5 \text{mg/kg}\)) as laid down in the general specifications and considerations for enzymes used in food processing (FAO/WHO, 2006).\(^{15}\)

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).\(^{16}\)

The presence of mycotoxins (T-2 toxin, total aflatoxin, ochratoxin, zearalenone, fumonisin and sterigmatocystin) was examined in the three food enzyme batches, and was below the limits of detection (LoDs) of the applied analytical methods.\(^{16}\)

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 recombinant DNA in the food enzyme was demonstrated\(^{17}\).

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.

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13 Technical dossier/Additional information July 2019/Annex AD.
14 LoD: 0.05 mg/kg.
15 Technical dossier/Annex F and Annex G.
16 LoDs: T2 toxin: 10 \(\mu\text{g/Kg}\); aflatoxin: 5 \(\mu\text{g/Kg}\); ochratoxin: 2 \(\mu\text{g/Kg}\); zearalenone: 25 \(\mu\text{g/Kg}\); fumonisin: 100 \(\mu\text{g/Kg}\); sterigmatocystin: 100 \(\mu\text{g/Kg}\).
17 Technical dossier/Annex F and Additional information July 2019/Annex AG.
18 Technical dossier/Additional information July 2019/Annex AH.
The potential allergenicity of the glucan 1,4-α-glucosidase produced with the genetically modified strain T. reesei DP-Nzh63 was assessed by comparison of 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, one match was found. The matching allergen Sch c1, a glucoamylase produced by the pathogenic fungus Schizophyllum commune.

No information is available on oral sensitisation or elicitation reactions of this glucoamylase from T. reesei.

Glucoamylase from S. commune is a known respiratory allergen associated with mycosis (Toyotome et al., 2014). 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; Poulsen, 2004; Armentia et al., 2009). Such information is not available for glucoamylases.

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 64.8 mg TOS/kg cereals.

In distilled alcohol production, the food enzyme is typically applied during the pre-saccharification together with other saccharification enzymes 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 glucan 1,4-α-glucosidase and other saccharification enzymes are added together with yeast at the beginning of fermenter fill.

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 glucan 1,4-α-glucosidase produced with the genetically modified T. reesei strain DP-Nzh63 does not give rise to safety concerns under the intended conditions of use.

The CEP Panel considers the food enzyme free from viable cells of the production organism and recombinant DNA.

### Documentation provided to EFSA

1) Dossier “Application for authorisation of an Glucan 1,4-alpha-glucosidase from a genetically modified strain of Trichoderma reesei, DP-Nzh63”. March 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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19 Technical dossier/Annex U.
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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 |
| EINECS       | European Inventory of Existing Commercial Chemical Substances |
| 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 |
| SSF          | simultaneous saccharification and fermentation |
| TOS          | Total Organic Solids |
| WGS          | whole genome sequence |
| WHO          | World Health Organization |