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## Safety evaluation of the food enzyme pullulanase from a genetically modified *Bacillus licheniformis* (strain DP-Dzp39)

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### Abstract

The food enzyme pullulanase (pullulan 6- $\alpha$ -glucanohydrolase; EC 3.2.1.41) is produced with a genetically modified *Bacillus licheniformis* (strain DP-Dzp39) 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 its recombinant DNA. This pullulanase is intended to be used in brewing processes, starch processing for glucose syrups production and distilled alcohol production. Residual amounts of total organic solids (TOS) are removed by distillation and by the purification steps applied during the production of glucose syrups, consequently, dietary exposure was not calculated for these food processes. For brewery products, based on the maximum use level 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 0.053 mg TOS/kg body weight (bw) per day in European populations. Genotoxicity tests with the food enzyme did not raise 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 at the highest dose of 500 mg TOS/kg bw per day that, compared to the estimated dietary exposure, results in sufficiently high margin of exposure (at least 9,400). The amino acid sequence of the food enzyme did not match those of known allergens. 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 data provided, the Panel concluded that this food enzyme does not raise safety concerns under the intended conditions of use.

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**Keywords:** food enzyme, pullulanase, pullulan 6- $\alpha$ -glucanohydrolase, EC 3.2.1.41, *Bacillus licheniformis*, genetically modified microorganism

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## 1. Introduction

Article 3 of the Regulation (EC) No 1332/2008<sup>1</sup> 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<sup>2</sup> 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 'Erbslöh 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 *Bacillus licheniformis* (strain DP-Dzp39).

Following the requirements of Article 12.1 of Commission Regulation (EU) No 234/2011<sup>3</sup> implementing Regulation (EC) No 1331/2008<sup>2</sup>, 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.

<sup>1</sup> 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.

<sup>2</sup> 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.

<sup>3</sup> 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 assessment 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 *Bacillus licheniformis* (strain DP-Dzp39) in accordance with the 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 pullulanase from a genetically modified strain of *B. licheniformis* (strain DP-Dzp39).

### 1.3. Information on existing authorisations and evaluations

The applicant reports that the Danish and French authorities have evaluated and authorised the use of the food enzyme from a genetically modified *B. licheniformis* in glucose syrup production.<sup>4</sup>

## 2. Data and methodologies

### 2.1. Data

The applicant has submitted a dossier in support of the application for authorisation of the food enzyme pullulanase from a genetically modified strain of *B. licheniformis* (strain DP-Dzp39).

Additional information was sought from the applicant during the assessment process in a request from EFSA sent on 15 November 2017 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) and following Scientific Opinion on Guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use (EFSA GMO Panel, 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: Pullulanase  
Systematic name: Pullulan 6- $\alpha$ -glucanohydrolase  
Synonyms:  $\alpha$ -Dextrin endo-1,6-alpha-glucosidase  
IUBMB No: EC 3.2.1.41  
CAS No: 9075-68-7  
EINECS No.: 232-983-9.

Pullulanase catalyses the hydrolysis of (1 $\rightarrow$ 6)- $\alpha$ -D-glucosidic linkages in pullulan, amylopectin and glycogen, and in the  $\alpha$ - and  $\beta$ -limit dextrins of amylopectin and glycogen. It is intended to be used in brewing processes, starch processing for glucose syrups production and distilled alcohol production.

### 3.1. Source of the food enzyme

The pullulanase is produced with a genetically modified strain of *B. licheniformis*.

<sup>4</sup> Technical dossier/2nd submission/Updated dossier/p. 68 and Technical dossier/First submission/Annex O.

The production strain *B. licheniformis* DP-Dzp39 is deposited in the international culture collection of Westerdijk Fungal Biodiversity Institute (Utrecht, the Netherlands) with the numbers: [REDACTED]

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

The parental strain [REDACTED]

The recipient strain [REDACTED]

### 3.1.2. Characteristics of the introduced sequences

The pullulanase-encoding gene from [REDACTED]

### 3.1.3. Description of the genetic modification process

The pullulanase [REDACTED]

### 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 recipient strain [REDACTED]

The production *B. licheniformis* strain DP-Dzp39 [REDACTED]

The absence of vector sequences including those used for constructing the recipient strain was confirmed by [REDACTED]

<sup>5</sup> Technical dossier/2nd submission/New or updated annexes/Annex X and Technical dossier/Additional information May 2018/Annex G.

