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# Safety evaluation of the food enzyme alpha-amylase from a genetically modified *Trichoderma reesei* (strain DP-Nzb48)

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## Abstract

The food enzyme alpha-amylase (4-α-D-glucan glucanohydrolase; EC 3.2.1.1) is produced with a genetically modified strain of *Trichoderma reesei* 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. This α-amylase is intended to be used in distilled alcohol production and brewing processes. Residual amounts of total organic solids (TOS) are removed by distillation; consequently, dietary exposure was not calculated for this use. Based on the maximum use levels recommended for the brewing processes and individual data from the EFSA Comprehensive European Food Consumption Database, dietary exposure to the food enzyme–TOS was estimated to be up to 1.701 mg TOS/kg body weight (bw) per day in European populations. Genotoxicity tests with the food enzyme did not indicate a genotoxic concern. The systemic toxicity was assessed by means of a repeated dose 90-day oral toxicity study in rats. The Panel identified a no-observed-adverse-effect level (NOAEL) at the highest dose of 230 mg TOS/kg bw per day. Similarity of the amino acid sequence to those of known allergens was searched and one match was found. The Panel considered 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 is considered low. Based on the removal of residues of the food enzyme during distillation, the Panel concluded that the use of this enzyme in the distilled alcohol production is safe. When used in brewing processes, the margin of exposure calculated from the data provided is only (at least) 135, but no safety issues were identified.



**Keywords:** food enzyme, α-amylase, 4-α-D-glucan glucanohydrolase, Amylase, glycogenase EC 3.2.1.1, *Trichoderma reesei*, genetically modified microorganism

**Requestor:** European Commission

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# 1. Introduction

Article 3 of the Regulation (EC) No 1332/2008[1] provides definitions 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 entered 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 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; and
iii) 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 an approval via a Union list.

The 'Guidance on submission of a dossier on a food enzyme for 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.

Five applications have been introduced by the companies 'Danisco US Inc.' for the authorisation of the food enzymes Alpha-amylase from a genetically modified strain of *Trichoderma reesei* (DP-Nzb48) and Thermolysin from *Geobacillus caldoproteolyticus* (DP-Fzj32), and 'Amano Enzyme Inc.' for the authorisation of the food enzymes AMP deaminase from *Streptomyces murinus* (strain AE-DNTS), Beta-galactosidase form *Aspergillus oryzae* (strain AE-LA) and Dextranase from *Chateomium erraticum* (strain AE-DX).

Following the requirements of Article 12.1 of Commission Regulation (EU) 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 contains 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 Alpha-amylase from a genetically modified strain of *Trichoderma reesei* (DP-Nzb48), Thermolysin from *Geobacillus caldoproteolyticus* (DP-Fzj32), AMP deaminase from

[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 1493/199, 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.

*Streptomyces murinus* (strain AE-DNTS), Beta-galactosidase form *Aspergillus oryzae* (strain AE-LA), Dextranase from *Chateomium erraticum* (strain AE-DX) 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 Alpha-amylase from a genetically modified strain of *T. reesei* (DP-Nzb48).

### 1.3. Information on existing authorisations and evaluations

The applicant indicates that the food enzyme object of the present dossier has not been evaluated by authorities in the EU. The applicant also reports that the Australian, New Zealand, Canadian, Danish, French, Brazilian, Mexican and American authorities have evaluated and authorised other enzymes than α-amylase from genetically modified strains of *T. reesei.*[3]

## 2. Data and methodologies

### 2.1. Data

The applicant has submitted a dossier supporting the application for authorisation of the food enzyme α-amylase from a genetically modified *T. reesei* strain DP-Nzb48.

Additional information was sought from the applicant during the assessment process in a request from EFSA sent on 12 January 2018, 27 June 2018 and 12 September 2018 and was consequently provided (see 'Documentation provided to EFSA').

Following the request for additional information data sent by EFSA on 12 January 2018, EFSA requested a clarification teleconference, which was held on 16 May 2018.

### 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 'Scientific Opinion on Guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use' (EFSA, 2011) and following the relevant existing guidances of EFSA Scientific Committee.

