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Safety evaluation of the food enzyme acetolactate decarboxylase from a genetically modified *Bacillus licheniformis* (strain NZYM-JB)

EFSA Panel on Food Contact Materials, Enzymes, Processing Aids (CEP),
Vittorio Silano, José Manuel Barat Baviera, Claudia Bolognesi, Beat Johannes Brüschweiler,
Pier Sandro Cocconcelli, Riccardo Crebelli, David Michael Gott, Konrad Grob, Evgenia Lampi,
Alicja Mortensen, Gilles Rivière, Inger-Lise Steffensen, Christina Tlustos, Henk van Loveren,
Laurence Vernis, Holger Zorn, Lieve Herman, Sirpa Kärenlampi*, André Penninks*,
Davor Želježic*, Magdalena Andryszkiewicz, Davide Arcella, Ana Gomes, Christine Horn,
Natália Kovalkovičová, Yi Liu, Karl-Heinz Engel* and Andrew Chesson

Abstract

The food enzyme acetolactate decarboxylase (α-acetolactate decarboxylase; EC 4.1.1.5) is produced with a genetically modified *Bacillus licheniformis* strain NZYM-JB by Novozymes A/S. The genetic modifications do not give rise to safety concerns. The food enzyme is free from viable cells of the production organism and recombinant DNA. This acetolactate decarboxylase is intended to be used in distilled alcohol production and brewing processes. Residual amounts of total organic solids (TOS) are removed by distillation; consequently, dietary exposure was not calculated for distilled alcohol products. For other brewery products, based on the maximum use level recommended for the brewing processes and individual data from the EFSA Comprehensive European Food Consumption Database, dietary exposure to the food enzyme–TOS was estimated to be up to 0.003 mg TOS/kg body weight (bw) per day in European populations. Genotoxicity tests with the food enzyme did not indicate a genotoxic concern. The systemic toxicity was assessed by means of a repeated dose 90-day oral toxicity study in rats. The Panel identified a no observed adverse effect level at the highest dose of 1,018 mg TOS/kg bw per day. When compared with the dietary exposure, this results in a sufficiently high margin of exposure (at least 300,000). The amino acid sequence of the food enzyme did not match to those of known allergens. The Panel considered that, under the intended condition of use, the risk of allergic sensitisation and elicitation reactions upon dietary exposure to this food enzyme cannot be excluded, but the likelihood is considered low. Based on the data provided, the Panel concluded that this food enzyme does not raise safety concerns under the intended conditions of use.

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Keywords: food enzyme, acetolactate decarboxylase, α-acetolactate decarboxylase, EC 4.1.1.5, *Bacillus licheniformis*, genetically modified microorganism

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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, Beat Johannes Brüschweiler, Andrew Chesson, Pier Sandro Cocconcelli, Riccardo Crebelli, David Michael Gott, Konrad Grob, Evgenia Lampi, Alicja Mortensen, Gilles Rivière, Vittorio Silano, Inger-Lise Steffensen, Christina Tlustos, Henk van Loveren, Laurence Vernis and Holger Zorn.

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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 entered 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 European Union 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; and
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 an approval via a Union list.

The 'Guidance on submission of a dossier on a food enzyme for 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.

Five applications have been introduced by the companies 'Danisco US Inc.' for the authorisation of the food enzyme Mucorpepsin from *Rhizomucor miehei*, 'Novozymes A/S' for the authorisation of the food enzymes Acetolactate decarboxylase from a genetically modified strain of *Bacillus licheniformis* (strain NZYM-JB) and Glucose isomerase from *Streptomyces murinus* (strain NZYM-GA), and 'Amano Enzyme Inc.' for the authorisation of the food enzymes 4-alpha-glucanotransferase from *Geobacillus pallidus* (strain AE-SAS) and Tannase from *Aspergillus niger* (strain AE-TAN).

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

1.1.2. Terms of Reference

The European Commission requests the European Food Safety Authority to carry out a safety assessments of the food enzymes Mucorpepsin from *Rhizomucor miehei*, Acetolactate decarboxylase from a genetically modified strain of *Bacillus licheniformis* (strain NZYM-JB), Glucose isomerase from

[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 1493/199, 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, p. 1–6.