<sup>6</sup> Technical dossier/Additional information May 2018.

<sup>7</sup> Technical dossier/2nd submission/New or updated annexes/Annex X.

<sup>8</sup> Technical dossier/1st submission/Annex Z.

Genotypic stability of the *B. licheniformis* DP-Dzp39 production strain was demonstrated by analysing the DNA from [REDACTED]

Besides the presence of [REDACTED], no other issues of concern arising from the genetic modifications were identified by the Panel.

### 3.2. Production of the food enzyme

The food enzyme is manufactured<sup>10</sup> according to Food Hygiene Regulation (EC) No 852/2004<sup>11</sup>, with food safety procedures based on Hazard Analysis and Critical Control Points (HACCP) and in accordance with current Good Manufacturing Practice (GMP).<sup>12</sup>

The production strain is grown as a pure culture using a typical industrial medium in a submerged, 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<sup>13</sup> 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 pullulanase is a single polypeptide chain of [REDACTED] amino acids.<sup>14</sup> The molecular mass, based on the amino acid sequence, was calculated to be [REDACTED] kDa. The homogeneity of the food enzyme was investigated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis. The gels presented for the three food enzyme batches are comparable and showed one main protein band at about [REDACTED] kDa and a number of other faint bands in all batches.<sup>15</sup> No side activities were reported.

The in-house determination of pullulanase activity is determined based on the hydrolysis of a commercial sample of dyed pullulan (red pullulan). Enzymatic hydrolysis releases the dye into solution which is measured spectrophotometrically (reaction conditions: reaction time 30 min, pH 5, temperature 40°C and 510 nm). The amount of dye released is proportional to the activity present and is related to a standard curve produced with known activities. Activity is expressed in pullulanase Acid Stable Pullulanase Units (ASPU)/g.<sup>16</sup>

The food enzyme has been characterised with regard to its temperature and pH profiles. It is active at temperatures below 70°C with an optimum range between 55°C and 60°C at pH 4.5. The optimum pH for activity lies between pH 4.0 and 5.0 at 50°C. Thermostability measured by activity remaining after a pre-incubation for 30 min at the optimum pH showed that activity is retained at temperatures up to 57°C but rapidly lost at higher temperatures.<sup>17</sup>

#### 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

<sup>9</sup> Technical dossier/2nd submission/New or updated annexes/Annex AC.

<sup>10</sup> Technical dossier/2nd submission/Updated dossier/p. 52.

<sup>11</sup> 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.

<sup>12</sup> Technical dossier/1st submission/Annex K.

<sup>13</sup> Technical dossier/1st submission/Annex M.

<sup>14</sup> Technical dossier/1st submission/Annex H.

<sup>15</sup> Technical dossier/2nd submission/Updated dossier/p. 38 and Additional information May 2018/Q3. Annex E and L.

<sup>16</sup> Technical dossier/1st submission/Annex D.

<sup>17</sup> Technical dossier/2nd submission/Updated dossier/p. 42 and Technical dossier/1st submission/Annex Y.



tests (Table 1).<sup>18</sup> The average total organic solids (TOS) content of the three commercial enzyme batches was 19.38% (range 18.11–21.64%). The average enzyme activity/TOS ratio of the three batches for commercialisation is 55.2 ASPU/mg TOS.

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

Parameter	Unit	Batch			
		1	2	3	4 <sup>(a)</sup>
Pullulanase activity <sup>19</sup>	ASPU/g batch <sup>(b)</sup>	10,513	11,024	10,314	8703
Protein	%	6.1	6.4	6.1	7.6
Ash	%	1.0	1.0	1.2	2.2
Water	%	80.9	77.4	80.4	84.0
Total organic solids (TOS) <sup>(c)</sup>	%	18.1	21.6	18.4	14.0
Activity/mg TOS	ASPU/mg TOS	58.6	50.9	56.1	62.3

(a): Batch used for the toxicological studies.

