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Safety evaluation of the food enzyme beta-galactosidase from *Bacillus* sp. (strain M3-1)

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Abstract

The food enzyme β -D-galactosidase galactohydrolase (EC 3.2.1.23) is produced with *Bacillus* sp. strain M3-1 by GenoFocus Inc. The food enzyme β -galactosidase is intended to be used in the manufacture of galactooligosaccharides (GOS). Since residual amounts of total organic solids are removed by the purification steps applied during the production of GOS, toxicological studies were considered not necessary and no dietary exposure was calculated. Similarity of the amino acid sequence of the food enzyme 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 of such reactions 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.

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1. Introduction

Article 3 of the Regulation (EC) No 1332/2008¹ 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 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 1331/2008² 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 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.

Four applications have been introduced by the companies 'Cargill R&D Centre Europe' for the authorisation of the food enzyme Alternansucrase from *Leuconostoc citreum* (NRRL B-30894), 'Intertek Scientific & Regulatory Consultancy' for the authorisation of the food enzymes Beta-galactosidase from *Bacillus circulans* (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 234/2011³ 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 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, 11.3.2011, p. 15–24.

1.1.2. Terms of Reference

The European Commission requests European Food Safety Authority to carry out the safety assessments of the food enzymes Alternansucrase from *Leuconostoc citreum* (NRRL B-30894), Beta-galactosidase from *Bacillus circulans* (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 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 β -galactosidase from *B. circulans* (strain M3-1).

2. Data and methodologies

2.1. Data

The applicant has submitted a dossier in support of the application for authorisation of the food enzyme β -galactosidase from *B. circulans* (strain M3-1).

Additional information was requested from the applicant during the assessment process on 1/7/2017, on 2/4/2019, 20/8/2019 and on 9/9/2019 and was consequently provided (see 'Documentation provided to EFSA').

Following the request for additional data sent by EFSA on 2/4/2019, EFSA requested a clarification conference, which was held on 20/8/2019.

2.2. Methodologies

The assessment was conducted in line with the principles described in the EFSA 'Guidance on transparency in the scientific aspects of risk assessment' (EFSA, 2009) as well as in the '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 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

IUBMB nomenclature:	β -galactosidase
Systematic name:	beta-D-galactoside galactohydrolase
Synonyms:	lactase, β -D-galactosidase galactohydrolase
IUBMB No.:	EC 3.2.1.23
CAS No.:	9031-11-2
EINECS No.:	232-864-1.

The β -galactosidase catalyses the hydrolysis of β -(1,4)-glycosidic linkage of lactose (β -D-galactosyl-1,4-D-glucoside) resulting in the generation of D-galactose and D-glucose. In the presence of high concentration of lactose, the enzyme will also act as a transgalactosylase. It is intended to be used in the manufacture of galactooligosaccharides (GOS).

3.1. Source of the food enzyme

The β -galactosidase is produced with a non-genetically modified *Bacillus* strain obtained by mutagenesis of the parental strain deposited in the collection at the [REDACTED] with the deposition number [REDACTED] as *B. circulans*. The production strain *B. circulans* M3-1 was isolated from the mutagenesis library of its parental strain on the basis of its higher β -galactosidase expression.⁴ The strain has been deposited in the collection at the [REDACTED] with the deposition number [REDACTED].⁵

⁴ Technical dossier/1st submission/Annex I; Additional information August 2017.

⁵ Technical dossier/1st submission/Annex I.4.

Although the parental strain is currently deposited as *B. circulans* in the [REDACTED], the 16S rDNA gene sequence and whole genome sequence analysis of the production strain indicates that the most closely related species is *Bacillus mesonae*.⁶ However, the average nucleotide identity (ANI) between the strain M3-1 and *B. mesonae* is below the defined threshold value of 95% used for species demarcation. Consequently, a conclusive taxonomical classification of production strain *Bacillus* sp. M3-1 was not achieved.

