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Safety and efficacy of alpha-amylase from *Bacillus amyloliquefaciens* DSM 9553, *Bacillus amyloliquefaciens* NCIMB 30251, *Aspergillus oryzae* CBS 585.94 and *Aspergillus oryzae* ATTC SD-5374, endo-1,4-beta-glucanase from *Trichoderma reesei* ATCC PTA-10001, *Trichoderma reesei* ATCC SD-6331 and *Aspergillus niger* CBS 120604, endo-1,4-beta-xylanase from *Trichoderma koningii* MUCL 39203 and *Trichoderma citrinoviride* CBS 614.94 and endo-1,3(4)-beta-glucanase from *Aspergillus tubingensis* MUCL 39199 as silage additives for all animal species

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Abstract

A total of 11 enzymes were assessed including alpha-amylase, endo-1,4-beta-glucanase, endo-1,4-beta-xylanase and endo-1,3(4)-beta-glucanase as silage additives for all animal species. These enzymes are obtained by fermentation of bacterial or fungi non-genetically modified production strains. Throughout information regarding the production strains of each product were provided, including the origin and history of modifications and allowing their identification. The identification was conclusive for 8 of 10 production strains. For three of the strains, more information/data would still be required in order to conclude. Three of the amylases are produced by bacterial strains that belong to a species that is considered by EFSA to be suitable for the Qualified Presumption of Safety approach to safety assessment. The identity of the strains has been established and the qualifications were met, and consequently, those products were regarded as safe. For the products derived from fungal strains, the strains or resulting products were tested for the presence of secondary metabolites which could be of toxicological concern. These were found to be below the limits of detection or the strain not capable of producing them. Considering all the information provided by the applicant, the Panel concluded that these products can be regarded as safe for the target species, consumer and the environment. In the absence of data, the Panel could not conclude on the skin and eye irritancy or skin sensitisation potential of the products under evaluation. These products should be considered to have the potential to be a respiratory sensitiser. For some of the products under evaluation, the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) concluded that they have a potential to improve the characteristic of the silage material; for some other products, the Panel could not conclude on their efficacy.

Keywords: technological additives, silage additive, safety, efficacy, enzymes

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Summary

Following a request from the European Commission, the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) was asked to deliver a scientific opinion on Safety and efficacy of alpha-amylases, cellulases, xylanases and a beta-glucanase from various sources as silage additives for all animal species.

A total of 11 enzymes were assessed including alpha-amylase (amylase; Enzyme Commission Number (EC) 3.2.1.1) from *Bacillus amyloliquefaciens* DSM 9553, *B. amyloliquefaciens* NCIMB 30251, *Aspergillus oryzae* CBS 585.94 and *A. oryzae* ATTC SD-5374, endo-1,4-beta-glucanase (cellulase; EC 3.2.1.4) from *Trichoderma reesei* American type culture collection (ATCC) PTA-10001, *T. reesei* ATCC SD-6331 and *Aspergillus niger* CBS 120604, endo-1,4-beta-xylanase (xylanase; EC 3.2.1.8) from *Trichoderma koningii* MUCL 39203 and *Trichoderma citrinoviride* CBS 614.94 and endo-1,3(4)-beta-glucanase (glucanase; EC 3.2.1.6) from *Aspergillus tubingensis* MUCL 39199 as silage additives for all animal species.

Throughout information regarding the production, strains of each product were provided. However, for the amylase produced with *A. oryzae* CBS 585.94, the information given was not descriptive enough to know if the production strain is CBS 585.94 or a derivative of it. Furthermore, the information regarding the identity of strains *T. koningii* MUCL 39203 and *A. tubingensis* MUCL 39199 should be completed in order to evaluate the adequacy of the methods used.

Three of the amylases are produced by bacterial strains that belong to a species (*B. amyloliquefaciens*) that is considered by European Food Safety Authority (EFSA) to be suitable for the Qualified Presumption of Safety approach to safety assessment. This approach requires the identity of the strain to be conclusively established and evidence that the strain does not show resistance to antimicrobials relevant for humans and animals and the absence of toxigenic activity. In the view of the FEEDAP Panel, the identity of the strains has been established and the qualifications were met, and consequently, products A, B and C are regarded as safe. For the products derived from fungal strains, the strains or resulting products were tested for the presence of secondary metabolites which could be of toxicological concern. These were found to be below the limits of detection or the strain not capable of producing them.