Streptomyces murinus (strain NZYM-GA), 4-alpha-glucanotransferase from *Geobacillus pallidus* (strain AE-SAS) and Tannase from *Aspergillus niger* (strain AE-TAN).

1.2. Interpretation of the Terms of Reference

The present scientific opinion addresses the European Commission request to carry out of the safety assessment of the food enzyme acetolactate decarboxylase from *B. licheniformis* (strain NZYM-JB).

1.3. Information on existing authorisations and evaluations

The applicant indicates that the food enzyme object of the present dossier has not been evaluated by authorities in the EU. The applicant also reports that the Australian, Brazilian, Canadian, Chinese, Danish, French, Mexican, Russian and South Korean authorities have evaluated and authorised acetolactate decarboxylases from genetically modified strains of *Bacillus subtilis.*[3]

2. Data and methodologies

2.1. Data

The applicant has submitted a dossier in support of the application for authorisation of the food enzyme acetolactate decarboxylase from *B. licheniformis* (strain NZYM-JB).

Additional information was sought from the applicant during the assessment process in response to a request from EFSA sent on 5 March 2018 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 Scientific Opinion on Guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use (EFSA GMO Panel, 2011).

The current 'Guidance on the submission of a dossier for safety evaluation of a food enzyme' (EFSA CEF Panel, 2009) has been followed for the evaluation of this 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:	Acetolactate decarboxylase
Systematic name:	(2*S*)-2-hydroxy-2-methyl-3-oxobutanoate carboxy-lyase [(3*R*)-3-hydroxybutan-2-one-forming]
Synonyms:	α-acetolactate decarboxylase; (*S*)-2-hydroxy-2-methyl-3-oxobutanoate carboxy-lyase
IUBMB No:	EC 4.1.1.5
CAS No:	9025-02-9
EINECS No:	618-520-7

Acetolactate decarboxylase catalyses the decarboxylation of α-acetolactate to acetoin. It is intended to be used in distilled alcohol production and brewing processes.

3.1. Source of the food enzyme

The acetolactate decarboxylase is produced with a genetically modified strain of *B. licheniformis*.

The production strain *B. licheniformis* NZYM-JB is deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Germany) with deposit number [redacted][4]

[3] Technical dossier/1st submission/p. 25 and 61.
[4] Technical dossier/1st submission/Annex A3.

3.1.1. Characteristics of the parental and recipient microorganisms

The parental strain was identified as *B. licheniformis* [5] The strain shows no cytotoxic activity in Chinese hamster ovary cells and VERO cells.[6] The absence of cytotoxicity has also been shown by the same methodology in strain an intermediate strain

The recipient strain,

Additionally,

For the development of the recipient strain

3.1.2. Characteristics of the introduced sequences

The sequence encoding for the mature acetolactate

3.1.3. Description of the genetic modification process

The purpose of the genetic modification was to enable the production strain to synthesise acetolactate decarboxylase

[5] Technical dossier/1st submission/Annex A2.
[6] Technical dossier/1st submission/Annex A4.

3.1.4. Safety aspects of the genetic modification

The technical dossier contains all necessary information on the recipient microorganism, the donor microorganism and the genetic modification process.

The production strain *B. licheniformis* NZYM-JB differs from the parental strain in its capability to produce high level of acetolactate decarboxylase from . The presence and the location of the enzyme gene were confirmed by Southern analyses.[7]

The phenotypic stability of the *B. licheniformis* NZYM-JB strain was confirmed by its capacity to produce a constant level of the enzyme acetolactate decarboxylase measured in relation to the TOS in three independent batches of the food enzyme and its genetic stability was demonstrated by Southern analysis with DNA isolated from end-of-production samples from three different batches.[9]

No antibiotic resistance genes used during the genetic modification were left in the genome.

