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Safety evaluation of the food enzyme alternansucrase from *Leuconostoc citreum* strain NRRL B-30894

EFSA Panel on Food Contact Materials, Enzymes and Processing Aids (CEP), Vittorio Silano, José Manuel Barat Baviera, Claudia Bolognesi, Pier Sandro Cocconcelli, Riccardo Crebelli, David Michael Gott, Konrad Grob, Claude Lambré, Evgenia Lampi, Marcel Mengelers, Alicja Mortensen, Gilles Rivière, Inger-Lise Steffensen, Christina Tlustos, Henk Van Loveren, Laurence Vernis, Holger Zorn, Boet Glandorf, Lieve Herman, André Penninks*, Jaime Aguilera, Margarita Aguilera-Gomez, Magdalena Andryszkiewicz, Natália Kovalkovičová, Yi Liu, Claudia Roncancio Peña and Andrew Chesson

Abstract

The food enzyme alternansucrase (sucrose:1,6(1,3)-α-D-glucan 6(3)-α-D-glucosyltransferase, EC 2.4.1.140) is produced with a non-genetically modified *Leuconostoc citreum* strain NRRL B-30894 by Cargill Incorporated. As a consequence of the absence of antimicrobial resistance genes identified in its genome, the production strain meets the criteria to qualify for the Qualified Presumption of Safety (QPS) approach to safety assessment. As no other concerns arising from the microbial source or from the manufacturing process have been identified, the Panel considers that toxicological tests are not needed for the assessment of this food enzyme. The alternansucrase food enzyme is intended to be used for the manufacture of α-D-glucan oligosaccharides as a sweetening agent. The purification processes applied to syrups produced from sucrose with alternansucrase are expected to largely remove the food enzyme. Any residual TOS remaining in the final product would consist of non-hazardous material. This is based on the QPS status of the production organism, the medium components and the identified material used in downstream processing. Consequently, the Panel decided that dietary exposure did not need to be calculated. 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. Based on the data provided, the Panel concluded that this food enzyme does not give rise to safety concerns under the intended conditions of use.

Keywords: food enzyme, alternansucrase, sucrose:1,6(1,3)-α-D-glucan 6(3)-α-D-glucosyltransferase, EC 2.4.1.140, *Leuconostoc citreum*, non-genetically modified microorganism

Requestor: European Commission

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Correspondence: fip@efsa.europa.eu

*: Member of the former Working Group on 'Enzymes' of the EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF).

Panel members: José Manuel Barat Baviera, Claudia Bolognesi, Andrew Chesson, Pier Sandro Cocconcelli, Riccardo Crebelli, David Michael Gott, Konrad Grob, Claude Lambré, Evgenia Lampi, Marcel Mengelers, Alicja Mortensen, Gilles Rivière, Vittorio Silano, Inger-Lise Steffensen, Christina Tlustos, Henk Van Loveren, Laurence Vernis and Holger Zorn.

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Table of contents

1. Introduction

Article 3 of the Regulation (EC) No 1332/2008[1] provides definition 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[1] 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:

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, 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 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[1] on food enzymes.

Four applications have been introduced by the companies 'Cargill R&D Centre Europe' for the authorisation of the food enzyme alternansucrase from *Leuconostoc citreum* (strain NRRL B-30894), 'Intertek Scientific&Regulatory Consultancy' for the authorisation of the food enzymes β-galactosidase from *Bacillus circulans* (strain M3-1) and D-fructose 3-epimerase from a genetically modified strain of *Escherichia coli* (W3110-TKO), and 'AB Enzymes GmbH' for the authorisation of the food enzyme triacylglycerol lipase from a genetically modified strain of *Trichoderma reesei* (RF10625).

Following the requirements of Article 12.1 of Commission Regulation (EU) No 234/2011[3] implementing Regulation (EC) No 1331/2008[2], the Commission has verified that the four applications fall within the scope of the food enzyme Regulation and contain all the elements required under Chapter II of that Regulation.

[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, pp. 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 flavourings. OJ L 64, 11.03.2011, pp. 15–24.

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 alternansucrase from *Leuconostoc citreum* (strain NRRL B-30894), β-galactosidase from *Bacillus circulans* (strain M3-1), D-fructose 3-epimerase from a genetically modified strain of *Escherichia coli* (W3110-TKO), and triacylglycerol lipase from a genetically modified strain of *Trichoderma reesei* (RF10625) in accordance with Article 17.3 of Regulation (EC) No 1332/2008[1] 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 alternansucrase from the non-genetically modified *L. citreum* (strain NRRL B-30894).

