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Safety evaluation of the food enzyme D-psicose 3-epimerase from the genetically modified *Escherichia coli* strain K-12 W3110 (pWKLP)

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Abstract

The food enzyme D-psicose 3-epimerase (EC 5.1.3.30) is produced with the genetically modified *Escherichia coli* strain K-12 W3110 (pWKLP) by Matsutani Chemical Industry Co., Ltd. The production strain of the food enzyme contains multiple copies of an antimicrobial resistance gene. However, based on the absence of viable cells and DNA from the production organism in the food enzyme, this is not considered to be a risk. The food enzyme is used as an immobilised preparation in processing fructose for the production of a speciality carbohydrate D-allulose (syn. D-psicose). Since residual amounts of total organic solids (TOS) are removed by the purification steps applied during the production of D-allulose, dietary exposure was not calculated and toxicological studies were not considered necessary. A search for similarity of the amino acid sequence of the enzyme to known allergens was made and no match was found. The Panel notes that the food enzyme may contain traces of protein, including a known allergen, after processing of the food enzyme. Therefore, allergenicity cannot be excluded, but the Panel considers that the likelihood of allergic reactions to occur is low. Based on the data provided, the immobilisation process and the removal of TOS during the production of D-allulose products, the Panel concluded that this food enzyme does not give rise to safety concerns when used in the immobilised form.

Keywords: food enzyme, D-psicose 3-epimerase, EC 5.1.3.30, D-allulose 3-epimerase, *Escherichia coli*, 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 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[2] established the European Union (EU) procedures for the safety assessment and the authorisation procedure of food additives, food enzymes and food flavourings. The use of a food enzyme shall be authorised only if it is demonstrated that:

- it does not pose a safety concern to the health of the consumer at the level of use proposed;
- there is a reasonable technological need;
- 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, 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 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.

An application has been introduced by the applicant "Matsutani Chemical Industry Co., Ltd." for the authorisation of the food enzyme D-allulose 3-epimerase from a genetically modified strain of *Escherichia coli* (strain K-12 W3110).

Following the requirements of Article 12.1 of Commission Regulation (EC) No 234/2011[3] implementing Regulation (EC) No 1331/2008, the Commission has verified that the application falls 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 assessment on the following food enzyme: D-allulose 3-epimerase from a genetically modified strain of *Escherichia coli* (strain K-12 W3110) in accordance with Article 17.3 of Regulation (EC) No 1332/2008 on food enzymes.

[1] Regulation (EC) No 1332/2008 of the European Parliament and of the Council of 16 December 2008 on Food Enzymes and Amending Council Directive 83/417/EEC, Council Regulation (EC) No 1493/1999, Directive 2000/13/EC, Council Directive 2001/112/EC and Regulation (EC) No 258/97. OJ L 354, 31.12.2008, 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, pp. 1–6.

[3] Commission Regulation (EU) No 234/2011 of 10 March 2011 implementing Regulation (EC) No 1331/2008 of the European Parliament and of the Council establishing a common authorisation procedure for food additives, food enzymes and food.

1.2. Interpretation of the Terms of Reference

The present scientific opinion addresses the European Commission's request to carry out the safety assessment of the food enzyme D-psicose 3-epimerase (systematic name of D-allulose 3-epimerase) from the genetically modified *E. coli* strain K-12 W3110 (pWKLP) (strain name as updated by applicant). The food enzyme is evaluated when used in the immobilised form only.

2. Data and methodologies

2.1. Data

The applicant has submitted a dossier in support of the application for authorisation of the food enzyme D-psicose 3-epimerase from the genetically modified *E. coli* strain K-12 W3110 (pWKLP).

Additional information was requested from the applicant during the assessment process on 3 March 2020 and 8 October 2020 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 of EFSA Scientific Committee.

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 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:	D-Psicose 3-epimerase
Systematic name:	D-Psicose 3-epimerase
Synonyms:	D-Allulose 3-epimerase, fructose epimerase
IUBMB No:	EC 5.1.3.30
CAS No:	1219591-85-1

D-Psicose 3-epimerase catalyses the epimerisation of D-fructose at the C3 position to produce D-allulose (also known as D-psicose) and vice versa. The enzyme is currently used in an immobilised form to produce D-allulose.[4]

3.1. Source of the food enzyme

The D-psicose 3-epimerase is produced with the genetically modified *E. coli* strain K-12 W3110 (pWKLP), which is deposited at the NITE Biological Resource Center (NBRC, Japan) with deposit number [redacted].[5]

The production strain was identified as a *E. coli* K-12 derivative [redacted].[6] No acquired antimicrobial resistance (AMR) genes were identified in the genome with the exception of the [redacted] gene used for the genetic modification.

