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## Safety evaluation of the food enzyme isoamylase from a *Dyella* sp. strain

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### Abstract

The food enzyme isoamylase (glycogen  $\alpha$ -1,6-glucanohydrolase, EC 3.2.1.68) is produced with *Dyella* sp. by Hayashibara Co. Ltd. Whole genome sequence analysis of the production strain identified a sequence with high homology with a gene conferring resistance to an antimicrobial, which may confer cross-resistance to a critically important antimicrobial, as defined by the World Health Organisation. This is a concern, since DNA from the production strain was detected in the food enzyme. The isoamylase food enzyme is intended to be used in starch processing for the production of various starch hydrolysates. Since residual amounts of total organic solids are removed by the purification steps applied during the production of saccharides from starch, dietary exposure was not calculated. The batch used for toxicological testing was not sufficiently characterised; therefore, the toxicological data provided were not considered. Similarity of the amino acid sequence to those of known allergens was searched and no match was found. The Panel considered that, under the intended conditions of use, the risk of allergic sensitisation and elicitation reactions by dietary exposure cannot be excluded, but the likelihood for this to occur is considered to be low. Overall, the Panel cannot conclude on the safety of the food enzyme isoamylase produced with *Dyella* sp.

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**Keywords:** food enzyme, Isoamylase, Glycogen  $\alpha$ -1,6-glucanohydrolase, EC 3.2.1.68, *Pseudomonas amyloclavata*, *Dyella* sp

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## 1. Introduction

Article 3 of the Regulation (EC) No 1332/2008<sup>1</sup> provides definition for 'food enzyme' and 'food enzyme preparation'.

'Food enzyme' means a product obtained from plants, animals or microorganisms or products thereof including a product 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<sup>2</sup> established the European Union (EU) procedures for the safety assessment and the authorisation procedure of food additives, food enzymes and food flavourings. The use of a food enzyme shall be authorised only if it is demonstrated that:

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

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

The 'Guidance on submission of a dossier on food enzymes for safety evaluation' (EFSA CEF Panel, 2009a) lays down the administrative, technical and toxicological data required.

### 1.1. Background and Terms of Reference as provided by the requestor

#### 1.1.1. Background as provided by the European Commission

Only food enzymes included in the European Union (EU) Community list may be placed on the market as such and used in foods, in accordance with the specifications and conditions of use provided for in Article 7(2) of Regulation (EC) No 1332/2008 on food enzymes.

Five applications have been introduced by the companies "Hayashibara Co. Ltd." for the authorisation of the food enzymes Alpha-amylase from *Bacillus circulans*/*Paenibacillus alginolyticus*, 1,4- $\alpha$ -glucan 6- $\alpha$ -glucosyltransferase from *Bacillus circulans*/*Paenibacillus alginolyticus*, Cyclomaltodextrin glucanotransferase from *Bacillus circulans*, Isoamylase from *Pseudomonas amylofermentosa* and "Intertek Scientific & Regulatory Consultancy" for the authorisation of the food enzyme D-Fructose 4-epimerase from a genetically modified strain of *Corynebacterium glutamicum* (strain FIS003).

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 Alpha-amylase from *Bacillus circulans*/*Paenibacillus alginolyticus*, 1,4- $\alpha$ -glucan 6- $\alpha$ -glucosyltransferase from *Bacillus circulans*/*Paenibacillus alginolyticus*, Cyclomaltodextrin

<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 1493/1999, Directive 2000/13/EC, Council Directive 2001/112/EC and Regulation (EC) No 258/97. OJ L 354, 31.12.2008, pp. 7–15.

<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, pp. 1–6.

glucanotransferase from *Bacillus circulans*, Isoamylase from *Pseudomonas amyloclavata*, D-Fructose 4-epimerase from a genetically modified strain of *Corynebacterium glutamicum* (strain FIS003) in accordance with Article 17.3 of Regulation (EC) No 1332/2008 on food enzymes.

## 1.2. Interpretation of the Terms of Reference

The present scientific opinion addresses the European Commission's request to carry out the safety assessment of food enzyme Isoamylase from a *Pseudomonas amyloclavata*.

## 2. Data and methodologies

### 2.1. Data

The applicant has submitted a dossier in support of the application for authorisation of the food enzyme Isoamylase from a *Pseudomonas amyloclavata*.

Additional information was requested from the applicant during the assessment process on 19 April 2018, 23 May 2019 and 16 October 2019, and was consequently provided (see 'Documentation provided to EFSA').