B. licheniformis is recommended for the Qualified Presumption of Safety (QPS) status, with the qualification that the absence of acquired antimicrobial resistance genes and toxigenic activity are verified for the specific strain used (EFSA BIOHAZ Panel, 2018). The parental strain *B. licheniformis* strain and an intermediate strain were shown not to be cytotoxic. The absence of acquired antibiotic resistance has not been shown for the parental/recipient strain, however taking into account the absence of production organism and DNA in the final product (see Section 3.1.5), the Panel did not consider this to be necessary. None of the introduced traits raise safety concerns and therefore the production strain can be presumed to be of no concern.

No issues of concern arising from the genetic modifications were identified.

3.2. Production of the food enzyme

The food enzyme is manufactured according to 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 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 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.

[7] Technical dossier/1st submission/Annex D1.
[8] Technical dossier/1st submission/Annex D2.
[9] Technical dossier/1st submission/Annex D3.
[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, 321 pp.
[11] Technical dossier/1st submission/p. 49.
[12] Technical dossier/1st submission/Annex 6.

3.3. Characteristics of the food enzyme

3.3.1. Properties of the food enzyme

The acetolactate decarboxylase is a single polypeptide chain of 261 amino acids. The molecular mass, based on the amino acid sequence, was calculated to be 29.1 kDa.[13] 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 the target protein migrating between the marker proteins of 31 and 36 kDa in all batches.[14] The food enzyme was tested for α-amylase, glucoamylase, protease and lipase activities and no relevant activities were detected.[15] No other enzymatic side activities were reported.

The in-house determination of acetolactate decarboxylase activity is based on the decarboxylation of α−acetolactate to acetoin which reacts with naphthol and creatine to produce a red colour which is determined spectrophotometrically at 510 nm (reaction conditions: 30°C, pH 6.0). The acetolactate decarboxylase activity is measured relative to internal enzyme standard and the result is given in α−Acetolactate-Decarboxylase Unit (L standard) (ADU(L)). One ADU(L) is defined as the amount of enzyme that, by decarboxylation of α−acetolactate, produces 1 μmol of acetoin per minute.[16]

The food enzyme has been characterised with regard to its temperature and pH profiles. It has a temperature optimum around 40°C (pH 6.0)[17] and a pH optimum around pH 6.0 (T 30°C). Thermostability was tested after a pre-incubation of the food enzyme for 30 min at different temperatures. Under the conditions (pH 4.5) of the applied temperature stability assay, the acetolactate decarboxylase activity showed 50% residual activity at 60°C and almost no residual activity at 80°C.[17]

3.3.2. Chemical parameters

Data on the chemical parameters of the food enzyme have been provided for four food enzyme batches, three batches used for commercialisation and one batch used for the toxicological tests (Table 1). The average total organic solids (TOS) content of the three commercial enzyme batches was 6.9% (range 6.1%–7.9%). The average enzyme activity/TOS ratio of the three batches for commercialisation is 377 ADU(L)/mg TOS.

Table 1: Compositional data provided for the food enzyme[(e)]

Parameter	Unit	Batch			
		1	2	3	4[(a),(f)]
Acetolactate decarboxylase	ADU(L)[(b)]/g	27,500	25,800	24,200	22,000
Protein	% w/w	7.5	7.3	7.0	NA[(c)]
Ash	% w/w	0.3	0.4	0.5	1.5
Water	% w/w	91.8	92.9	93.4	88.7
Total organic solids (TOS)[(d)]	% w/w	7.9	6.7	6.1	9.8
Acetolactate decarboxylase activity/mg TOS	ADU(L)/mg TOS	348	385	397	224

(a): Batch used for toxicological tests (Ames, micronucleus and 90-day oral toxicity study).
(b): ADU(L): Acetolactate Decarboxylase Unit L standard (see Section 3.3.1). Enzyme activity was measured in ADU/g. The correlation factor between ADU(L) and ADU is 1 (1 ADU(L) = 1ADU).
(c): NA: not available.
(d): TOS calculated as 100% - % water - % ash.
(e): Technical dossier/1st submission/p. 34 and 64.
(f): Technical dossier/1st submission/Annex 7.02 and Additional information March 2018.

[13] Technical dossier/1st submission/p. 32.
[14] Technical dossier/1st submission/p. 34.
[15] Technical dossier/1st submission/p. 42.
[16] Technical dossier/1st submission/Annex 3.01.
[17] Technical dossier/1st submission/Annex 9.