3.1.1. Characteristics of the parental and recipient microorganisms

The recipient strain is *E. coli* K-12 W3110 ([redacted]), a well-known *E. coli* strain derived from the parental *E. coli* K-12 strain (Gorbach, 1978). The genomes of both strains have been sequenced and are publicly available (Hayashi et al., 2006). Sequencing data of the recipient strain's genome is available at the NCBI GeneBank under the accession number NC_007779.1 (https://www.ncbi.nlm.nih.gov/nuccore/NC_007779).

[4] Technical dossier/Additional data August 2020.
[5] Technical dossier/Additional data August 2020/Annex 1.
[6] Technical dossier/Additional data August 2020/Annex 2a.

3.1.2. Characteristics of the introduced sequences

The gene encoding the D-psicose 3-epimerase ([redacted]) is from the bacterium *Arthrobacter globiformis*, strain Ag M30, which was isolated from soil. [redacted]

3.1.3. Description of the genetic modification process

The purpose of genetic modification was to enable the production strain to synthesise the D-psicose 3-epimerase from *A. globiformis*. For this, the [redacted] gene was amplified from the *A. globiformis* M30 chromosome, [redacted]

The recipient strain *E. coli* K-12 W3110 was transformed with the replicative plasmid pWKLP. The production strain K-12 W3110 (pWKLP) contains multiple copies of the plasmid pWKLP, each carrying one copy of the [redacted] gene and one copy of the [redacted] gene.

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 K-12 W3110 (pWKLP) differs from the recipient strain K-12 W3110 in its capacity to produce the D-psicose 3-epimerase from *A. globiformis* [redacted]

The production strain contains the [redacted] gene on a multicopy replicative plasmid, conferring resistance to [redacted], an antibiotic listed as a critically important antimicrobial (WHO, 2018), which is considered a hazard.

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 according to the Food Hygiene Regulation (EC) No 852/2004[9], with food safety procedures based on Hazard Analysis and Critical Control Points, and in accordance with current Good Manufacturing Practice.[10]

The production strain is grown as a pure culture using a typical industrial medium in a submerged fermentation system with conventional process controls in place. After completion of the fermentation and treatment with [redacted],[11] 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 the 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.[12]

The applicant describes three different food enzyme preparations. These are a liquid preparation, where the food enzyme is stabilised with [redacted], a solid preparation, where the food enzyme is lyophilised without the addition of [redacted], and an immobilised preparation. The immobilised form of the food enzyme is said by the applicant to be the only form currently in use.[4] The food enzyme is immobilised [redacted]

[7] Technical dossier/2nd submission/Confidential/Main text and Reference 13.
[8] Technical dossier/2nd submission/Confidential/Main text, Reference 13 and Annex 3.
[9] 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.
[10] Technical dossier/Additional data August 2020/Annex 5.
[11] Technical dossier/Additional data August 2020/Annexes 6a and 6b.
[12] Technical dossier/2nd submission/p. 22–23 and Annex 4.

[redacted][13]

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 D-psicose 3-epimerase is a single polypeptide chain of 289 amino acids. The molecular mass of the mature protein, derived from the amino acid sequence, was calculated to be 31 kDa.[4] The food enzyme was analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). A consistent protein pattern was observed across all batches. The gels showed a single major protein band migrating between the marker proteins of 25 and 37 kDa in all batches, consistent with the expected mass of the enzyme. No other enzymatic activities were reported.