### 2.2. Methodologies

The assessment was conducted in line with the principles described in the EFSA Guidance on transparency in the scientific aspects of risk assessment (EFSA, 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: | Isoamylase                              |
| Systematic name:    | Glycogen $\alpha$ -1,6-glucanohydrolase |
| Synonyms:           | Debranching enzyme                      |
| IUBMB No.:          | EC 3.2.1.68                             |
| CAS No.:            | 9067-73-6                               |

The enzyme catalyses the hydrolysis of 1,6- $\alpha$ -D-glucosidic branch linkages in  $\alpha$ -glucans, such as glycogen and amylopectin, releasing amylose. It is intended to be used in starching processing for the production of various starch hydrolysates.<sup>3</sup>

### 3.1. Source of the food enzyme

The isoamylase is produced with a non-genetically modified bacterium strain, which was deposited in the American Type Culture Collection as *Pseudomonas amyloclavata* [redacted].<sup>4</sup> The strain was obtained by mutagenesis from strain SB-15 (ATCC 21262).<sup>5</sup> *P. amyloclavata* is a species name without standing in nomenclature. Phylogenetic analysis of the 16S rRNA gene sequence identified strain SB-15 as closely related to *Dyella terrae*. Identification data based on whole genome sequence (WGS) analysis were requested but the limited data provided did not allow to refine further the taxonomic identity of the strain.<sup>6</sup>

The applicant provided a study on antimicrobial susceptibility of a strain named *P. amyloclavata* [redacted], according to the recommendations of the Guidance on the characterisation of microorganism used as feed additives or as production organisms (EFSA FEEDAP Panel, 2018). The antimicrobials tested were those recommended for Enterobacteriaceae. The strain was sensitive to all antimicrobials tested except [redacted], for which the minimum inhibitory concentration (MIC) was [redacted] (cut-off value= 8 mg/L). The relationship between strain [redacted] and the production strain was not provided.<sup>7</sup>

<sup>3</sup> 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 adoption of this opinion.

<sup>4</sup> Technical dossier/Additional information July 2018/Annex 10-1.

<sup>5</sup> Technical dossier/Additional information February 2019/Annex 7.

<sup>6</sup> Technical dossier/Additional information February 2019/Annexes 6 and 8.

<sup>7</sup> Technical dossier/Additional information February 2019/Annex 12.

Further WGS analysis of the production strain identified a sequence with high homology with a gene conferring resistance to [REDACTED], which may show cross-resistance to [REDACTED], which is a critically important antimicrobial according to the World Health Organisation (WHO, 2016).<sup>8</sup> Moreover, the WGS obtained was incomplete and the database used for the search no longer maintained. Overall, the limited data provided suggest that the presence of at least one antimicrobial resistance gene in the production strain could give rise to a safety concern.

### 3.2. Production of the food enzyme

The food enzyme is manufactured according to the Food Hygiene Regulation (EC) No 852/2004<sup>9</sup>, 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, [REDACTED] 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.<sup>10</sup>

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 isoamylase is a single polypeptide chain of 750 amino acids. The molecular mass of the mature protein was calculated to be ca. [REDACTED] kDa. The food enzyme was analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. A consistent protein pattern was observed across all batches. The gels showed a single major protein band corresponding to an apparent molecular mass of about [REDACTED] kDa.<sup>11</sup> The food enzyme preparation was tested for cellulase, lipase and protease activities. Only low levels of activity were detected.<sup>12</sup>

The in-house determination of isoamylase activity is based on hydrolysis of waxy corn starch (reaction conditions: pH 3.5, 40°C, 30 min). The reaction is stopped by the addition of sulfuric acid solution, and then, an iodine solution is added (reaction conditions: 25°C, 15 min). The enzymatic activity is determined by measuring the absorbance at 720 nm. One isoamylase Unit (U) is defined as the amount of isoamylase which increases the absorbance by 0.004 in 30 min under the conditions of the assay.<sup>13</sup>

The food enzyme has been characterised with regard to its temperature and pH profiles in the literature (Yokobayashi et al., 1970). It has a temperature optimum around 52°C (pH 3.5) and a pH optimum around pH 3–4 (40°C). Thermostability was tested after a pre-incubation of the food enzyme for 10 min at different temperatures. Under the conditions (pH 3.5) of the applied temperature stability assay, isoamylase activity decreased above 42°C showing no residual activity above 62°C.<sup>14</sup>

#### 3.3.2. Chemical parameters

Data on the chemical parameters of the food enzyme preparation were provided for five food enzyme preparation batches used for commercialisation (Table 1).<sup>15</sup> The mean total organic solids (TOS) of the five batches was 4.04 %. The mean enzyme activity/TOS ratio of the five batches is 35,974 U/mg TOS. Further data provided on three other batches had similar values.<sup>16</sup>

<sup>8</sup> Technical dossier/Additional information September 2019/Annex Q6a.

<sup>9</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, pp. 3–21.

<sup>10</sup> Technical dossier/Section 3.2.1.2.2 and Additional data February 2019.

