

Safety evaluation of an extension of use of the food enzyme pullulanase from the non-genetically modified *Pullulanibacillus naganoensis* strain AE-PL

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Abstract

The food enzyme pullulanase (pullulan 6- α -glucanohydrolase; EC 3.2.1.41) is produced with the non-genetically modified *Pullulanibacillus naganoensis* strain AE-PL by Amano Enzyme Inc. A safety evaluation of this food enzyme was made previously, in which EFSA concluded that this food enzyme did not give rise to safety concerns when used in one food manufacturing process. Subsequently, the applicant has requested to extend its use to include seven additional processes and to revise the previous use level. In this assessment, EFSA updated the safety evaluation of this food enzyme when used in a total of eight food manufacturing processes. As the food enzyme-total organic solids (TOS) are not carried into the final foods in two food manufacturing processes, the dietary exposure was estimated only for the remaining six processes. The dietary exposure was calculated to be up to 0.004 mg TOS/kg body weight (bw) per day in European populations. The Panel evaluated the repeated dose 90-day oral toxicity study in rats submitted in the previous application and identified a no observed adverse effect level of 643 mg TOS/kg bw per day, the highest dose tested. When compared with the calculated dietary exposure, this resulted in a margin of exposure of at least 160,750. Based on the data provided for the previous evaluation and the revised margin of exposure in the present evaluation, the Panel concluded that this food enzyme does not give rise to safety concerns under the revised intended conditions of use.

KEYWORDS

EC 3.2.1.41, EFSA-Q-2015-00451, EFSA-Q-2023-00662, food enzyme, non-genetically modified microorganism, pullulanase

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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 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 1331/2008² 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:

- 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.

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.

Pullulanase from a non-genetically modified strain of *Pullulanibacillus naganensis* (strain AE-PL) is a food enzyme included in the Register of food enzymes³ to be considered for inclusion in the Union list and thus subject to a risk assessment by the European Food Safety Authority (EFSA).

On 15 May 2023, a new application has been introduced by the applicant “Amano Enzyme Inc.” for an extension of the condition of the use of the food enzyme pullulanase from a non-genetically modified strain of *Pullulanibacillus naganensis* (strain AE-PL).

1.1.2 | Terms of Reference

The European Commission requests the European Food Safety Authority to carry out the safety assessment and the assessment of possible confidentiality requests of the extension of use for the following food enzyme: pullulanase from a non-genetically modified strain of *Pullulanibacillus naganensis* (strain AE-PL), in accordance with Regulation (EC) No 1331/2008 establishing a common authorisation procedure for food additives, food enzymes and food flavourings.⁴

1.1.3 | Interpretation of the Terms of Reference

The present scientific opinion addresses the European Commission's request to carry out the safety assessment of an extension of the conditions of use for the pullulanase from the non-genetically modified *Pullulanibacillus naganensis* strain AE-PL.

¹Regulation (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.

²Regulation (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.

³https://food.ec.europa.eu/safety/food-improvement-agents/enzymes/eu-list-and-applications_en.

⁴OJ L 354, 31.12.2008, p. 1.

2 | DATA AND METHODOLOGIES

2.1 | Data

The applicant has submitted a dossier in support of the application for the authorisation of the extension of use of food enzyme pullulanase from a non-genetically modified *Pullulanibacillus naganoensis* strain AE-PL.

Additional information, requested from the applicant during the assessment process on 6 February 2024, were received on 27 February 2024 (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 guidance documents of EFSA Scientific Committee.

The 'Scientific Guidance for the submission of dossiers on food enzymes' (EFSA CEP Panel, 2021) and the 'Food manufacturing processes and technical data used in the exposure assessment of food enzymes' (EFSA CEP Panel, 2023) have been followed for the evaluation of the application.

2.3 | Public consultation

According to Article 32c(2) of Regulation (EC) No 178/2002⁵ and to the Decision of EFSA's Executive Director laying down the practical arrangements on pre-submission phase and public consultations, EFSA carried out a public consultation on the non-confidential version of the technical dossier from 01 February to 22 February 2024.⁶ No comments were received.