For the assessment of the safety of the product for the target species, consumer and environment, the Panel took into consideration (i) the nature of the active substances, (ii) the characteristics of the production strains and the resulting products (including purity parameters), (iii) the conditions of use and the intended use in silage and concluded that the enzyme products used as silage may be regarded as safe for the target species, consumer and the environment. For product D, the conclusion would apply only in the case that the production strain was *A. oryzae* CBS 585.94.

In the absence of data, the Panel could not conclude on the skin and eye irritancy or skin sensitisation potential of the products under evaluation. Owing to the nature of the active substance, these products should be considered to have the potential to be a respiratory sensitiser.

The FEEDAP Panel concluded that amylase from *B. amyloliquefaciens* DSM 9553, from *B. amyloliquefaciens* NCIMB 30251 (in product B), from *A. oryzae* CBS 585.94 and from *A. oryzae* ATTC SD-5374 and cellulase from *Trichoderma longibrachiatum* ATCC PTA-100001 have the potential to improve the characteristics of the silage material prepared from easy, moderate and difficult to ensile material. The Panel also concluded that the xylanase from *T. longibrachiatum* CBS 614.94 (product K) has a potential to increase the preservation of easy to ensile material when used in combination with *Pediococcus pentosaceus* NCIMB 30238 and *Lactobacillus plantarum* NCIMB 30237, with or without product H. The comparison of the results obtained when adding K compared to the microbial inoculant alone showed an added effect of enzyme K. However, the Panel could not conclude on the efficacy of the enzyme K when used alone. The Panel could not conclude on the efficacy for amylase from *B. amyloliquefaciens* NCIMB 30251 (in product C), cellulase from *T. reesei* ATCC PTA-10001 (product G), cellulase from *A. niger* CBS 120604 (product H), xylanase from *T. koningii* MUCL 39203 (product J) and glucanase from *A. tubingensis* MUCL 39199 (product L).

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1. Introduction

1.1. Background and Terms of Reference

Regulation (EC) No 1831/2003[1] establishes the rules governing the Community authorisation of additives for use in animal nutrition. In particular, Article 10(2)(7) of that Regulation specifies that for existing products within the meaning of Article 10(1), an application shall be submitted in accordance with Article 7, within a maximum of seven years after the entry into force of this Regulation.

The European Commission received a request from SILAC EEIG[2] for re-evaluation of the authorisation of the product α-amylase (Enzyme Commission Number (EC) 3.2.1.1) from *Aspergillus oryzae,* from *Bacillus amyloliquefaciens* and *Bacillus subtilis*, cellulase (EC 3.2.1.4) from *Trichoderma longibrachiatum* and *Aspergillus niger*, β-glucanase (EC 3.2.1.6) from *Aspergillus niger* and xylanase (EC 3.2.1.8) from *Trichoderma longibrachiatum*, when used as a feed additive for all animal species (category: Technological additives; functional group: silage additives).

According to Article 7(1) of Regulation (EC) No 1831/2003, the Commission forwarded the application to the European Food Safety Authority (EFSA) as an application under under Article 10(2)(7) (re-evaluation of an authorised feed additive). EFSA received directly from the applicant the technical dossier in support of this application. The particulars and documents in support of the application were considered valid by EFSA as of 26 April 2012.

According to Article 8 of Regulation (EC) No 1831/2003, EFSA, after verifying the particulars and documents submitted by the applicant, shall undertake an assessment in order to determine whether the feed additive complies with the conditions laid down in Article 5. EFSA shall deliver an opinion on the safety for the target animals, consumer, user and the environment and on the efficacy of the product α-amylase, cellulase, xylanase and β-glucanase, when used under the proposed conditions of use (see Section 3.1.5).