(b): ASPU: Acid Stable Pullulanase 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)<sup>20</sup> as laid down in the general specifications and considerations for enzymes used in food processing (FAO/WHO, 2006).<sup>21</sup>

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.<sup>22</sup>

No antimicrobial activity was detected in any of these batches (FAO/WHO, 2006).<sup>23</sup>

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

The production strain could not be detected

.<sup>24</sup>

No recombinant DNA was detected

.<sup>25</sup>

## 3.4. Toxicological data

A battery of toxicological tests including a bacterial gene mutation assay (Ames test), an *in vitro* mammalian chromosomal aberration test and a repeated dose 90-day toxicity study in rats has been provided. The batch 4 (Table 1) used in these studies has similar protein pattern<sup>26</sup> as the batches used for commercialisation<sup>27</sup> and similar chemical purity, and thus is considered suitable as a test item.

### 3.4.1. Genotoxicity

#### 3.4.1.1. Bacterial reverse mutation test

A bacterial reverse mutation assay (Ames test) was performed according to OECD Test Guideline 471 (OECD, 1997a) and following Good Laboratory Practice (GLP).<sup>28</sup> Four strains of *Salmonella*

<sup>18</sup> Technical dossier/2nd submission/Updated dossier/p. 37 and p. 76; Technical dossier/1st submission/Annexes F, G, T and S; Technical dossier/Additional information May 2018/Q1.Annex A, B, C.

<sup>19</sup> Technical dossier/Additional info May 2018/Q2.Annex D.

<sup>20</sup> LOD: Pb = 5 mg/kg.

<sup>21</sup> Technical dossier/2nd submission/Updated dossier/p. 40 and p. 76; Technical dossier/1st and 2nd submissions/Annexes: F, G and T.

<sup>22</sup> Technical dossier/2nd submission/Updated dossier/p. 40 and p. 76.

<sup>23</sup> Technical dossier/1st submission/Annex F.

<sup>24</sup> Technical dossier/1st submission/Annex F, Annex Z; Technical dossier/Additional information May 2018/Q11.Annex I.

<sup>25</sup> Technical dossier/1st submission/Annex AA; Technical dossier/Additional information May 2018/Q.10.Annex H.

<sup>26</sup> Technical dossier/Additional information May 2018/Q3.Annex\_L.

<sup>27</sup> Technical dossier/Additional information May 2018/Q3.Annex\_E.

<sup>28</sup> Technical dossier/2nd submission/Updated dossier/p. 70 and Technical dossier/1st submission/Annex Q.



Typhimurium (TA98, TA100, TA1535 and TA1537) and *E. coli* WP2uvrA were used, applying the treat and plate method both in the presence and absence of metabolic activation. Two experiments were performed at least in duplicate plates. Based on the results obtained in an initial test, the amounts selected for the confirmatory test were 1.5, 5, 15, 50, 150, 500, 1,500 and 5,000 µg total protein/plate (corresponding to 2.7, 9.2, 27, 92, 275, 916, 2,748, and 9,159 µg TOS/plate, respectively) with and without S9-mix. No precipitation and growth inhibition were observed in any strain at any dose level tested. However, a reduction in revertant counts was observed in the presence of S9-mix with TA98 at 150 µg total protein/plate and above as well as with TA1535 at 5,000 µg total protein/plate; this effect was not reproducible. No statistically significant increases in the number of revertant colonies were observed in any tester strain, in the absence or presence of metabolic activation.

Therefore, the Panel concluded that the food enzyme did not induce gene mutations under the conditions of the 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.<sup>29</sup> Based on the results of a preliminary toxicity assay, the cells were exposed to the food enzyme at 2,450, 3,500 and 5,000 µg total protein/mL (corresponding to 4,488, 6,412 and 9,159 µg TOS/mL) in a short-term treatment (4 + 20 h of recovery) both in the presence and absence of S9-mix, and at 250, 500 and 1,000 µg total protein/mL (corresponding to 458, 916 and 1,832 µg TOS/mL) in a continuous treatment (20 h) in the absence of S9-mix. A reduction in the mitotic index of 26%, 3% and 55% of negative control values were observed at the highest dose in the short-term treatments (-S9, +S9) and continuous treatment, respectively. The frequency of chromosomal aberrations in treated cultures was comparable to the values detected in negative controls and within the range of the laboratory historical solvent control data.