3.3.3. Purity

The food enzyme complies with the specification for lead (not more than 5 mg/kg)[18] as laid down in the general specifications and considerations for enzymes used in food processing (FAO/WHO, 2006). In addition, the applicant provided that the levels of arsenic, cadmium and mercury were below the limits of detection of the employed methods.[19,20]

The food enzyme complies with the microbiological criteria 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 are not more than 30 colony forming units (CFU) per gram.[21] No antimicrobial activity was detected in any of these batches (FAO/WHO 2006).[22]

3.3.4. Viable cells and DNA of the production strain

The absence of the production strain in the product was demonstrated [redacted] Three independent solid production batches were analysed in triplicate. For each batch, a positive product control spiked with reference strain (NZYM-JB) was included to verify growth of the production strain.[23]

A test for recombinant DNA in the food enzyme was made by polymerase chain reaction (PCR) analysis of three batches in triplicate. No DNA was detected [redacted] in the production strain.[24] [redacted]

3.4. Toxicological data

A battery of toxicological tests including a bacterial gene mutation assay (Ames test), an *in vitro* mammalian micronucleus test and a repeated dose 90-day toxicity study in rats has been provided. The batch 4 (Table 1) used in these studies has similar protein pattern[14] as the batches used for commercialisation, but has lower chemical purity, and thus is considered suitable as a test item.

3.4.1. Genotoxicity

3.4.1.1. Bacterial reverse mutation test

A bacterial reverse mutation assay (Ames test) was performed according to OECD Test Guideline 471 (OECD, 1997) and following Good Laboratory Practice (GLP) in four strains of *Salmonella* Typhimurium (TA1535, TA1537, TA98 and TA100) and *E. coli* WP2 uvrA (pKM101) in the presence or absence of metabolic activation (S9-mix) applying a 'treat and plate' assay. Two separate experiments were carried out using six final concentrations (156, 313, 625, 1,250, 2,500 and 5,000 μg TOS/mL) of the food enzyme. The food enzyme treatment induced significant decrease in cell viability in the absence of S9-mix at 1,250, 2,500 and 5,000 μg TOS/mL for *S*. Typhimurium strains TA 100 and TA 1535. However, the effect was not reproducible or concentration dependant. No statistically significant or biological relevant increases in revertant colonies were observed in any of the tester strains at any concentration of the food enzyme and test conditions.[25]

Therefore, the Panel concluded that the food enzyme did not induce gene mutations under the conditions of the study.

3.4.1.2. *In vitro* mammalian cell micronucleus test

The *in vitro* micronucleus assay was carried out according to the OECD Test Guideline 487 (OECD, 2010) and following GLP in human peripheral whole blood lymphocyte cultures. A short treatment (3 + 21 h) in the presence and absence of S9-mix at 3,000, 4,000 and 5,000 μg TOS/mL and a continuous treatment (24 + 24 h) without S9-mix at 100, 500, 1,000 and 3,000 μg TOS/mL were

[18] Technical dossier/1st submission/p. 35 and Additional information March 2018.
[19] LOD: As = 0.3 mg/kg, Cd and Hg = 0.05 mg/kg.
[20] Technical dossier/1st submission/p. 35.
[21] Technical dossier/1st submission/p. 37.
[22] Technical dossier/1st submission/p. 36 and Additional information March 2018.
[23] Technical dossier/1st submission/Annex E1.
[24] Technical dossier/1st submission/Annex E2.
[25] Technical dossier/1st submission/Annex 7.01 and Additional information March 2018.

performed. Highest cytotoxicity in pulse treatment was observed in the absence of S9-mix at the concentration of 3,000 μg TOS/mL. In the continuous treatment, cytotoxicity was clearly concentration dependant reaching the highest value of 60% at 3,000 μg TOS/mL. Statistically significant increases in the frequency of micronucleated binucleate cells (MNBN) were observed at 4,000 μg TOS/mL following 3 + 21 h treatment without S9-mix ($p \leq 0.05$) and at 500 μg TOS/mL following 24 + 24 h treatment without S9-mix ($p \leq 0.05$). However, in both cases, the observed mean MNBN cell frequencies were in the range of historical negative control values and not concentration dependant. No statistically significant increase in the MNBN cell frequencies was reported after treatment with the food enzyme at all other concentrations analysed in comparison to concurrent vehicle controls. Therefore, the observed increases were not considered to be biologically relevant.[26] The Panel concluded that the food enzyme acetolactate decarboxylase did not induce an increase in the frequency of MNBNs in cultured human peripheral blood lymphocytes when tested up to 5,000 μg TOS/mL in the test conditions employed.