2. Data and methodologies

2.1. Data

The applicant has submitted a dossier in support of the application for authorisation of the food enzyme alternansucrase from the non-genetically modified *L. citreum* (strain NRRL B-30894).

Additional information was requested from the applicant during the assessment process on 17 May 2019 and 2 April 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, 2009) and following the relevant existing guidances of EFSA Scientific Committee.

The current 'Guidance on the submission of a dossier on food enzymes for safety evaluation' (EFSA CEF Panel, 2009) 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[4]

IUBMB nomenclature:	alternansucrase
Systematic name:	sucrose:1,6(1,3)-α-D-glucan 6(3)-α-D-glucosyltransferase
Synonyms: sucrose:	1,6-, 1,3-α-D-glucan 3-α- and 6-α-D-glucosyltransferase
IUBMB No.:	EC 2.4.1.140
CAS No.:	100630-46-4

The food enzyme alternansucrase is a glucosyl transferase catalysing the transfer of the glucose moiety in sucrose to the non-reducing terminal of α-D-glucans via alternating α-1,6- and α-1,3-glycosidic linkages with the release of free fructose.[5] It is intended to be used in the manufacture of α-D-glucan oligosaccharides.

3.1. Source of the food enzyme[6]

The food enzyme is produced with a non-genetically modified strain of the bacterium *L. citreum*, which is deposited at the USDA Northern Regional Research Laboratory (NRRL) with the accession number NRRL B 30894.[7] The [redacted], deduced from [redacted] of the production strain,[8] confirms that the production strain is correctly identified as *L. citreum*.[9]

[4] Technical dossier/p. 21.
[5] Technical dossier/p. 25.
[6] Technical dossier/p. 28–31; Technical dossier/Additional information, 2 October 2020.
[7] Technical dossier/Annex A3.2.
[8] Technical dossier/Additional information, 2 October 2020/Annex A1.
[9] Technical dossier/p. 28–29.

L. citreum NRRL B-30894 is an alternansucrase-overproducing mutant obtained by [redacted] from the parent strain [redacted] It is available as GenBank accession number [redacted] through the U.S. National Library of Medicine.[4]

The species *L. citreum* is included in the Qualified Presumption of Safety (QPS) list with the qualification that no acquired antimicrobial resistance (AMR) genes are present in the specific strain under application (EFSA, 2007; EFSA BIOHAZ Panel, 2017a,b). Phenotypic testing showed that the production strain (*Leuconostoc citreum* strain NRRL B-30894) was resistant to a number of aminoglycoside antibiotics, particularly kanamycin, when judged against the minimum inhibitory concentration (MIC) cut-off values used by EFSA to determine sensitivity.[11] A further search for AMR genes in the genome of the production strain was performed by the applicant using eight different databases of known resistance determinants.[12] No antibiotic resistance or virulence genes were detected.[8]

Consequently, the production organism meets the criteria of the QPS approach to safety assessment.

3.2. Production of the food enzyme[13]

The food enzyme is manufactured according to the Food Hygiene Regulation (EC) No 852/2004[14], with food safety procedures based on hazard analysis and critical control points,[15] and in accordance with current Good Manufacturing Practice.[16]

The production strain is grown as a pure culture using a typical industrial medium in [redacted] with conventional process controls in place. After completion of the fermentation, the solid biomass is removed from the fermentation broth [redacted] The final stage in the production process is [redacted] the food enzyme. The applicant provided information on the identity and analysis of the substances used to control the fermentation and in the subsequent downstream processing of the food enzyme.

The Panel considered that sufficient information has been provided on the manufacturing process and the quality assurance system implemented by the applicant to exclude issues of concern.

3.3. Characteristics of the food enzyme

3.3.1. Properties of the food enzyme

The food enzyme is a single polypeptide chain of [redacted][17] amino acids, excluding the signal sequence of [redacted] amino acids. The molecular mass of the mature protein, derived from the amino acid sequence, was calculated to be 229 kDa.[18] The food enzyme was analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). This showed the presence of three major protein bands at about 220 kDa (corresponding to the determined mass of alternansucrase), 48 kDa and 45 kDa (both ascribed to amylase proteins), in all four of the commercial batches examined. Other bands of minor staining intensity were also found.[19] Amylase activity was detected in the food enzyme at approximately 1 U/g. Protease and lipase activities were below the respective limits of detection (LODs).[20]

[10] Technical dossier/p. 28.
[11] Technical dossier/Additional information, 9 March 2020/Annex 4.
[12] Technical dossier/Additional information, 9 March 2020; Technical dossier/Additional information, 2 October 2020.
[13] Technical dossier/p. 31–34.
[14] 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.
[15] Technical dossier/Annex A4.3.
[16] Technical dossier/Annex A4.
[17] Technical dossier/p. 21–22 and http://www.ncbi.nlm.nih.gov/protein/q9re05
[18] Technical dossier/Additional information, 9 March 2020.
[19] Technical dossier/Additional information, 9 March 2020/Annex 2.
[20] Technical dossier/p. 27 and Annex 2.4.