The antimicrobial susceptibility of *Bacillus* sp. M3-1 was tested via broth dilution tests against the antimicrobial vancomycin, gentamicin, kanamycin, streptomycin, erythromycin, clindamycin, tetracycline and chloramphenicol.⁷ The minimum inhibitory concentrations (MICs) were below the cut-off values established for the *Bacillus* genus by EFSA (EFSA CEP Panel, 2019). The absence of acquired genes coding for resistance to antimicrobial was demonstrated by analysing the whole genome sequencing (WGS).⁸

The data provided demonstrated that the food enzyme lacks antibacterial activity.⁹

3.2. Production of the food enzyme

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

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 final product is freeze dried and stabilised with [REDACTED].¹² 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.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 β -galactosidase is composed of [REDACTED] amino acids.¹⁴ The molecular mass, derived from the amino acid sequence, was calculated to be [REDACTED] and [REDACTED].¹⁴ 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 two major protein bands corresponding to an apparent molecular mass of about [REDACTED] and [REDACTED].¹⁵ No other enzymatic side activities were reported.¹⁶

The in-house determination of β -galactosidase activity is based on hydrolysis of a synthetic substrate (*o*-nitrophenol β -D-galactopyranoside (oNPG)), (reaction conditions: pH 6.0, 37°C).¹⁷ The enzymatic activity is determined by measuring the release of the yellow-coloured *p*-nitrophenol spectrophotometrically at 420 nm. The enzyme activity is expressed in U/g. One unit of activity is

⁶ Technical dossier/1st submission/Additional information July 2019, Attachment 1.

⁷ Technical dossier/1st submission/Additional information July 2019, Attachment 3.

⁸ Technical dossier/1st submission/Additional information July 2019, Attachment 4.

⁹ Technical dossier/1st submission/p.28 and Annex I.6.

¹⁰ 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.

¹¹ Technical dossier/1st submission/p.29-34/Annex IV.1.

¹² Technical dossier/1st submission/p.49 and Annex V.3.

¹³ Technical dossier/1st submission/Annex V.2.

¹⁴ Technical dossier/1st submission/p. 16.

¹⁵ Technical dossier/1st submission/p. 18.

¹⁶ Technical dossier/1st submission/p. 24.

¹⁷ Technical dossier/1st submission/Annex II.8.

defined as the amount of enzyme that will release one μ mole of o-nitrophenol per minute under the conditions of the assay.¹⁸

The food enzyme has a temperature optimum around 50°C (pH 6.0) and a pH optimum around pH 6 (37°C).¹⁹ Thermostability was tested after a pre-incubation of the food enzyme for 60 min at different temperatures. Under the conditions of the applied temperature stability assay, β -galactosidase was stable at 50°C, but lost 50% of its activity at 60°C after 1 h.²⁰

3.3.2. Chemical parameters

Data on the chemical parameters of the food enzyme were provided for eight food enzyme batches, three batches used for commercialisation and five batches produced for the toxicological tests (Table 1).²¹ The average Total Organic Solids (TOS) of the three food enzyme batches for commercialisation was 30.93%. The average enzyme activity/TOS ratio of the three food enzyme batches for commercialisation was 7.69 U/mg TOS.

Table 1: Compositional data of three commercial batches of food enzyme preparation

Parameter	Unit	Batches		
		1	2	3
β -galactosidase activity	U/g ^(a)	2,370	2,410	2,320
Protein	%	10.95	11.33	10.65
Ash	%	3.20	2.50	3.60
Water	%	5.9	4.3	4.7
Total Organic Solids (TOS) ^(b)	%	32.9	28.2	31.7
Activity/mg TOS	U/mg TOS	7.20	8.55	7.32
Lactose (excipient)	%	58.0	65.0	60.0

(a): U/g: Unit/g (see Section 3.3.1).

(b): TOS calculated as 100% - % water - % ash - % diluent.

3.3.3. Purity

The lead content in the three commercial batches was below 0.1 mg/kg²² 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 arsenic were below the limits of detection (LOD) of the employed methodologies.²³

The food enzyme preparation complies with the microbiological criteria²⁴ 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 should not exceed 30 CFU/g. No antimicrobial activity was detected in any of the tested batches (FAO/WHO, 2006).²⁵

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

3.4. Toxicological data

The food enzyme is intended to be used in GOS production. In the course of this process, the food enzyme is removed by the applied purification steps during manufacture of GOS (see Section 3.6) and, consequently, toxicological data although provided were considered not necessary.

¹⁸ Technical dossier/1st submission/p. 20.

¹⁹ Technical dossier/1st submission/p. 22.

²⁰ Technical dossier/1st submission/p. 23.

²¹ Technical dossier/1st submission/p. 33; Additional information August 2017.

²² LOD: Pb = 0.1 mg/kg; Additional information August 2017.

²³ LOD: As = 0.1 mg/kg; Additional information August 2017.

²⁴ Technical dossier/1st submission/p.33, 51/Annexes: VI.2, VI.3, VI.4, VII.6a, VII.6B, VII.6c; Additional information August 2017.

²⁵ Technical dossier/1st submission/p.36/Annex 2.04; Additional information August 2017.