The in-house determination of D-psicose 3-epimerase activity is based on the conversion of the substrate D-allulose to D-fructose (reaction conditions: pH 8.0, 50°C, 10 min). The enzymatic activity is determined by measuring the release of D-fructose by high-performance liquid chromatography (HPLC) with refractive index detection. The D-psicose 3-epimerase activity is expressed in Units/g (U/g). One Unit is defined as the amount of enzyme that epimerises 1 μmol of substrate per minute.[14]

The food enzyme has a temperature optimum around 50°C (pH 7.5) and a pH optimum between 7.0 and 8.0 (55°C). Thermostability was tested after a pre-incubation of the food enzyme for 60 min at different temperatures (pH 7.5). The D-psicose 3-epimerase activity decreased above 60°C, showing no residual activity at 80°C.[15]

3.3.2. Chemical parameters

Data on the chemical parameters of the food enzyme were provided for three batches of the liquid food enzyme preparation[16] used for commercialisation and the solid preparation used for the toxicological tests (Table 1).[17] The mean total organic solids (TOS) of the three liquid food enzyme preparation batches was 4.75% and the mean enzyme activity/TOS ratio was 8.53 U/mg TOS.

Table 1: Compositional data of three batches of the liquid food enzyme preparation and the single batch of the solid food enzyme preparation used for the toxicological studies

Parameter	Unit	Batches			
		1	2	3	4[(a)]
D-psicose 3-epimerase activity	U/g batch [(b)]	432	337	446	31,237
Protein	%	0.46	0.36	0.48	58.8
Ash	%	0.14	0.08	0.13	5.5
Water	%	49.4	49.9	49.4	11.0
[redacted] (excipient)	%	45.67	45.32	45.72	-
Total organic solids (TOS)[(c)]	%	4.79	4.70	4.75	83.5
Activity/mg TOS	U/mg TOS	9.02	7.17	9.39	37.4

(a): Batch used for the toxicological studies.
(b): U/g: D-psicose 3-epimerase units (see Section 3.3.1).
(c): TOS calculated as 100% – % water – % ash – % excipient.

3.3.3. Purity

The lead content in the three liquid batches and in the batch used for the toxicological studies was below 0.1 mg/kg, which complies with the specification for lead (≤ 5 mg/kg) as laid down in the

[13] Additional data November 2020/Annexes: 19, 20b and 20c.
[14] Technical dossier/2nd submission/p. 16–18 and Annex 2.
[15] Technical dossier/2nd submission/p. 16.
[16] Technical dossier/Additional data August 2020/Annexes 11a and 14.
[17] Technical dossier/Additional data August 2020/Annex 11b; Additional data November 2020.

general specifications for enzymes used in food processing (FAO/WHO, 2006). In addition, the levels of arsenic and mercury were below the limits of detection (LODs) of the employed methodologies.[18,19]

The food enzyme preparation complies with the microbiological criteria for total coliforms, *E, coli* and *Salmonella*, as laid down in the general specifications for enzymes used in food processing (FAO/WHO, 2006).[20] No antimicrobial activity was detected in any of the tested batches (FAO/WHO, 2006).[20]

The presence of mycotoxins (aflatoxins B1, B2, G1 and G2) was examined in the three food enzyme preparation batches and were below the LOD of the applied analytical methods.[20,21]

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 was demonstrated in three independent batches of the formulated liquid enzyme, analysed in triplicate. [redacted] No colonies were produced. A positive control was included.[22]

The absence of recombinant DNA in the food enzyme was demonstrated by polymerase chain reaction (PCR) analysis of three batches of the formulated liquid enzyme analysed in triplicate. No DNA was detected [redacted].[23]

3.4. Toxicological data

Taking into account the intended use and the fact that the enzyme is currently used in the immobilised form only, the exposure is negligible (see Section 3.5.1). Therefore, toxicological tests are not needed for the assessment of this food enzyme.

A battery of toxicological tests, including a bacterial gene mutation assay (Ames test), an *in vitro* micronucleus test and a repeated dose 90-day oral toxicity study in rats, has been provided. These tests have not been considered, even as supporting evidence, as the item used for toxicological testing (see batch 4, Table 1) has a significantly higher chemical purity (as indicated by the higher activity/mg TOS) and is not considered suitable to evaluate the toxicity of this food enzyme.

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 the D-psicose 3-epimerase produced with the genetically modified *E. coli* strain K-12 W3110 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.[24]

No information is available on oral or respiratory sensitisation and elicitation reactions to this D-psicose 3-epimerase. In addition, no allergic reactions to ingestion or respiratory exposure to epimerases have been reported in the literature.