1.2. Additional information

The following enzymes were authorised as silage additives under the provisions of Article 10(1) of Regulation (EC) No 1831/2003: α-amylase (amylase; Enzyme Commission Number (EC) 3.2.1.1) obtained from the following strains *B. amyloliquefaciens* (DSM 9553), *B. subtilis* (DS098), *B. amyloliquefaciens* (SD80), *A. oryzae* (CBS 585.94) and *A. oryzae* (DS 114), endo-1,4-β-glucanase (cellulase; EC 3.2.1.4) from *T. longibrachiatum* (American type culture collection (ATCC) PTA-10001), *T. longibrachiatum* (ATCC 74252) and *A. niger* (CBS 120604 294), endo-1,4-β-xylanase (xylanase; EC 3.2.1.8) from *T. longibrachiatum* (MUCL 39203) and *T. longibrachiatum* (CBS 614.94) and endo1,3(4)-β-glucanase (glucanase; EC 3.2.1.6) from *A. niger* (MUCL 39199).[1]

2. Data and methodologies

2.1. Data

The present assessment is based on data submitted by the applicant in the form of a technical dossier[3] in support of the authorisation request for the use of enzyme products as a feed additive. The technical dossier was prepared following the provisions of Article 7 of Regulation (EC) No 1831/2003, Regulation (EC) No 429/2008[4] and the applicable EFSA guidance documents.

EFSA has verified the European Union Reference Laboratory (EURL) report as it relates to the methods used for the control of the active substances in animal feed. The Executive Summary of the EURL report can be found in Annex A.[5]

2.2. Methodologies

The approach followed by the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) to assess the safety and the efficacy of the active substances is in line with the principles laid

[1] Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. OJ L 268, 18.10.2003, p. 29.
[2] SILAC EEIG, Avenue Louise 120, 1050 Brussels, Belgium.
[3] FEED dossier reference: FAD-2010-0367.
[4] Commission Regulation (EC) No 429/2008 of 25 April 2008 on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the preparation and the presentation of applications and the assessment and the authorisation of feed additives. OJ L 133, 22.5.2008, p. 1.
[5] The full report is available on the EURL website: https://ec.europa.eu/jrc/sites/jrcsh/files/finrep_fad-2010-0367_enzyme-group.pdf

down in Regulation (EC) No 429/2008 and the relevant guidance documents: Guidance on technological additives (EFSA FEEDAP Panel, 2012a), Technical Guidance for assessing the safety of feed additives for the environment (EFSA, 2008), Guidance for establishing the safety of additives for the consumer (EFSA FEEDAP Panel, 2012b), Guidance on studies concerning the safety of use of the additive for users/ workers (EFSA FEEDAP Panel, 2012c), Guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition (EFSA FEEDAP Panel, 2014) and Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance (EFSA FEEDAP Panel, 2012d).

3. Assessment

This opinion deals with the assessment of the safety and efficacy of four enzymes, produced by fermentation when used as a technological additive, silage additive for all animal species.

3.1. Characterisation[6]

3.1.1. Characterisation of active substances and production strains

The 11 additives included in this assessment are listed in Table 1 along with the microbial strains (defined by their accession numbers) from which they are obtained. There are four types of enzymes α-amylase (amylase; Enzyme Commission Number (EC) 3.2.1.1), endo-1,4-β-glucanase (cellulase; EC 3.2.1.4), endo-1,4-β-xylanase (xylanase; EC 3.2.1.8) and endo1,3(4)-β-glucanase (glucanase; EC 3.2.1.6). Each additive has been given a reference letter which, for convenience, will be used throughout this Opinion.

Table 1: The nature and source of the 11 enzymes that are the subject of assessment

Ref letter	Enzyme	Microbial source
A	Amylase	*Bacillus amyloliquefaciens* DSM 9553
B	Amylase	*Bacillus amyloliquefaciens* NCIMB 30251
C	Amylase	*Bacillus amyloliquefaciens* NCIMB 30251
D	Amylase	*Aspergillus oryzae* CBS 585.94
E	Amylase	*Aspergillus oryzae* ATTC SD-5374
F	Cellulase	*Trichoderma reesei* ATCC PTA-10001
G	Cellulase	*Trichoderma reesei* ATCC SD-6331
H	Cellulase	*Aspergillus niger* CBS 120604
J	Xylanase	*Trichoderma koningii* MUCL 39203
K	Xylanase	*Trichoderma citrinoviride* CBS 614.94
L	Glucanase	*Aspergillus tubingensis* MUCL 39199

The production strain of the amylase contained in product A is a strain of *B. amyloliquefaciens* deposited at the Deutsche Sammlung von Mikroorganisms and Zellkulturen (DSMZ) with the accession number DSM 9553.[7] The strain was identified.[8] The absence of toxigenic potential of the strain was demonstrated following the guidance for the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition (EFSA FEEDAP Panel, 2014).[9] The susceptibility of the strain to the antibiotics recommended by EFSA (EFSA FEEDAP Panel, 2012d),[10] the strain is considered to be susceptible to all relevant antibiotics.