<sup>11</sup> Technical dossier/Additional information February 2019/Annex 3.

<sup>12</sup> Technical dossier/Annex 4.

<sup>13</sup> Technical dossier/Annex 3.

<sup>14</sup> Technical dossier/Additional information February 2019/Annex 5a and 5b.

<sup>15</sup> Technical dossier/page 36.

<sup>16</sup> Technical dossier/Additional information February 2019/Annex 1 and Additional information September 2019.

**Table 1:** Compositional data of the food enzyme preparation

| Parameter                                 | Unit                     | Mean      | Maximum   | Minimum   |
|-------------------------------------------|--------------------------|-----------|-----------|-----------|
| Isoamylase activity                       | U/g batch <sup>(a)</sup> | 1,326,000 | 1,350,000 | 1,300,000 |
| Protein                                   | %                        | 1.90      | 1.90      | 1.90      |
| Ash                                       | %                        | 0.24      | 0.34      | 0.13      |
| Water                                     | %                        | 58.37     | 59.92     | 55.73     |
| Diluents <sup>(b)</sup>                   | %                        | 37.34     | 37.34     | 37.34     |
| Total organic solids (TOS) <sup>(c)</sup> | %                        | 4.04      | 6.59      | 2.54      |
| Activity/mg TOS                           | U/mg TOS                 | 35,974    | 51,181    | 20,030    |

(a): U: Isoamylase Unit (see Section 3.3.1).

(b): Diluents: [REDACTED].

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

### 3.3.3. Purity

The lead content of three of the five batches described in Table 1 was up to 0.02 mg/kg which complies with the specification for lead ( $\leq 5$  mg/kg) as laid down in the general specifications and considerations for enzymes used in food processing (FAO/WHO, 2006).<sup>17</sup> In addition, the level of total mercury was below the limit of detection of the employed methodology.<sup>18</sup> For arsenic and cadmium, the concentrations determined in the commercial batches were up to 0.016 and 0.001 mg/kg, respectively.<sup>18</sup> The Panel considered these concentrations as not of concern.

The food enzyme preparation complies with the microbiological criteria (for total coliforms, *Escherichia coli* and *Salmonella*) as laid down in the general specifications and considerations for enzymes used in food processing (FAO/WHO, 2006). No antimicrobial activity was detected in any of these batches (FAO/WHO, 2006).<sup>17</sup>

The presence of mycotoxins (aflatoxins B1, B2, G1 and G2, sterigmatocystin, zearalenone and ochratoxin) was examined in three food enzyme batches. All were below the LODs of the applied analytical methods.<sup>19, 17</sup>

The Panel considered that the information provided on the purity of the food enzyme is sufficient.

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

The absence of the production strain in the food enzyme preparation was studied [REDACTED].<sup>20</sup>

[REDACTED]. No colonies of the production strain were produced.

The possible presence of DNA from the production strain was studied [REDACTED].

[REDACTED], two of the three samples were positive.<sup>21</sup> The Panel notes that the starting volume was much lower than the recommended 1 mL sample (EFSA CEP Panel, 2019).

## 3.4. Toxicological data

The applicant provided a set of toxicological data in support of the safety of the food enzyme under assessment. However, the batch used for toxicological examination was not sufficiently characterised. Only data on protein content (1.9%) and enzyme activity (1,362,500 U/g of enzyme solution) were provided in the study report. From this, it is not possible to calculate the TOS value in the batch used for toxicological examination. Consequently, it is not possible to ascertain that the test item is representative of the commercial product, and therefore, the toxicological studies provided were not further considered.

<sup>17</sup> Technical dossier/Annex 1.

<sup>18</sup> LODs: Pb = 0.005 mg/kg; As = 0.005 mg/kg; Cd = 0.001 mg/kg; Hg = 0.005 mg/kg.

<sup>19</sup> LOD: Aflatoxins (B1, B2, G1 and G2) = 0.001 µg/g each; Sterigmatocystin = 0.005 µg/g; Zearalenone = 0.02 µg/g; Ochratoxin = 0.001 µg/g.

<sup>20</sup> Technical dossier/Additional information September 2019/Annex Q7.

<sup>21</sup> Technical dossier/Additional information February 2019/Annex 11.

### 3.4.1. 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 isoamylase produced with *Dyella sp.* was assessed by comparing its amino acid sequence with those of known allergens according to the scientific opinion on the assessment of allergenicity of GM plants and microorganisms and derived food and feed of the Scientific Panel on Genetically Modified Organisms (EFSA GMO Panel, 2010). Using higher than 35% identity in a sliding window of 80 amino acids as the criterion, no match was found.<sup>22</sup>

No information is available on oral sensitisation or elicitation reactions of this isoamylase. The Panel is not aware of reports of allergenicity of isoamylase enzymes.