The current 'Guidance on the submission of a dossier for safety evaluation of a food enzyme' (EFSA CEF Panel, 2009) has been followed for the evaluation of this 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: 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.

α-Amylase catalyses the hydrolysis of 1,4-α-glycosidic linkages in starch (amylose and amylopectin), resulting in the generation of oligosaccharides. It is intended to be used in distilled alcohol production and brewing processes.

### 3.1. Source of the food enzyme

The α-amylase is produced with a genetically modified strain of *T. reesei* (DP-Nzb48[redacted]), which is deposited at the Westerdijk Fungal Biodiversity Institute (CBS, the Netherlands) with deposit number [redacted].[4]

[3] Technical dossier/2nd submission/Updated dossier/p. 22, 25 and 66.
[4] Technical dossier/Additional information April 2018.

### 3.1.1. Characteristics of the parental and recipient microorganisms

The parental strain RL-P37 (Sheir-Neiss and Montenecourt, 1984). (Kuhls et al., 1996).

The recipient strain .

### 3.1.2. Characteristics of the introduced sequences

The α-amylase .

### 3.1.3. Description of the genetic modification process

The purpose of genetic modification was to enable the production strain to synthesise α-amylase

### 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.

The production strain differs from the parental strain . The strain overproduces α-amylase. The absence of antimicrobial resistance genes used during the genetic modification was demonstrated in the production strain.

Genotypic stability was demonstrated .[5] Phenotypic stability of the production strain was demonstrated by stable production of α-amylase following industrial fermentation.

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

[5] Technical dossier/2nd submission/New or updated annexes/Annex AB.

## 3.2. Production of the food enzyme

The food enzyme is manufactured according to Food Hygiene Regulation (EC) No 852/2004[6], with food safety procedures based on Hazard Analysis and Critical Control Points (HACCP), and in accordance with current Good Manufacturing Practice (GMP).[7]

The production strain is grown as a pure culture using a typical industrial medium in a 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.[8]

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 α-amylase is a single polypeptide chain of [redacted] amino acids, including a signal sequence of [redacted] amino acids, which is cleaved off during the secretion of the enzyme. The molecular mass of the mature protein, based on the amino acid sequence, was calculated to be [redacted] kDa. The apparent molecular mass based on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) pattern is about 75 kDa[9] as reported in the dossier. The presence of other enzyme activities was not reported by the applicant.[10]

The α-amylase activity is quantified based on the hydrolysis of non-reducing-end blocked *p*-nitrophenyl maltoheptaoside (BPNPG7) substrate combined with excess levels of α-glucosidase and glucoamylase and is expressed Soluble Starch Unit/g (SSU/g). One SSU is defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenol per minute from BPNPG7 under the conditions described for the assay (reaction conditions: pH 4.5, temperature 30°C, reaction time 5 min).[11]

The food enzyme has been characterised with regard to its temperature and pH profiles. It has a temperature optimum in the temperature range between 60°C and 70°C (pH 3.5) and a pH optimum between pH 2.5 and 6.0 (temperature 32°C). Thermostability was tested after a pre-incubation of the food enzyme for 20 min at different temperatures. Under the conditions (pH 5.0) of the applied temperature stability assay, the α-amylase activity decreased rapidly above 70°C and showed almost no residual activity at 90°C.[12]

### 3.3.2. Chemical parameters

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).[13] The average total organic solids (TOS) content of the three commercial enzyme batches was 29.3% (range 28.7–30.4%). The average enzyme activity/TOS ratio of the three batches for commercialisation is 56 α-amylase Units/mg TOS.

[6] 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, 321 pp.
[7] Technical dossier/2nd submission/Updated dossier/p. 51–59.
[8] Technical dossier/1st submission/Annex N and Additional information April 2018.
[9] Technical dossier/2nd submission/Updated dossier/p. 39.
[10] Technical dossier/2nd submission/Updated dossier/p. 42.
[11] Technical dossier/1st submission/Annex E.
[12] Technical dossier/2nd submission/Updated dossier/p. 43–45 and Technical dossier/1st submission/Annex J.
[13] Technical dossier/2nd submission/Updated dossier/p. 38 and 72 and Additional information April 2018.