3 | ASSESSMENT

IUBMB nomenclature	Pullulanase
Systematic name	Pullulan 6- α -glucanohydrolase
Synonyms	α -dextrin endo-1,6- α -glucosidase
IUBMB No	3.2.1.41
CAS No	9075-68-7
EINECS No	232-983-9

Pullulanases catalyse the hydrolysis of (1–6)- α -D-glucosidic linkages in pullulan, amylopectin and glycogen as well as in α - and β -limit dextrins of amylopectin and glycogen. All aspects concerning the safety of this food enzyme were evaluated in September 2017, when used in one food manufacturing process (EFSA CEF Panel, 2017). However, although a repeated dose 90-day oral toxicity study in rodents was described, a no observed adverse effect level (NOAEL) was not identified due to the removal of the food enzyme-total organic solids (TOS) in the single food manufacturing process considered.

Following a request to update the intended uses (adding seven food manufacturing processes and revising the use levels), EFSA revises the exposure assessment, evaluates the repeated dose 90-day oral toxicity study in rodents described in the previous evaluation (EFSA CEF Panel, 2017), identifies a NOAEL and updates the safety evaluation of this food enzyme when used in eight food manufacturing processes.

3.1 | Dietary exposure

The current dietary exposure supersedes section 3.5 in the previous evaluation (EFSA CEF Panel, 2017).

3.1.1 | Revised intended use of the food enzyme

The food enzyme is intended to be used in eight food manufacturing processes at the revised use levels summarised in [Table 1](#).

⁵Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. OJ L 31, 1.2.2002, p. 1–24.

⁶Accessible at <https://connect.efsa.europa.eu/RM/s/consultations/publicconsultation2/a0lTk0000006hbm/pc0807>.

TABLE 1 Updated intended uses and use levels of the food enzyme.⁷

Food manufacturing process ^a	Raw material (RM)	Maximum recommended use level (mg TOS/kg RM)	
		Current evaluation ^b	Previous evaluation ^{b,c}
Processing of cereals and other grains			
• Production of baked products	Flour	0.1	
• Production of cereal-based products other than baked	Cereals	0.3	
• Production of brewed products	Cereals	0.03	
• Production of glucose syrups and other starch hydrolysates	Starch slurry	0.3	79
• Production of distilled alcohol	Cereals, potato, tapioca etc.	11.7	
Processing of fruits and vegetables			
• Production of non-wine vinegar	Cereals	0.3	
Processing of plant and fungal derived products			
• Production of coffee substitutes	Barley	0.3	
• Production of plant-based analogues of milk and milk products	Cereals, legumes, oilseeds, nuts etc.	0.3	

^aThe name has been harmonised by EFSA according to the 'Food manufacturing processes and technical data used in the exposure assessment of food enzymes' (EFSA CEP Panel, 2023).

^bThe numbers in bold were used for calculation.

^cThe previous evaluation is made for the food enzyme application EFSA-Q-2015-00451.

The panel noted a substantial decrease in use level recommended in the current assessment when compared to the previously reported level. The applicant ascribes this change to the availability of more recent information on actual use levels.⁸

The additional seven uses of the food enzyme are described below.

In the production of baked products, the food enzyme is added to flour during the preparation of the dough.⁹ This pullulanase is used in conjunction with other enzymes to hydrolyse starch, which retards starch retrogradation.¹⁰ The food enzyme-TOS remain in the baked products.

In the production of cereal-based products other than baked, the food enzyme is added in the production of cooked rice, or to cereals such as corn, wheat and barley for the production of processed cereal products.¹¹ The pullulanase hydrolyses starch within the raw materials to retard starch retrogradation and reduce product viscosity.¹² The food enzyme-TOS remain in the cereal-based products.

In the production of brewed products, the food enzyme is added during mashing and liquefaction in the production of beer.¹³ In the production of fermented beverages such as sake or rice wine, it may be added during the slurry mixing, the liquefaction, the pre-saccharification or the fermentation steps.¹⁴ This reduces the mashing time and improves the alcohol yield. The food enzyme-TOS remains in the beer and cereal-based liquors.

In the production of distilled alcohol, the food enzyme may be added during slurry mixing, liquefaction, pre-saccharification or fermentation of the cereals.¹⁵ In combination with other starch-degrading enzymes, it improves the hydrolysis of starch, which increases fermentation rate, increases alcohol yield and improves performance in the pre-saccharification process. The food enzyme-TOS are not carried over with the distilled alcohols (EFSA CEP Panel, 2023).