3.4.2. Repeated dose 90-day oral toxicity study in rodents

A repeated dose 90-day oral toxicity study with acetolactate decarboxylase was performed according to the OECD Test Guideline 408 (OECD, 1998) and following GLP. Groups of 10 male and 10 female Sprague–Dawley rats received by gavage 0% (vehicle control), 10%, 33% or 100% of the food enzyme in doses corresponding to 102, 336 and 1,018 mg TOS/kg bw per day at a volume of 10 mL/kg bw per day. Reverse osmotic water served as a vehicle.

No mortality was observed.

Clinical appearance, feed and water intake, body weight of males in all dose groups and of females in the low- and mid-dose groups were similar to controls throughout the study. The mean body weight of high-dose females at the end of week 13 was 12% lower than that of the control group due to low body weight gain of two females, while the body weight gain of other high-dose females was in the range recorded for the controls. Therefore, the Panel considered that body weight was not affected by treatment.

The group mean scores for both high and low beams (rearing and cage floor activity, respectively) were statistically significantly increased at all doses in males during the initial 6-min interval without a dose response. Furthermore, isolated statistically significantly decreased group mean scores for low and high beams were observed in the high-dose females during the 12-min interval. In view of the different directions of change between males and females, scores in the concurrent male control below the historical control range, and that most of the scores for the treated animals were within the historical control range, these intergroup differences were considered by the authors of the study to reflect normal biological variation and therefore of no toxicological significance.

Statistically significant differences from controls in haematological parameters included: increased neutrophil count at all doses in males and decreased platelet count in low-dose males, decreased haematocrit and haemoglobin concentrations in low-dose females and reduced mean activated partial thromboplastin times in the mid- and high-dose females. As the differences to controls in the values of these parameters were minor, confined to one sex or lacked dose relationship, and the values were within the historical control range, they were considered by the authors of the study to represent normal biological variation and as such of no toxicological significance.

Clinical chemistry examination revealed a small but statistically significant increase of plasma creatinine concentration in the mid- and high-dose males and of the plasma glucose concentration in the high-dose females. As the differences to controls in the values were minor, confined to one sex or lacked dose relationship, and the values were within the historical control range they were attributed by the authors of the study to normal biological variation and therefore of no toxicological importance.

No other significant effects were observed.[27]

The Panel agreed with the evaluation of the authors of the study and with the no-observed-adverse-effect level (NOAEL) of 1,018 mg TOS/kg bw per day, the highest dose tested.

3.4.3. Allergenicity

The allergenicity assessment considers only the food enzyme and not any carrier or other excipient, which may be used in the final formulation.

[26] Technical dossier/1st submission/Annex 7.02.
[27] Technical dossier/Additional information March 2018.

The potential allergenicity of the acetolactate decarboxylase produced with the genetically modified strain *B. licheniformis* NZYM-JB was assessed by comparison of 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, 2011). Using higher than 35% identity in a window of 80 amino acids as the criterion. No match was found.[28]

No oral or respiratory sensitisation and elicitation reactions to the bacterial acetolactate decarboxylase under evaluation have been reported. Therefore, it can be concluded that an allergic reaction upon oral ingestion of acetolactate decarboxylase produced by the genetically modified *B. licheniformis* NZYM-JB cannot be excluded, but the likelihood of such reactions to occur is considered to be low.

According to the information provided, substances or products that may cause allergies or intolerances (Regulation (EU) No 1169/2011[29]) are used as raw materials (█████████) in the growth medium of the production organisms. However, during the fermentation process, these products will be degraded and utilised by the bacteria for cell growth, cell maintenance and production of enzyme protein. In addition, the bacterial 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.