**Table 1:** Compositional data provided for the food enzyme

| Parameter | Unit | Batch | | | |
|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4[(a)] |
| α-amylase activity | SSU/g batch[(b)] | 16,371 | 16,878 | 16,298 | 3,161 |
| Protein | % | 24.4 | 22.6 | 22.1 | 4.51 |
| Ash | % | 0.4 | 0.4 | 0.3 | 0.1 |
| Water | % | 69.2 | 70.7 | 71.0 | 94.2 |
| Total organic solids (TOS)[(c)] | % | 30.4 | 28.9 | 28.7 | 5.7 |
| α-amylase Units/mg TOS | Units/mg TOS | 54 | 58 | 57 | 55.1 |

(a): Batch used for toxicological tests.
(b): SSU: Soluble Starch Units (see Section 3.3.1).
(c): TOS calculated as 100% − % water − % ash.

### 3.3.3. Purity

The food enzyme complies with the specification for lead (not more than 5 mg/kg) as laid down in the general specifications and considerations for enzymes used in food processing (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.[14]

No antimicrobial activity was detected in any of these batches (FAO/WHO 2006).[14]

The presence of mycotoxins (aflatoxins, sterigmatocystin, ochratoxin, T2 toxin and zearalenone) was examined in the food enzyme batch used for toxicological testing. The levels of these mycotoxins were found to be below the limits of detection.[15,16]

Strains of *Trichoderma*, in common with most filamentous fungi, have the capacity to elaborate a range of secondary metabolites (Blumenthal, 2004) including trichodermin (Watts et al., 1988). The applicant did not provide information on possible secondary metabolites produced under the conditions of fermentation which might contribute to the food enzyme TOS. This issue is addressed by the toxicological examination of the food enzyme TOS.

### 3.3.4. Viable cells and DNA of the production strain

The absence of the production microorganism in the food enzyme was demonstrated in [redacted].[17]

No recombinant DNA was detected in [redacted].[18]

## 3.4. Toxicological data

The batch used for the toxicological assays is described in Table 1. The toxicological batch is an enzyme concentrate without addition of additives or other standardisation or stabilisation ingredients and is representative of the commercial food enzyme.

### 3.4.1. Genotoxicity

#### 3.4.1.1. Bacterial reverse mutation test

The Ames test was performed according to Organisation for Economic Co-operation and Development (OECD) Test Guideline of Chemicals No 471, Bacterial Reverse Mutation Test (OECD, 1997a) and following Good Laboratory Practice (GLP), in *Salmonella* Typhimurium (strains TA1535,

[14] Technical dossier/2nd submission/Updated dossier/p. 41 and Technical dossier/1st submission/Annex G
[15] Technical dossier/Additional information June 2018/Annex O.
[16] LOD: aflatoxins = 5 ppb; sterigmatocystin = 100 ppb; ochratoxin = 10 ppb; T-2 toxin = 25 ppb; zearalenone = 25 ppb.
[17] Additional information April 2018.
[18] Technical dossier/1st submission/Annex Z.

TA100, TA102, TA1537, TA98) in the presence or absence of metabolic activation by S9-mix.[19] Two experiments in triplicate were carried out using five different concentrations of the food enzyme (50, 160, 500, 1,500, 1,600 and 5,000 μg total protein/plate corresponding to 63, 202, 631, 1,892, 2,018 and 6,307 μg TOS/plate). The 'treat and plate' assay was performed in both experiments. Upon treatment with the food enzyme, there was no increase in revertant colony numbers or in cytotoxicity. Therefore, the Panel concluded that the food enzyme has no mutagenic activity under the conditions employed in this study.

#### 3.4.1.2. *In vitro* mammalian chromosomal aberration test

The *in vitro* chromosome aberration test was carried out according to the OECD Test Guideline 473 (OECD, 1997b) and following GLP in human peripheral blood lymphocytes.[20] Two experiments were performed. In the first experiment, whole blood cultures were exposed to the food enzyme α-amylase in the presence and absence of S9-mix for 3 + 16 h, and in the second experiment with S9-mix for 3 + 16 and without 18 + 0 h. Based on the results of the cell-growth inhibition test, the dose levels for the chromosome aberration assay were set at 1,250, 2,500 and 5,000 μg total protein/mL (corresponding to 1,577, 3,154 and 6,307 μg TOS/mL, respectively) for both short-term treatments with and without metabolic activation and 18 h continuous treatment. In the chromosomal aberration test, no cytotoxicity was observed in most of the experimental points. The statistically significant increase in the frequency of aberrant metaphases observed in a single culture in test 1 (5,000 μg/mL with S9-mix) and in test 2 (1,250 μg/mL without S9-mix) was not considered biologically relevant because it was not reproduced in the replicate culture or test.