In the production of non-wine vinegar, the food enzyme is added to milled cereals during slurry mixing.¹⁶ The food enzyme-TOS remain in the non-wine vinegar.

In the production of coffee substitutes, the food enzyme is added to barley and water after milling.¹⁷ It hydrolyses the starch, which decreases viscosity.¹⁸ The food enzyme-TOS remain in the final coffee substitutes.

⁷Technical dossier/Intended use(s) in food and use level(s) (Proposed normal and maximum use levels)/4–11 Proposed conditions of use/p. 5.

⁸Additional information February 2024.

⁹Technical dossier/Intended use(s) in food and use level(s) (Proposed normal and maximum use levels)/Annex: Flow Chart of Each application/p. 2.

¹⁰Technical dossier/Intended use(s) in food and use level(s) (Proposed normal and maximum use levels)/4–11 Proposed conditions of use/p.1.

¹¹Technical dossier/Intended use(s) in food and use level(s) (Proposed normal and maximum use levels)/Annex: Flow Chart of Each application/p. 8.

¹²Technical dossier/Intended use(s) in food and use level(s) (Proposed normal and maximum use levels)/4–11 Proposed conditions of use/p. 3.

¹³Technical dossier/Intended use(s) in food and use level(s) (Proposed normal and maximum use levels)/Annex: Flow Chart of Each application/p. 3.

¹⁴Technical dossier/Intended use(s) in food and use level(s) (Proposed normal and maximum use levels)/Annex: Flow Chart of Each application/p. 4.

¹⁵Technical dossier/Intended use(s) in food and use level(s) (Proposed normal and maximum use levels)/Annex: Flow Chart of Each application/p. 6.

¹⁶Technical dossier/Intended use(s) in food and use level(s) (Proposed normal and maximum use levels)/Annex: Flow Chart of Each application/p. 5.

¹⁷Technical dossier/Intended use(s) in food and use level(s) (Proposed normal and maximum use levels)/Annex: Flow Chart of Each application/p. 9.

¹⁸Technical dossier/Intended use(s) in food and use level(s) (Proposed normal and maximum use levels)/4–11 Proposed conditions of use/p. 4.

In the production of plant-based analogues of milk and milk products, the food enzyme is added to the slurry of plant materials.¹⁹ It hydrolyses the starch for the production of malto-oligosaccharides.²⁰ The food enzyme-TOS remain in the enzymatically treated plant-based dairy analogues.

Based on the thermostability evaluated previously (EFSA CEF Panel, 2017) and the thermal treatments applied during food processing, it is expected that the enzyme is inactivated or removed in the food manufacturing processes listed in Table 1.

3.1.2 | Dietary exposure estimation

In accordance with the guidance document (EFSA CEP Panel, 2021), dietary exposure was calculated for the six food manufacturing processes where the food enzyme-TOS remain in the final foods.

Chronic exposure to the food enzyme-TOS was calculated by combining the maximum recommended use level with individual consumption data (EFSA CEP Panel, 2021). The estimation involved selection of relevant food categories and application of technical conversion factors (EFSA CEP Panel, 2023). Exposure from all FoodEx categories was subsequently summed up, averaged over the total survey period (days) and normalised for body weight. This was done for all individuals across all surveys, resulting in distributions of individual average exposure. Based on these distributions, the mean and 95th percentile exposures were calculated per survey for the total population and per age class. Surveys with only one day per subject were excluded and high-level exposure/intake was calculated for only those population groups in which the sample size was sufficiently large to allow calculation of the 95th percentile (EFSA, 2011).

Table 2 provides an overview of the derived exposure estimates across all surveys. Detailed mean and 95th percentile exposure to the food enzyme-TOS per age class, country and survey, as well as contribution from each FoodEx category to the total dietary exposure, are reported in Appendix A – Tables 1 and 2. For the present assessment, food consumption data were available from 48 dietary surveys (covering infants, toddlers, children, adolescents, adults and the elderly), carried out in 26 European countries (Appendix B). The highest dietary exposure was estimated to be about 0.004 mg TOS/kg body weight (bw) per day in infants at the 95th percentile.

TABLE 2 Updated dietary exposure to the food enzyme-TOS in six population groups.