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 (e.g. in distilled alcohol production).

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 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 food enzyme is intended to be used in two food manufacturing processes at the recommended use levels summarised in Table 2.

Table 2: Intended uses and recommended use levels of the food enzyme as provided by the applicant[(b)]

Food manufacturing process[(a)]	Raw material	Recommended dosage of the food enzyme
Distilled alcohol production	Fermenting wort	0.133 mg TOS/L fermenting wort (up to 50 ADU(L)/L fermenting wort)
Brewing processes	Fermenting wort	0.133 mg TOS/L fermenting wort (up to 50 ADU(L)/L fermenting wort)

TOS: total organic solids.

(a): The description provided by the applicant has been harmonised by EFSA according to the 'EC working document describing the food processes in which food enzymes are intended to be used' - not yet published at the time of adoption of this opinion.

(b): Technical dossier/1st submission/p. 59.

In beverage alcohol (distilling) and brewing processes, the food enzyme is added to the fermenting wort. Acetolactate decarboxylase is intended to be used to convert α-acetolactate directly into acetoin and as such to reduce the amount of diacetyl (influencing the organoleptic properties of the final product) formed during fermentation and maturation. This is to reduce the length of the maturation step, and thus to ensure that the level of diacetyl is acceptably low.

Experimental data have been provided on the removal (> 99%) of protein in the course of distilled alcohol production (Documentation provided to EFSA No 3). The Panel considered the evidence as sufficient to conclude that residual amounts of TOS (including substances other than proteins) are removed by distillation.

[28] Technical dossier/1st submission/Annex 8.

[29] 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.

The food enzyme remains in the beer. Based on data provided on thermostability (see Section 3.3.1), it is anticipated that the acetolactate decarboxylase is inactivated during brewing processes.

3.5.2. Dietary exposure estimation

As residual amounts of TOS are removed by distillation, a dietary exposure to the food enzyme resulting from the intended use 'Distilled alcohol production' was not calculated.

For brewing processes, exposure estimates were calculated using the methodology described in the CEF Panel statement on the exposure assessment of food enzymes (EFSA CEF Panel, 2016). The assessment of the food process covered in this opinion involved the selection of relevant food categories from the Comprehensive Database and application of process and technical conversion factors (Annex B in EFSA CEF Panel, 2016). The selected food categories were assumed to always contain the food enzyme–TOS at the maximum recommended use level (Table 2).

Chronic exposure was calculated by combining the maximum recommended use level provided by the applicant (see Table 2) with the relevant FoodEx categories (Annex B in EFSA CEF Panel, 2016), based on individual consumption data. Exposure from all FoodEx categories was subsequently summed up, averaged over the total survey period and normalised for bodyweight. 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 3 provides an overview of the derived exposure estimates across all surveys. Detailed average 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 35 different dietary surveys (covering infants, toddlers, children, adolescents, adults and the elderly), carried out in 22 European countries (Appendix B).

Table 3: Summary of estimated dietary exposure to food enzyme–TOS in six population groups

Population group	Estimated exposure (mg/kg bw 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 (6)	0 (10)	0 (18)	0 (17)	0.000–0.001 (17)	0 (14)
Min–max 95th percentile (number of surveys)	0 (5)	0 (7)	0 (18)	0.000–0.001 (17)	0.000–0.003 (17)	0.000–0.001 (14)

3.5.3. Uncertainty analysis

In accordance with the guidance provided in the EFSA opinion related to uncertainties in dietary exposure assessment (EFSA, 2007), the following sources of uncertainties have been considered and are summarised in Table 4.

Table 4: Qualitative evaluation of the influence of uncertainties on the dietary exposure estimate

Sources of uncertainties	Direction of impact exposure to food enzyme–TOS
Model input data	
Consumption data: different methodologies/representativeness/ underreporting/misreporting/no portion size standard	+/–
Use of data from food consumption survey 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	
FoodEx categories included in the exposure assessment were assumed to always contain the food enzyme–TOS	+
Exposure to food enzyme–TOS was always calculated based on the recommended maximum use level	+
Selection of broad FoodEx categories for the exposure assessment	+
Assumption of 1 L fermenting wort = 1 kg of beer, without considering e.g. trub loss, before beers reach the consumers	+

TOS: total organic solids.
+: uncertainty with potential to cause overestimation of exposure; –: uncertainty with potential to cause underestimation of exposure.