The Panel concluded that the food enzyme pullulanase 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

The repeated dose 90-day oral toxicity study was performed in accordance with OECD Test Guideline 408 (OECD, 1998) and following GLP.<sup>30</sup> Groups of 10 male and 10 female CrI:CD(SD) rats received by gavage the food enzyme diluted in deionised water for 90 days, at doses of 100, 200 and 500 mg TOS/kg body weight (bw) per day (referred to as the low-, mid- and high-dose groups) (5 mL/kg bw). Controls group received the deionised water alone.

One high-dose male and one low-dose male were sacrificed on days 82 and 87, respectively, due to a gavage trauma.

No effects on clinical signs, body weight, body weight gain, food consumption, food efficiency, ophthalmology observations, neurobehavioral parameters, haematology, clinical chemistry, macroscopic or histological changes were observed in any dosed-groups and gender.

In high-dose males, mean daily food consumption was statistically significantly higher (11%) when compared to the control group during test days 85–90. In high-dose females, mean daily food consumption was statistically significantly higher (< 10%) when compared to the control group during test days 8–15, and overall test days 1–29.

Among haematology parameters mean corpuscular haemoglobin concentration (MCHC) was statistically lower in high-dose females (1.8% below control), but not associated with changes in other red cell mass parameters.

In the clinical chemistry examination, a total protein in low-dose males was found to be 5.9% above the vehicle control value.

The Panel considered that none of these findings were of toxicological significance.

The Panel concluded that the no-observed-adverse-effect level (NOAEL) in this study was 500 mg TOS/kg bw per day, the highest dose tested.

<sup>29</sup> Technical dossier/2nd submission/Updated dossier/p. 72 and Technical dossier/1st submission/Annex R.

<sup>30</sup> Technical dossier/2nd submission/Updated dossier/p. 74 and Technical dossier/1st submission/Annex S.

### 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 pullulanase produced with *B. licheniformis* strain DP-Dzp39 has been assessed by comparing of its amino acid sequence with those of known allergens according to the EFSA 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 criterion, no match was found.<sup>31</sup>

Pullulanase from *B. licheniformis* strain DP-Dzp39 is not described as a potential allergen and no food allergic reactions to this pullulanase have been reported, so there is no evidence for potential allergenicity of this food enzyme.

According to the information provided substances or products that may cause allergies or intolerances (Regulation EU 1169/2011<sup>32</sup>) are used as raw materials (██████████) in the media fed to the microorganisms in the course of the production of the enzyme. However, the proteins will be digested during the fermentation process and consumed by the microorganisms for cell growth, cell maintenance and production of enzyme protein. In addition, the microbial biomass and fermentation solids will be removed. Therefore, potentially allergenic residues of these foods employed as protein sources are not expected to be present.

The Panel considers 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 reactions to occur 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 three food manufacturing processes at the recommended use levels summarised in Table 2.<sup>33</sup>

**Table 2:** Intended uses and recommended use levels of the food enzyme as provided by the applicant

Food manufacturing process <sup>(a)</sup>	Raw material	Recommended dosage of the food enzyme
Brewing processes (beer)	Cereals	Up to 11.5 mg TOS/kg cereals
Distilled alcohol production	Cereals	Up to 1.4 mg TOS/kg cereals
Starch processing for the production of glucose syrups	Starch	Up to 0.05 mg TOS/kg starch

TOS: total organic solids.

(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.

In distilled alcohol production, the food enzyme is added to the raw materials during liquefaction, pre-saccharification (optional) or the fermentation stage. It is used to increase fermentation rate, increased ethanol yield and improve performance in pre-saccharification process.

In starch processing for glucose syrups production, the food enzyme is added during the saccharification step. It is used to degrade starch polysaccharides into maltose and glucose in an efficient way.

Experimental data have been provided on the removal (> 99%) of protein in the course of distilled alcohol production and starch processing for the production of glucose syrups (Documentation provided to EFSA No. 3). The Panel considered the evidence as sufficient to conclude that residual

<sup>31</sup> Technical dossier/2nd submission/Updated dossier/p. 77 and Technical dossier/1st submission/Annex U.