The Panel concluded that the food enzyme α-amylase did not induce chromosome aberrations under the test conditions employed for this study.

### 3.4.2. Repeated dose 90-day oral toxicity study in rodents

A repeated dose 90-day oral toxicity study in rats with α-amylase was performed according to OECD Test Guideline 408 (OECD, 1981), and following GLP.[21] Three groups of 10 male and 10 female SPF Sprague–Dawley (Ntac:SD strain) rats received by gavage food enzyme in doses of 57, 115 and 230 mg TOS/kg body weight (bw) per day in a volume of 5 mL/kg bw. A control group received 0.9% saline which served as a vehicle.

One low-dose male died on day 13 due to misdoing (water-like fluid in chest cavity at necropsy).

In open field testing, a reduced time of moving in low-dose males and a reduced total distance completed in low-dose males and females were recorded. Despite these findings being statistically significant, they were considered to be incidental and not treatment-related as they were only observed in the low-dose group.

Water consumption was statistically significantly increased in low-dose females in weeks 1, 6 and 8–9 and statistically significantly decreased in mid-dose females in weeks 11–12. These findings were considered incidental and not treatment-related as they were only observed for limited timeframes, with no dose relationship and in one sex.

Statistically significant differences to controls in haematological parameters were limited to an increase in the absolute number and percentage of reticulocytes in high-dose females, a slight increase in haemoglobin in low- and mid-dose females and haematocrit in mid-dose females. As no dose relationship was observed and these findings were not consistent between the sexes they were considered as incidental and not of toxicological importance.

Clinical chemistry examination revealed a statistically significantly decreased level of total protein in serum in the low-, mid- and high-dose females. As the decreases were slight, not dose related and the levels were within the historical control range for the laboratory, they were considered not to be of toxicological significance.

In urinalysis, statistically significant differences in specific gravity were observed in low- and mid-dose males and an increased pH in low-dose males as compared to controls. These differences were considered incidental and not related to treatment as they occurred with no dose relationship and in one sex only.

[19] Technical dossier/2nd submission/Updated dossier/p. 67 and Technical dossier/1st submission/Annex R.
[20] Technical dossier/1st submission/Annex S.
[21] Technical dossier/1st submission/Annex T.

At microscopic urinary examination, statistically significantly lower amounts of leucocytes in low-, mid- and high-dose males, of epithelial cells in high-dose females and crystals in mid-dose males were observed. These findings were considered not to be of toxicological significance.

At necropsy, statistically significantly increases in absolute weights of spleen in high-dose males, of testes in mid-dose males and ovary weight in low-dose females were observed. Since the microscopic examination did not reveal any test substance related changes in these organs, the relative weights were not statistically significantly different from the controls, and all these absolute weights were within the usual ranges of the strain, these findings were considered as incidental.

Based on the analysis of the results, the Panel identified a no-observed-adverse-effect level (NOAEL) of 230 mg TOS/kg bw per day, the highest dose tested.

### 3.4.3. 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 α-amylase produced with the genetically modified *T. reesei* strain DP-Nzb48 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 micro-organisms 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, one match was found with TAKA-amylase-A, also called Asp o 21 an α-amylase from *A. oryzae.*[22]

No information is available on oral and respiratory sensitisation or elicitation reactions of this α-amylase from *T. reesei* strain DP-Nzb48. α-amylase from *A. oryzae* (Brisman and Belin, 1991; Quirce et al., 1992, 2002; Sander et al., 1998; Brisman, 2002) is described as an occupational respiratory allergen 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*) may be able to ingest the corresponding allergen without acquiring clinical symptoms of food allergy (Cullinan et al., 1997; Brisman, 2002; Poulsen, 2004; Armentia et al., 2009). Taking into account the wide use of α-amylase, only a low number of case reports has been described in literature focussed on allergic reactions upon oral exposure to α-amylase in individuals sensitised by inhalation 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). Therefore, it can be concluded that an allergic reaction upon oral ingestion of this α-amylase, produced with the genetically modified *T. reesei* strain DP-Nzb48, in individuals sensitised by inhalation to α-amylase cannot be ruled out, but the likelihood of such reaction to occur is considered to be low.