[18] LODs: Pb = 0.05 mg/kg; As = 0.75 mg/kg; Hg = 0.01 mg/kg.
[19] Technical dossier/Additional data August 2020/Annex 11a and 11b.
[20] Technical dossier/Additional data August 2020/Annexes 11a and 11b.
[21] LOD: aflatoxins B1, B2, G1 and G2 = 1 μg/kg each.
[22] Technical dossier/Additional data August 2020/Annex 3.
[23] Technical dossier/Additional data August 2020/Annex 4.
[24] Technical dossier/2nd submission/References 15 and 43 and Additional data November 2020/Annex 23.

According to the information provided, substances or products that may cause allergies or intolerances (Regulation EU 1169/2011)[25] are used as raw materials ([redacted]). In addition, [redacted], a known source of allergen, is also present in the media fed to the microorganisms. However, during the fermentation process, these products will be degraded and utilised 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 materials employed as nitrogen sources are not expected to be present in the food enzyme.

The Panel notes that [redacted], a known allergen, is used during the downstream processing of the food enzyme.

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. However, traces of protein could be present in the D-allulose product.

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, in particular, due to the potential presence of [redacted], but the likelihood of such reactions to occur is low.

3.5. Dietary exposure

3.5.1. Intended use of the food enzyme

Currently, the food enzyme is intended to be used only in immobilised form for the production of allulose. A calculation of a use level up to [redacted] mg TOS/kg D-fructose was provided for the solid form of the food enzyme.[26]

When the food enzyme is used in immobilised form, the transfer of TOS into the final product, i.e. highly purified D-allulose, is expected to be negligible. No hazard was identified during the immobilisation process (see Section 3.2). [redacted] the reaction product (non-purified D-allulose) is subjected to a series of purification steps ([redacted]),[27] which are expected to eliminate any residual food enzyme from the final product D-allulose. The applicant measured the total nitrogen content in six batches of crystalline allulose and the results were below the limit of quantification (LOQ).[28] [redacted] was also not detected in three batches of allulose by HPLC analysis.[29]

The Panel accepted the evidence provided as sufficient to conclude that the presence of residual amounts of TOS after processing of D-allulose is negligible.

3.5.2. Dietary exposure estimation

The technical information and experimental data provided on the removal of food enzyme TOS during the production of D-allulose were considered by the Panel as sufficient to exclude this process from the exposure assessment (Annex B in EFSA CEF Panel, 2016). Consequently, a dietary exposure was not calculated.

4. Conclusions

Based on the data provided and the removal of TOS during the purification steps applied during the production of D-allulose, the Panel concluded that the food enzyme D-psicose 3-epimerase produced with the genetically modified *E. coli* strain K-12 W3110 (pWKLP) does not give rise to safety concerns when the food enzyme is used in the immobilised form.

The production strain of the food enzyme contains multiple copies of an AMR gene in a self-replicating plasmid. However, based on the absence of viable cells and DNA from the production organism in the food enzyme, this is not considered to be a risk.

[25] 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.

[26] Technical dossier/Additional data November 2020/Annexes 24a and 24b.

[27] Additional data August 2020/Annex 18.

[28] Additional data August 2020/Annex 8; LOQ = 0.1 g/100 g & November 2020/Annex 2a, LOQ = 0.01 g/100 g.

[29] Additional data November 2020/Annex 20d.

Documentation provided to EFSA

1) Dossier "Application for the authorisation of the food enzyme: D-allulose 3-epimerase", July 2019. Submitted by Matsutani Chemical Industry Co., Ltd.
2) Additional information. August 2020. Submitted by Matsutani Chemical Industry Co., Ltd.
3) Additional information. November 2020. Submitted by Matsutani Chemical Industry Co., Ltd.

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Abbreviations

AMR	antimicrobial resistance
CAS	Chemical Abstracts Service
CEF	EFSA Panel on Food Contact Material, Enzymes, Flavourings and Processing Aids
CEP	EFSA Panel on Food Contact Materials, Enzymes and Processing Aids
FAO	Food and Agriculture Organization of the United Nations
GMO	genetically modified organisms
HPLC	high-performance liquid chromatography
IUBMB	International Union of Biochemistry and Molecular Biology
LOD	limit of detection
LOQ	limit of quantification
MLST	multilocus sequence typing
NBRC	NITE Biological Resource Center
PCR	polymerase chain reaction
SDS–PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
TOS	total organic solids
WGS	whole-genome sequencing
WHO	World Health Organization