<sup>32</sup> REGULATION (EU) No 1169/2011 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 25 October 2011 on the provision of food information to consumers, amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) No 608/2004.

<sup>33</sup> Technical dossier/Section 3.2.1.4.

amounts of TOS (including substances other than proteins) are removed by distillation. In addition, taking into account the purification steps applied to the production of glucose syrups, i.e. filtration, ion exchange chromatography, treatment with active carbon, the Panel also considers that the amount of TOS in the final glucose syrup will be removed to a similar degree.

In brewing processes, the food enzyme is added at the beginning of the mashing, where it takes part in the degradation of starch into hydrolysis products of various chain lengths. It facilitates achieving of more uniform and predictable production process with higher brewing yield due to efficient degradation of starch and thereby reduced use of raw materials.

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

### 3.5.2. Dietary exposure estimation

As residual amounts of TOS are removed by distillation and by the purification steps applied during the production of glucose syrups (by > 99%), foods/ingredients derived through these two processes, i.e., distilled alcohols and glucose syrups were excluded from the estimation.

For brewing processes, chronic exposure was calculated using the methodology described in the CEF Panel statement on the exposure assessment of food enzymes (EFSA CEF Panel, 2016). The assessment involved selection of relevant food categories from the EFSA Comprehensive European Food Consumption Database<sup>34</sup> and application of process and technical conversion factors (Annex B in EFSA CEF Panel, 2016).

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 (10)	0 (14)	0 (19)	0.000–0.002 (18)	0.001–0.012 (19)	0.000–0.006 (18)
Min–max 95th percentile (number of surveys)	0 (8)	0 (12)	0 (19)	0.000–0.014 (17)	0.007–0.053 (19)	0.001–0.024 (18)

TOS: total organic solids.

<sup>34</sup> <http://www.efsa.europa.eu/en/food-consumption/comprehensive-database>

### 3.5.3. Uncertainty analysis

In accordance with the guidance provided in the EFSA opinion related to uncertainties in dietary exposure assessment (EFSA, 2006), 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
<b>Model input data</b>	
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	+/-
<b>Model assumptions and factors</b>	
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	+
Use of recipe fractions in disaggregation FoodEx categories	+/-
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 (500 mg TOS/kg bw per day) from the 90-day study with the derived exposure estimates in six human population groups of 0–0.012 mg TOS/kg bw per day at the mean and from 0 to 0.053 mg TOS/kg bw per day at the 95th percentile, resulted in margins of exposure (MOE) above 9,400 indicating that there is no safety concern.

## 4. Conclusions

Based on the data provided, the removal of TOS during the distilled alcohol production and glucose syrup production and the MOE calculated when used in brewing processes, the Panel concludes that the food enzyme pullulanase produced with the genetically modified *B. licheniformis* strain DP-Dzp39 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 'Pullulanase from a genetically modified strain of *Bacillus licheniformis* (strain DP-Dzp39)'. March 2015. Submitted by Danisco US Inc.
- 2) Additional information, May 2018. Submitted by Danisco US Inc.
- 3) Additional information on 'Food enzyme removal during the production of cereal-based distilled alcoholic beverages' and 'Food enzyme carry-over in glucose syrups'. February 2017. Provided by Associations of Manufacturers and Formulators of Enzyme Products.