The in-house determination of alternansucrase activity is based on the release of fructose from sucrose. [redacted] is determined by high-performance liquid chromatography. One unit of alternansucrase activity is defined as the amount of enzyme that liberates 1 μmoL of fructose per minute under the conditions of the assay.[21]

The food enzyme shows a temperature optimum at [redacted]°C[22] and a pH optimum between [redacted][22] It is unstable when stored at pH values [redacted] Studies relevant to the practical use of the enzyme showed that activity was lost at temperatures [redacted][23]

3.3.2. Chemical parameters

Data on the chemical parameters of the food enzyme were provided for three food enzyme batches used for commercialisation (Table 1). The average total organic solids (TOS) of the three food enzyme batches for commercialisation was 3.69% and the average enzyme activity/mg TOS ratio was 1.55 U/mg TOS.

Table 1: Compositional data of the three batches of the food enzyme[24]

Parameter	Unit	Batches		
		1	2	3
Alternansucrase activity	U/mL[(a)]	56.96	56.50	58.71
Protein	%	n.a.	n.a.	n.a.
Ash	%	1.29	0.88	1.54
Water	%	94.9	95.5	94.8
Total organic solids(TOS)[(b)]	%	3.81	3.62	3.66
Alternansucrase activity/mg TOS	U/mg TOS	1.50	1.56	1.60

n.a.: not analysed.
(a): U: Alternansucrase unit (see Section 3.3.1).
(b): TOS calculated as 100% – % water – % ash.

3.3.3. Purity

The lead[25] content in the three commercial batches was below 5 mg/kg which complies with the specification for lead (≤ 5 mg/kg) as laid down in the general specifications and considerations for enzymes used in food processing (FAO/WHO, 2006). In addition, the levels of heavy metals (cadmium and mercury) were below the LODs of the employed methodologies.[26] Trace amounts of arsenic was detected in one of the three commercial batches ([redacted]mg/kg),[27] well below the specification set (not more than 3 mg/kg for arsenic) by Regulation (EU) No 231/2012 for food additives.[28]

The food enzyme 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). Detection of *E. coli* used a recognised AOAC method with a minimum specification set at < [redacted]/g food enzyme. Analytical reports of the three commercial batches include additional microbiological parameters (numbers of yeasts and filamentous fungi, sulfite-reducing bacteria and *Staphylococcus aureus*).[29] None were found in the three batches of the food enzyme tested.

No antimicrobial activity[30] was detected in the three batches intended for commercialisation described in Table 1 (FAO/WHO, 2006).

[21] Technical dossier/p. 25; Technical dossier/Annex A2.5.
[22] Technical dossier/p. 25–26.
[23] Technical dossier/Annex A5.1.
[24] Technical dossier/p. 24; Technical dossier/Annex A2.4.
[25] Technical dossier/p. 25; Technical dossier/Annex A2.1; LOD: Pb = [redacted] μg/kg.
[26] Technical dossier/p. 25; Technical dossier/Annex A2.1; LODs: Cd = [redacted] mg/kg; Hg = [redacted] mg/kg.
[27] Technical dossier/Annex A2.1.
[28] Regulation (EU) No 231/2012 of the European Parliament and of the Council of 9 March 2012 laying down specifications for food additives listed in Annexes II and III to Regulation (EC) No 1333/2008 of the European Parliament and of the Council. OJ L 83/1, 22.3.2012.
[29] Technical dossier/p. 25; Technical dossier/Annex A2.2.
[30] Technical dossier/p. 24.

Analysis for the presence of mycotoxins (aflatoxins B1, B2, G1 and G2, deoxynivalenol, fumonisins B1, B2 and B3, and zearalenone) was made for the three food enzyme preparation batches. Results were below the LOD of the applied analytical methods.[31]

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[32]

The absence of the production strain in the food enzyme preparation was demonstrated in 15 samples coming from five production batches, using [redacted] no colonies were produced.[33]

3.4. Toxicological data

The Panel considers that no toxicological studies other than assessment of allergenicity are necessary for this food enzyme. This is based on the QPS status of the production strain and the absence of any identified hazards arising from the production and downstream processing of the 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.