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. In the starch processing for the production of starch hydrolysates, experimental data showed a significant removal (> 99%) of protein.<sup>23</sup> However, traces of protein could be present in starch hydrolysates.

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 for 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 starch processing for the production of various starch hydrolysates at a recommended use level of up to 50 mg TOS/kg starch.<sup>24</sup>

Flow charts depicting the manufacturing process of trehalose,<sup>25</sup> maltose,<sup>26</sup> glucose syrups,<sup>27</sup> maltosyltrehalose syrups<sup>28</sup> and ascorbic acid derivative<sup>29</sup> have been provided. After liquefaction, the isoamylase is added to the starch slurry together with other enzymes during the saccharification step, where it degrades gelatinised starch into saccharides by debranching amylopectin into linear  $\alpha$ -glucans.

The resulting starch hydrolysates are treated with heat to inactivate enzymes,<sup>30</sup> and are further purified in multiple steps, including decolourisation, deionisation and crystallisation as appropriate.

The Panel considered that the purification steps in the production of these starch hydrolysates are essentially the same as those in the production of glucose syrups. The removal of food enzyme TOS was supported by the virtual absence of nitrogen in the resulting trehalose (< 0.1%), maltose (< 0.1%), glucose syrups (< 0.1%), maltose-trehalose syrup (< 0.1%) and ascorbic acid derivative (< 0.1%),<sup>31,32</sup> and by ELISA using the isoamylase-specific antibody.<sup>33</sup> DNA from the production strain was not found in samples of starch hydrolysis produced by the applicant [REDACTED]

[REDACTED]<sup>34</sup>

### 3.5.2. Dietary exposure estimation

The technical information and experimental data provided on the removal of food enzyme TOS during starch processing to produce various starch hydrolysates were considered by the Panel as sufficient to exclude this process from the exposure estimation (Annex B in EFSA CEF Panel, 2016). Consequently, dietary exposure was not calculated.

<sup>22</sup> Technical dossier/Section 3.2.2.2 and Annex 24.

<sup>23</sup> Technical dossier/Annex 9 and Additional information June 2020/Table 2.

<sup>24</sup> Technical dossier/pp. 52–56, Additional information July 2018/A8 and Additional information February 2019.

<sup>25</sup> Additional information April 2020/Annex3-1.

<sup>26</sup> Additional information April 2020/Annex3-2.

<sup>27</sup> Additional information April 2020/Annex3-3.

<sup>28</sup> Additional information April 2020/Annex3-4.

<sup>29</sup> Additional information April 2020/Annex3-5.

<sup>30</sup> Technical dossier/Annex 9, Table 4.

<sup>31</sup> Technical dossier/Annex 9 and Additional information June 2020/Table 2.

<sup>32</sup> Additional information June 2020/Table 1, LoD = 0.005% by Kjeldahl method.

<sup>33</sup> Technical dossier/Annex 9, Table 2.

<sup>34</sup> Technical dossier/Additional information June 2020.

## 4. Conclusions

Insufficient data were provided to complete a risk assessment of the food enzyme. In particular, uncertainty remains about the presence of a gene conferring resistance to antimicrobials in the genome of the production strain and its transfer to the food enzyme. Consequently, the Panel cannot conclude on the safety of the food enzyme isoamylase produced with *Dyella* sp. strain [REDACTED].

## Documentation provided to EFSA

- 1) Application for authorisation of Isoamylase preparation from *Pseudomonas amyloclavata* (microorganism's species-specific authorisation) in accordance with Regulation (EC) No. 1331/2008. August 2016. Submitted by Hayashibara Co., Ltd.
- 2) Additional information. July 2018. Submitted by Hayashibara Co., Ltd.
- 3) Additional information. February 2019. Submitted by Hayashibara Co., Ltd.
- 4) Additional information. September 2019. Submitted by Hayashibara Co., Ltd.
- 5) Additional information. June 2020. Submitted by Hayashibara Co., Ltd.

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## Abbreviations

|       |                                                                   |
|-------|-------------------------------------------------------------------|
| CAS   | Chemical Abstracts Service                                        |
| CEP   | EFSA Panel on Food Contact Materials, Enzymes and Processing Aids |
| FAO   | Food and Agricultural Organization of the United Nations          |
| GMM   | Genetically Modified Microorganism                                |
| GMO   | Genetically Modified Organism                                     |
| IUBMB | International Union of Biochemistry and Molecular Biology         |
| kDa   | Kilo Dalton                                                       |

|          |                                                           |
|----------|-----------------------------------------------------------|
| LOD      | Limit of Detection                                        |
| SDS-PAGE | Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis |
| TOS      | Total Organic Solids                                      |
| WGS      | Whole Genome Sequence                                     |
| WHO      | World Health Organization                                 |