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).

The Panel considered that under the intended conditions 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 reaction occurring is considered to be low.

## 3.5. Dietary exposure

### 3.5.1. Intended use of the food enzyme

The food enzyme is intended to be used in distilled alcohol production and brewing processes at the recommended use levels summarised in Table 2.

**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 |
|---|---|---|
| Brewing processes (beer) | Cereals | Up to 371 mg TOS/kg cereals |
| Distilled alcohol production | Cereals | Up to 116 mg TOS/kg cereals |

(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 time of adoption of this opinion.

[22] Technical dossier/1st submission/Annex U.

In distilled alcohol production, the food enzyme is added during the slurry mixing step, in the liquefaction step and if needed in the pre-saccharification step. α-Amylase is intended to be used to convert liquefied starch into a maltose-rich solution, to increase the amounts of fermentable sugars which results in higher alcohol yields.

In brewing processes, the food enzyme is added during the mashing step. The α-amylase is used to convert liquefied starch into a maltose-rich solution, improving the amounts of fermentable sugars and thus increasing brewing yield.

Experimental data have been provided on the removal (> 99%) of protein in the course of distilled alcohol production (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.

The food enzyme remains in the beer. Based on data provided on thermostability (see Section 3.3.1), it is anticipated that the α-amylase is inactivated during brewing processes.

### 3.5.2. Dietary exposure estimation

As residual amounts of TOS are removed by distillation (by > 99%), foods/ingredients derived through this process, i.e. distilled alcohol, were excluded from the estimation.

For brewing processes, 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 process covered in this opinion involved the selection of relevant food categories from the Comprehensive Database and application of process and technical conversion factors (Annex B in EFSA CEF Panel, 2016). The selected food categories were assumed to always contain the food enzyme–TOS at the maximum recommended use level (Table 2).

Chronic exposure was calculated by combining the maximum recommended use level provided by the applicant (see Table 2) with the relevant FoodEx categories (Annex B in EFSA CEF Panel, 2016), based on individual consumption data. Exposure from individual FoodEx categories was subsequently summed up, averaged over the total survey period and normalised for bodyweight. This was done for all individuals across all surveys, resulting in distributions of individual average exposure. Based on these distributions, the mean and 95th percentile exposures were calculated per survey for the total population and per age class. Surveys with only one day per subject were excluded and 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, 2011).

Table 3 provides an overview of the derived exposure estimates across all surveys. Detailed average and 95th percentile exposure to the food enzyme-TOS per age class, country and survey, as well as contribution from each FoodEx category to the total dietary exposure are reported in Appendix A – Tables 1 and 2. For the present assessment, food consumption data were available from 35 different dietary surveys (covering infants, toddlers, children, adolescents, adults and the elderly), carried out in 22 European countries (Appendix B).

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

| Population group | Estimated exposure (mg TOS/kg body weight 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) | 0–0 (10) | 0–0.005 (14) | 0–0.009 (19) | 0–0.072 (18) | 0.029–0.378 (19) | 0.007–0.186 (18) |
| Min–max 95th percentile (number of surveys) | 0–0 (8) | 0–0 (12) | 0–0 (19) | 0–0.449 (17) | 0.210–1.701 (19) | 0.047–0.778 (18) |

TOS: total organic solid.

### 3.5.3. Uncertainty analysis

In accordance with the guidance provided in the EFSA opinion 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<br>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 based on the description of the food process provided by the applicant (based on examples given by applicant) | + |
| Use of recipe fractions in disaggregation FoodEx categories likely to contain the food enzyme. | +/– |
| Use of technical factors in the exposure model | +/– |

TOS: total organic solids.
+: 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.