Potential allergenicity of alternansucrase produced with *Leuconostoc citreum* NRRL B-30894 strain 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, 2017). Using higher than 35% identity in a sliding window of 80 amino acids as the criterion, no match was found.[34]

No information is available on oral sensitisation or elicitation reactions of this alternansucrase under evaluation. There are also no reports in the literature on allergenicity of other alternansucrases.

Yeast extract, a known allergen, is present in the fermentation medium. During the fermentation process, this product 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 are removed. Taking into account the fermentation process and downstream processing, the Panel considered that potentially allergenic residues of this food employed as protein source is not expected to be present.

The Panel considered that, under the intended conditions of use, the risk of allergic sensitisation and elicitation reactions upon dietary exposure to this food enzyme cannot be excluded but the likelihood of such reactions to occur is considered to be low.

3.5. Dietary exposure

3.5.1. Intended use of the food enzyme

The alternansucrase food enzyme is intended to be used in the manufacture of α-D-glucan oligosaccharides. The syrup produced by the food enzyme consists mainly of gluco-oligosaccharides up to a degree of polymerisation of 12 with application as a low glycaemic index sweetening agent in a wide variety of foods (e.g. replacing sucrose, high fructose corn syrup, corn syrup, brown rice syrup, and malt syrup). The typical use level of the food enzyme is between 0.2 and 1.5 mg TOS/kg sugar.[35]

Evidence was provided to show that enzyme activity could not be detected in syrup produced with alternansucrase.[36]

[31] Technical dossier/p. 25 and 35; Technical dossier/Annex A2.3.; LODs: aflatoxin B1 = [redacted] µg/kg, aflatoxin B2 = [redacted] µg/kg, aflatoxin G1 = [redacted] µg/kg, aflatoxin G2 = [redacted] µg/kg, deoxynivalenol = [redacted] µg/kg, fumonisin B1 = [redacted] µg/kg, fumonisin B2 = [redacted] µg/kg, fumonisin B3 = [redacted] µg/kg and zearalenone = [redacted] µg/kg.

[32] Technical dossier/Additional information, 2 October 2020.

[33] Technical dossier/Additional information, 2 October 2020/Annex A2.

[34] Technical dossier/p. 44; Technical dossier/Annex A7.2.

[35] Technical dossier/p. 37.

[36] Technical dossier/p. 36/Figure 9 and Table 10; Technical dossier/Annex A.5.

Since the purification processes applied to syrups produced from sucrose with alternansucrase included decolourisation and ion-exchange, the food enzyme is expected to be largely removed. Although, no experimental data were provided to support this view, any residual TOS remaining in the final product would consist of non-hazardous material. This is based on the QPS status of the production organism, the medium components and the identified material used in downstream processing. Consequently, the Panel decided that dietary exposure did not need to be calculated.

4. Conclusions

Based on the data provided and the QPS status of the production strain, the Panel concluded that the food enzyme alternansucrase produced with the non-genetically modified *L. citreum* strain NRRL B-30894 does not give rise to safety concerns under the intended conditions of use.

Documentation provided to EFSA

1) Technical dossier `An alternansucrase preparation from *Leuconostoc citreum* NRRL B-30894 for use as a food processing aid´. March 2015. Submitted by Cargill R&D Centre Europe.
2) Additional information. 9 March 2020. Submitted by Cargill R&D Centre Europe.
3) Additional information. 2 October 2020. Submitted by Cargill R&D Centre Europe.
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Abbreviations

AMFEP	Association of Manufacturers and Formulators of Enzyme Products
AMR	antimicrobial resistance genes
AOAC	Association of Official Analytical Chemists
CAS	Chemical Abstracts Service
EC	Enzyme Commission
EFSA BIOHAZ Panel	EFSA Panel on Biological Hazards
EFSA CEF Panel	EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
EFSA CEP Panel	EFSA Panel on Food Contact Materials, Enzymes and Processing Aids
EFSA GMO Panel	EFSA Panel on Genetically Modified Organisms
FAO	Food and Agricultural Organization of the United Nations
GM	genetically modified
GMO	genetically modified organism
IUBMB	International Union of Biochemistry and Molecular Biology
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LOD	limit of detection
MIC	minimum inhibitory concentration
[redacted]	[redacted]
n.a.	not analysed
NCBI	National Center for Biotechnology Information
NRRL	Northern Regional Research Laboratory
QPS	Qualified Presumption of Safety
[redacted]	[redacted]
SDS–PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
TOS	total organic solids
U	alternansucrase enzymatic unit
USDA	United States Department of Agriculture
WHO	World Health Organization