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

3.6. Margin of exposure

A comparison of the NOAEL (1,018 mg TOS/kg bw per day) from the 90-day study with the exposure estimates of 0.000–0.001 mg TOS/kg bw per day at the mean and from 0.000 to 0.003 mg TOS/kg bw per day at the 95th percentile, resulted in margin of exposure (MOE) at least 300,000.

4. Conclusions

Based on the data provided, the removal of TOS during the distilled alcohol production and the MOE calculated when used in brewing processes, the Panel concludes that the food enzyme acetolactate decarboxylase produced with the genetically modified *B. licheniformis* strain NZYM-JB does not give rise to safety concerns under the intended conditions of use.

The CEP Panel considers the food enzyme free from viable cells of the production organism and recombinant DNA.

Documentation provided to EFSA

1) Dossier 'Application for authorisation of acetolactate decarboxylase produced by a genetically modified strain of *Bacillus licheniformis* (strain NZYM-JB)', March 2015. Submitted by Novozymes A/S.
2) Additional information, March 2018. Submitted by Novozymes A/S.
3) Additional information on 'Food enzyme removal during the production of cereal based distilled alcoholic beverages'. February 2017. Provided by the Association of Manufacturers and Formulators of Enzyme Products.

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Abbreviations

ADU(L)	Acetolactate Decarboxylase Unit L standard
bp	base pair
bw	body weight
CAS	Chemical Abstracts Service
CFU	colony forming units
EC	Enzyme Commission
EINECS	European Inventory of Existing Commercial Chemical Substances
FAO	Food and Agricultural Organization
GLP	Good Laboratory Practice
GM	genetically modified
GMP	Good Manufacturing Practice
HACCP	Hazard Analysis and Critical Control Points
IUBMB	International Union of Biochemistry and Molecular Biology
JECFA	Joint FAO/WHO Expert Committee on Food Additives
MNBN	micronucleated binucleated
MOE	margin of Exposure
NOAEL	no-observed-adverse-effect level
OECD	Organisation for Economic Cooperation and Development
PCR	polymerase chain reaction
QPS	Qualified Presumption of Safety
SDS-PAGE	sodium dodecyl sulfate-poly acrylamide gel electrophoresis
TOS	total organic solids
WHO	World Health Organization

Appendix A – Dietary exposure estimates to the food enzyme–TOS in details

Information provided in this appendix is shown in an excel file (downloadable here).

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: The contribution of FoodEx categories to the food enzyme–TOS dietary exposure.

Appendix B – Population groups considered for the exposure assessment

Population	Age range	Countries with food consumption surveys covering more than one day
Infants	From 12 weeks on up to and including 11 months of age	Bulgaria, Denmark, Estonia, Finland, France, Germany, Italy, Latvia, Portugal, United Kingdom
Toddlers	From 12 months up to and including 35 months of age	Belgium, Bulgaria, Denmark, Estonia, Finland, France, Germany, Italy, Latvia, Netherlands, Portugal, Spain, United Kingdom
Children[(a)]	From 36 months up to and including 9 years of age	Austria, Belgium, Bulgaria, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Italy, Latvia, Netherlands, Portugal, Spain, Sweden, United Kingdom
Adolescents	From 10 years up to and including 17 years of age	Austria, Belgium, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Italy, Latvia, Netherlands, Portugal, Spain, Sweden, United Kingdom
Adults	From 18 years up to and including 64 years of age	Austria, Belgium, Croatia, Czech Republic, Denmark, Estonia, Finland, France, Germany, Hungary, Ireland, Italy, Latvia, Netherlands, Portugal, Romania, Spain, Sweden, United Kingdom
The elderly[(a)]	From 65 years of age and older	Austria, Belgium, Denmark, Estonia, Finland, France, Germany, Hungary, Ireland, Italy, Latvia, Netherlands, Portugal, Romania, Spain, Sweden, United Kingdom

(a): The 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).