Population group	Estimated exposure (mg TOS/kg body weight per day)					
	Infants	Toddlers	Children	Adolescents	Adults	The elderly
Age range	3–11 months	12–35 months	3–9 years	10–17 years	18–64 years	≥ 65 years
Min–max mean (number of surveys)	0–0.001 (12)	0.001–0.001 (15)	0–0.001 (19)	0–0.001 (21)	0–0 (22)	0–0 (23)
Min–max 95th percentile (number of surveys)	0.001–0.004 (11)	0.001–0.003 (14)	0.001–0.002 (19)	0–0.001 (20)	0–0.001 (22)	0–0.001 (22)

3.1.3 | Uncertainty analysis

In accordance with the guidance provided in the EFSA opinion related to uncertainties in dietary exposure assessment (EFSA, 2006), the following sources of uncertainties have been considered and are summarised in Table 3.

TABLE 3 Qualitative evaluation of the influence of uncertainties on the dietary exposure estimate.

Sources of uncertainties	Direction of impact
Model input data	
Consumption data: different methodologies/representativeness/underreporting/misreporting/no portion size standard	+/-
Use of data from food consumption surveys of a few days to estimate long-term (chronic) exposure for high percentiles (95th percentile)	+
Possible national differences in categorisation and classification of food	+/-
Model assumptions and factors	
Exposure to food enzyme-TOS was always calculated based on the recommended maximum use level	+
Selection of broad FoodEx categories for the exposure assessment	+
Use of recipe fractions to disaggregate FoodEx categories	+/-
Use of technical factors in the exposure model	+/-
Exclusion of two processes from the exposure estimation: - Production of glucose syrups and other starch hydrolysates - Production of distilled alcohol	-

Abbreviations: +, uncertainty with potential to cause overestimation of exposure; -, uncertainty with potential to cause underestimation of exposure.

¹⁹Technical dossier/Intended use(s) in food and use level(s) (Proposed normal and maximum use levels)/Annex: Flow Chart of Each application/p. 7.

²⁰Technical dossier/Intended use(s) in food and use level(s) (Proposed normal and maximum use levels)/4–11 Proposed conditions of use/p. 3.

The conservative approach applied to estimate the exposure to the food enzyme-TOS, in particular assumptions made on the occurrence and use levels of this specific food enzyme, is likely to have led to an overestimation of the exposure.

The exclusion of two food manufacturing processes from the exposure estimation was based on > 99% of TOS removal. This is not expected to impact the overall estimate derived.

3.2 | Repeated dose 90-day oral toxicity study in rodents

In the previous evaluation (EFSA CEF Panel, 2017), a 90-day study was described, but a NOAEL was not identified as the food enzyme-TOS were removed during the single food manufacturing process considered. Since the applicant introduced additional food manufacturing processes in which the food enzyme-TOS remain in the final foods, this introduced the need for a dietary exposure estimation. Consequently, the 90-day study is evaluated to identify a NOAEL.

The repeated dose 90-day oral toxicity study was performed following good laboratory practice (GLP) and in accordance with guidelines of the Japanese Ministry of Health and Welfare (1999). The study is in accordance with OECD Test Guideline 408 (OECD, 1998) with the following deviation: plasmatic urea was not determined. The Panel considered that this deviation is minor and does not impact the evaluation of the study.

Groups of 12 male and 12 female Sprague–Dawley (Crj:CD(SD)) rats received the food enzyme by gavage in doses of 2.1, 4.3, 8.5 mL/kg bw per day, corresponding to 159, 325 and 643 mg TOS/kg bw per day. Controls received the vehicle (water for injection). No mortality was observed.

The feed consumption was statistically significantly decreased on days 26, 42, 56, 67 and 70 of administration in mid-dose males (–7% in all occasions). The Panel considered the changes as not toxicologically relevant as they were recorded sporadically, were only observed in one sex, there was no dose–response relationship, and there was no statistically significant change in the final feed consumption, the body weight and/or the body weight gain.

Haematological investigations revealed a statistically significant decrease in prothrombin time (PT) in high-dose males (–11%), in activated partial thromboplastin time (APTT) in low- and high-dose males (–10% and –12%) and in total protein (TP) in high-dose males (–2%). The Panel considered the changes as not toxicologically relevant, as they were only observed in one sex (all) and there was no dose-response relationship (APTT).