## References

- EFSA (European Food Safety Authority), 2006. Opinion of the Scientific Committee related to uncertainties in dietary exposure assessment. EFSA Journal 2006;5(1):438, 54 pp. <https://doi.org/10.2903/j.efsa.2007.438>
- EFSA (European Food Safety Authority), 2009. Guidance of the Scientific Committee on transparency in the scientific aspects of risk assessments carried out by EFSA. Part 2: general principles. EFSA Journal 2009;7(5):1051, 22 pp. <https://doi.org/10.2903/j.efsa.2009.1051>
- EFSA (European Food Safety Authority), 2011. Use of the EFSA Comprehensive European Food Consumption Database in Exposure Assessment. EFSA Journal 2011;9(3):2097, 34 pp. <https://doi.org/10.2903/j.efsa.2011.2097>
- EFSA CEF Panel (EFSA Panel on Food Contact Material, Enzymes, Flavourings and Processing Aids), 2009. Guidance of the Scientific Panel of Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF) on the Submission of a Dossier on Food Enzymes for Safety Evaluation by the Scientific Panel of Food Contact Material, Enzymes, Flavourings and Processing Aids. EFSA Journal 2009;7(8):1305, 26 pp. <https://doi.org/10.2903/j.efsa.2009.1305>
- EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids), Silano V, Bolognesi C, Castle L, Cravedi JP, Fowler P, Franz R, Grob K, Gürtler R, Husøy T, Kärenlampi S, Mennes W, Milana MR, Penninks A, Smith A, Tavares Poças MF, Tlustos C, Wölfle D, Zorn H, Zugravu CA, Arcella D, Liu Y and Engel KH, 2016. Panel statement on the exposure assessment of food enzymes. EFSA Journal 2016; 10(10):4581, 11 pp. <https://doi.org/10.2903/j.efsa.2016.4581>
- EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms), 2011. Scientific Opinion on Guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use. EFSA Journal 2011;9(6):2193, 54 pp. <https://doi.org/10.2903/j.efsa.2011.2193>
- EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms), Naegeli H, Birch AN, Casacuberta J, De Schrijver A, Galak MA, Guerche P, Jones H, Manachini B, Messean A, Nielsen EE, Nogue F, Robaglia C, Rostoks N, Sweet J, Tebbe C, Visioli F, Wal J-M, Eigenmann P, Epstein M, Hoffmann-Sommergruber K, Koning F, Lovik M, Mills C, Moreno FJ, van Loveren H, Selb R and Fernandez Dumont A, 2017. Guidance on allergenicity assessment of genetically modified plants. EFSA Journal 2017;15(5):4862, 49 pp. <https://doi.org/10.2903/j.efsa.2017.4862>
- FAO/WHO (Food and Agriculture Organization of the United States/World Health Organization), 2006. General specifications and considerations for enzyme preparations used in food processing in Compendium of food additive specifications. 67th meeting. FAO JECFA Monographs 3, 63–67. Available online: <http://www.fao.org/3/a-a0675e.pdf>
- OECD (Organisation for Economic Co-Operation and Development), 1997a. OECD Guideline for the testing of chemicals, Section 4 Health effects, Test No. 471: Bacterial reverse mutation test. 21 July 1997. 11 pp. Available online: [http://www.oecd-ilibrary.org/environment/test-no-471-bacterial-reverse-mutation-test\\_9789264071247-en;jsessionid=9zfgzu35paaq.x-oecd-live-01](http://www.oecd-ilibrary.org/environment/test-no-471-bacterial-reverse-mutation-test_9789264071247-en;jsessionid=9zfgzu35paaq.x-oecd-live-01)
- OECD (Organisation for Economic Co-Operation and Development), 1997b. OECD Guideline for the testing of chemicals, Section 4 Health effects, Test No. 473: *In vitro* mammalian chromosomal aberration test. 21 July 1997. 10 pp. Available online: [https://www.oecd-ilibrary.org/environment/test-no-473-in-vitro-mammalian-chromosome-aberration-test\\_9789264071261-en](https://www.oecd-ilibrary.org/environment/test-no-473-in-vitro-mammalian-chromosome-aberration-test_9789264071261-en)
- OECD, 1998. Repeated Dose 90-day Oral Toxicity Study in Rodents. Guideline 408, adopted 21.09.1998. Available online: [http://www.oecd-ilibrary.org/environment/test-no-408-repeated-dose-90-day-oral-toxicity-study-in-rodents\\_9789264070707-en](http://www.oecd-ilibrary.org/environment/test-no-408-repeated-dose-90-day-oral-toxicity-study-in-rodents_9789264070707-en)

## Abbreviations

ASPU	Acid Stable Pullulanase Units
bp	Base pair
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 Organisation
GLP	Good Laboratory Practice
GM	genetically Modified
GMP	Good Manufacturing Practice
HACCP	Hazard Analysis and Critical Control Points
IUBMB	International Union of Biochemistry and Molecular Biology
JECFA	Joint FAO/WHO Expert Committee on Food Additives
MCHC	mean corpuscular haemoglobin concentration
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
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 <sup>(a)</sup>	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 <sup>(a)</sup>	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.5554>).

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