3.5. 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 β -galactosidase produced with the *Bacillus* sp. strain M3-1 was assessed by comparing its amino acid sequence with those of known allergens according to the scientific opinion on the assessment of allergenicity of genetically modified plants and 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.²⁶

No information is available on oral and respiratory sensitisation or elicitation reactions of this β -galactosidase.

Cases of occupational allergy following exposure by inhalation of β -galactosidase have been reported (Stöcker et al., 2016). However, several studies have shown that adults with occupational asthma can ingest respiratory allergens without acquiring clinical symptoms of food allergy (Brisman, 2002; Poulsen, 2004; Armentia et al., 2009). In addition, two case reports describing allergic reactions (swollen throat, shortness of breath and difficulty in swallowing) following ingestion of β -galactosidase pills, and confirmation by antigen challenge, have been reported (Binkley, 1996; Voisin and Borici-Mazi, 2016).

According to the information provided, substances or products that may cause allergies or intolerances (Regulation (EU) No 1169/2011²⁷) are used as raw materials () 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 are removed. Taking into account the fermentation process and downstream processing, the Panel considered that potentially allergenic residues of these foods employed as protein sources are 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.

3.6. Dietary exposure

The food enzyme is intended to be used in the production of GOS at a recommended use level of up to 700 mg TOS/kg lactose (2 g food enzyme preparation/kg lactose).²⁸

Flow charts depicting manufacturing of GOS syrup were provided by the applicant.²⁹ The food enzyme is added to lactose and held at 55°C to allow the reaction to occur after which the enzyme is heat inactivated. At this stage, yeast ()³⁰ may be added to remove low molecular weight saccharides. When used, the yeast is killed by heat and the syrup is filtered. The resulting GOS syrup is then treated with activated carbon, followed by passage through ion exchange columns.

Analytical data provided for three batches of the resulting GOS syrup showed the absence of viable microorganisms and only trace amounts of nitrogen.³¹ The Panel considers this information as sufficient to conclude that residual amounts of TOS (including substances other than proteins) are removed by the purification steps applied to the production of GOS.

Consequently, a dietary exposure to the food enzyme–TOS was not calculated.

²⁶ Technical dossier/1st submission/p.52/Annex VIII.1.

²⁷ 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.

²⁸ Technical dossier/1st submission/p. 34–36; Additional information August 2017.

²⁹ Technical dossier/Additional information July 2019/Attachment 5.

³⁰ Technical dossier/Additional information September 2019.

³¹ Technical dossier/Additional information August 2019.

4. Conclusions

Based on the data provided, in particular, considering the removal of TOS during the production of GOS, the Panel concluded that the food enzyme β -galactosidase produced with the *Bacillus* sp. strain M3-1 does not give rise to safety concerns under the intended conditions of use.

Documentation provided to EFSA

- 1) Dossier 'Application for authorisation of β -galactosidase from *Bacillus circulans* (strain M3-1)'. March 2015. Submitted by Intertek Scientific & Regulatory Consultancy on behalf the company GenoFocus Inc.
- 2) Additional information. August 2017. Submitted by Intertek Scientific & Regulatory Consultancy on behalf the company GenoFocus Inc.
- 3) Additional information. July 2019. Submitted by Intertek Scientific & Regulatory Consultancy on behalf the company GenoFocus Inc.
- 4) Additional information. August 2019. Submitted by Intertek Scientific & Regulatory Consultancy on behalf the company GenoFocus Inc.
- 5) Additional information. September 2019. Submitted by Intertek Scientific & Regulatory Consultancy on behalf the company GenoFocus Inc.

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Abbreviations

ANI	average nucleotide identity
ATCC	American Type Culture Collection
CAS	Chemical Abstracts Service
CEF	EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
CEP	EFSA Panel on Food Contact Materials, Enzymes and Processing Aids
CFU	colony forming units
EC	Enzyme Commission
EINECS	European Inventory of Existing Commercial Chemical Substances
FAO	Food and Agricultural Organization of the United Nations
GMO	genetically modified organism
GMP	Good Manufacturing Practice
GOS	galactooligosaccharides
HACCP	Hazard Analysis and Critical Control Points
IUBMB	International Union of Biochemistry and Molecular Biology
JECFA	Joint FAO/WHO Expert Committee on Food Additives
KCCM	Korean Culture Centre of Microorganisms
LOD	limit of detection
MIC	minimum inhibitory concentration
oNPG	o-nitrophenol β -D-galactopyranoside
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TOS	Total Organic Solids
WGS	Whole genome sequencing
WHO	World Health Organization