The only statistically significant change in organ weight detected was an increase in relative brain to body weight ratio in mid-dose males (+ 10%). The Panel considered the change as not toxicologically relevant, as it was only observed in one sex, there was no dose–response relationship, and there were no histopathological changes at the highest dose.

No other statistically significant or biologically relevant differences from controls were reported.

The Panel identified a NOAEL of 643 mg TOS/kg bw per day, the highest dose tested.

3.3 | Margin of exposure

A comparison of the NOAEL with the newly derived exposure estimates of 0–0.001 mg TOS/kg bw per day at the mean and from 0.001 to 0.004 at the 95th percentile resulted in a margin of exposure of at least 160,750.

4 | CONCLUSION

Based on the data provided for the previous evaluation and the derived margin of exposure estimation, the Panel concluded that the food enzyme pullulanase produced with the non-genetically modified *Pullulanibacillus naganoensis* strain AE-PL does not give rise to safety concerns under the revised intended conditions of use.

5 | DOCUMENTATION AS PROVIDED TO EFSA

Application for the extension of use of the food enzyme pullulanase from *Pullulanibacillus naganoensis* strain AE-PL in accordance with Regulation (EC) No 1331/2008. August 2023. Submitted by Amano Enzyme Inc.

Additional information. February 2024. Submitted by Amano Enzyme Inc.

ABBREVIATIONS

APTT	activated partial thromboplastin
bw	body weight
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
EC	European Commission
EINECS	European Inventory of Existing Commercial Chemical Substances
EU	European Union

FEZ	EFSA Panel on Food Enzymes
GLP	good laboratory practice
IUBMB	International Union of Biochemistry and Molecular Biology
NOAEL	no observed adverse effect level
PT	prothrombin time
RM	raw material
TOS	total organic solids
TP	total protein

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CONFLICT OF INTEREST

If you wish to access the declaration of interests of any expert contributing to an EFSA scientific assessment, please contact interestmanagement@efsa.europa.eu.

REQUESTOR

European Commission

QUESTION NUMBER

EFSA-Q-2023-00662

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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APPENDIX A

Dietary exposure estimates to the food enzyme-TOS in details

Appendix A can be found in the online version of this output (in the 'Supporting information' section). The file contains two sheets, corresponding to two tables.

Table 1: Average and 95th percentile exposure to the food enzyme-TOS per age class, country and survey.

Table 2: Contribution of food categories to the dietary exposure to the food enzyme-TOS per age class, country and survey.

APPENDIX B

Population groups considered for the exposure assessment

Population	Age range	Countries with food consumption surveys covering more than 1 day
Infants	From 12 weeks on up to and including 11 months of age	Bulgaria, Cyprus, Denmark, Estonia, Finland, France, Germany, Italy, Latvia, Portugal, Slovenia, Spain
Toddlers	From 12 months up to and including 35 months of age	Belgium, Bulgaria, Cyprus, Denmark, Estonia, Finland, France, Germany, Hungary, Italy, Latvia, Netherlands, Portugal, Republic of North Macedonia*, Serbia*, Slovenia, Spain
Children	From 36 months up to and including 9 years of age	Austria, Belgium, Bulgaria, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Italy, Latvia, Netherlands, Portugal, Republic of North Macedonia*, Serbia*, Spain, Sweden
Adolescents	From 10 years up to and including 17 years of age	Austria, Belgium, Bosnia and Herzegovina*, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Italy, Latvia, Montenegro*, Netherlands, Portugal, Romania, Serbia*, Slovenia, Spain, Sweden
Adults	From 18 years up to and including 64 years of age	Austria, Belgium, Bosnia and Herzegovina*, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Montenegro*, Netherlands, Portugal, Romania, Serbia*, Slovenia, Spain, Sweden
The elderly ^a	From 65 years of age and older	Austria, Belgium, Cyprus, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Montenegro*, Netherlands, Portugal, Romania, Serbia*, Slovenia, Spain, Sweden

*Consumption data from these pre-accession countries are not reported in Table 2 of this opinion. However, they are included in Appendix A for testing purpose.

^aThe terms 'children' and 'the elderly' correspond, respectively, to 'other children' and the merge of 'elderly' and 'very elderly' in the Guidance of EFSA on the 'Use of the EFSA Comprehensive European Food Consumption Database in Exposure Assessment' (EFSA, 2011).