### 3.6. Margin of exposure

A comparison of the NOAEL (230 mg TOS/kg bw per day) from the 90-day study with the calculated exposure estimates of 0.000–0.378 mg TOS/kg bw per day at the mean and of 0.000–1.701 mg TOS/kg bw per day at 95th percentile, resulted in a margin of exposure (MOE) of at least 135. This is considered by the Panel as insufficient to conclude on the safety of the food enzyme when used in brewing processes.

The Panel recognises that it is probable that the calculated MOE is an underestimate of the real value as:

- the NOAEL identified in the repeated dose study is the highest dose tested;
- the exposure estimate is likely to be an overestimate of the real exposure due to the conservative approach applied (e.g. the assumption that the food enzyme is used in all brewing processes, and at the highest dose recommended; extrapolation from consumption of only a few days to long-term intake, etc.; see Table 4).

However, the Panel was not able to quantify the extent to which these factors might contribute to a higher MOE.

## 4. Conclusions

Based on the removal of residues of the food enzyme during the distillation, the Panel concludes that the α-amylase produced with a genetically modified strain of *T. reesei* (strain DP-Nzb48) is safe when used in the production of distilled alcohol.

The MOE calculated from the data provided is insufficient to conclude on the safety of the food enzyme when used in brewing processes. While the Panel recognises that it is probable that the calculated MOE is underestimated, it is not in a position to quantify the degree of underestimation. However, no safety issues were identified by the Panel.

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

## Documentation provided to EFSA

1) Dossier 'α-amylase produced by a genetically modified strain of *Trichoderma reesei* strain DP-Nzb48'. February 2015. Submitted by Danisco US Inc.
2) Additional information, April 2018. Submitted by Danisco US Inc.
3) Additional information, June 2018. Submitted by Danisco US Inc.
4) Additional information, September 2018. Submitted by Danisco US Inc.
5) 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

| | |
|---|---|
| bw | body weight |
| CAS | Chemical Abstracts Service |
| CFU | colony forming units |
| EC | Enzyme Commission |
| EINECS | European Inventory of Existing Commercial Chemical Substances |
| FAO | Food and Agricultural Organization |
| GLP | Good Laboratory Practice |
| GMP | Good Manufacturing Practice |
| IUBMB | International Union of Biochemistry and Molecular Biology |
| ITS | internal transcribed spacer |
| JECFA | Joint FAO/WHO Expert Committee on Food Additives |
| MOE | Margin of Exposure |
| NOAEL | no-observed-adverse-effect level |
| OECD | Organisation for Economic Cooperation and Development |
| SDS–PAGE | sodium dodecyl sulfate–poly acrylamide gel electrophoresis |
| SSU | Soluble Starch Units |
| TOS | total organic solids |
| WHO | World Health Organization |

# 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, Estonia, Finland, France, Germany, Italy, Latvia, Portugal, United Kingdom |
| Toddlers | From 12 months up to and including 35 months of age | Belgium, Bulgaria, Denmark, Estonia, Finland, France, Germany, Italy, Latvia, Netherlands, Portugal, Spain, United Kingdom |
| Children[(a)] | From 36 months up to and including 9 years of age | Austria, Belgium, Bulgaria, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Italy, Latvia, Netherlands, Portugal, Spain, Sweden, United Kingdom |
| Adolescents | From 10 years up to and including 17 years of age | Austria, Belgium, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Italy, Latvia, Netherlands, Portugal, Spain, Sweden, United Kingdom |
| Adults | From 18 years up to and including 64 years of age | Austria, Belgium, Croatia, Czech Republic, Denmark, Estonia, Finland, France, Germany, Hungary, Ireland, Italy, Latvia, Netherlands, Portugal, Romania, Spain, Sweden, United Kingdom |
| The elderly[(a)] | From 65 years of age and older | Austria, Belgium, Denmark, Estonia, Finland, France, Germany, Hungary, Ireland, Italy, Latvia, Netherlands, Portugal, Romania, Spain, 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, 2011).

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

Information provided in this appendix is shown in an excel file (downloadable https://efsa.onlinelibrary.wiley.com/doi/10.2903/j.efsa.5553).

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: Contribution of food categories to the dietary exposure to the food enzyme–TOS per age class, country and survey.