The production strain of the amylase contained in product B and C is a strain of *B. amyloliquefaciens* deposited at the National Collection of Industrial Food and Marine Bacteria (NCIMB Ltd) with the accession number 30251 (originally notified for product B as *B. subtilis* DS098 and for product C as *B. amyloliquefaciens* SD80).[11] The strain was identified as *B. amyloliquefaciens*.[12] The absence of toxigenic potential of the strain was demonstrated following the guidance for the

[6] This Section has been amended following the provisions of Article 8(6) and Article 18 of Regulation (EC) No 1831/2003.
[7] Technical dossier/Section II/Annex II.2.2.1 and Supplementary information February 2015/Annex iib-1.
[8] Technical dossier/Supplementary information February 2015/Annex iib-1.
[9] Technical dossier/Supplementary information/February 2015/Annex viiia-1.
[10] Technical dossier/Supplementary information/May 2017/Annex Q2_MIC determination DSM9553.
[11] Technical dossier/Supplementary information May 2017/Annex Q1 and clarifications June 2017.
[12] Technical dossier/Supplementary information May 2017/Annex Q1a and Q1b.

assessment of the toxigenic potential of *Bacillus* species used in animal nutrition (EFSA FEEDAP Panel, 2014).[13] The susceptibility of the strain to the antibiotics recommended by EFSA (EFSA FEEDAP Panel, 2012d)[14] showed that the MIC values were below the corresponding cut-off values defined by the Panel.

With regard to product D, in principle, the production strain should be *A. oryzae* deposited at the Centraalbureau voor Schimmelcultures with the deposition number CBS 585.94.[15] The applicant provided the analysis supporting the identification and the deposit certificate for this strain.[16] However, in some passages of the dossier, it is mentioned that the production strain is a derivative of CBS 585.94,[17] and the clarifications provided at this regard were not sufficient.[18] Therefore, the Panel cannot conclude on the identity and origin of the production strain of the amylase contained in product D.

The production strain of the amylase contained in product E is a strain of *A. oryzae* deposited at the ATCC with the accession number SD-5374 (formerly deposited at DSMZ as *A. oryzae* DS 114).[19] The strain was identified as *A. oryzae*.[20]

The production strain of the cellulase contained in product F is a strain of *Trichoderma reesei*[21] deposited at the ATCC with the accession number PTA-10001 (originally notified as *T. longibrachiatum* PTA-10001).[22] The immediate precursor of the production strain was identified as *Trichoderma reesei*.[23]

The production strain of the cellulase contained in product G was originally *T. longibrachiatum* ATCC 74252 and now has been changed to *T. reesei* at the ATCC with the accession number SD 6331.[24] The strain was identified as *T. reesei*.[25]

The production strain of the cellullase contained in product H is a strain of *A. niger* deposited at the CBS with the accession number CBS 120604.[26] The strain was identified (and deposited) as *A. niger*.[27]

The production strain of the xylanase contained in product J is a strain of *Trichoderma koningii* that is deposited at the Culture collection Mycotheque de l'Université Catholique de Louvain with the number MUCL 39203 (originally notified as *T. longibrachiatum* MUCL 39203).[28] The strain was identified as *T. koningii* but since no details were provided on how the analysis was conducted that the Panel cannot conclude on the identity of this production strain.

The production strain of the xylanase contained in product K is a strain of *Trichoderma citrinoviride* (originally notified as *T. longibrachiatum*) and is deposited at the CBS with the accession number 614.94.[29] The strain was identified as *T. citrinoviride*.[30]

The production strain of the glucanase contained in product L is a strain of *Aspergillus tubingensis* that is deposited at the Culture collection Mycotheque de l'Université Catholique de Louvain with the number MUCL 39199 (originally notified as *A. niger*).[31] The strain was identified as *A. tubingensis* but since no details were provided on how the analysis was conducted the Panel cannot conclude on the identity of this production strain.

[13] Technical dossier/Supplementary information February 2015/Annex viiia-2 and 3.
[14] Technical dossier/Supplementary information May 2017/Annex Q2.
[15] Technical dossier/Supplementary information May 2017/Annex Q1f.
[16] Technical dossier/Supplementary information February 2015/Annex ivb2-2 and supplementary information May 2017/Annex Q1f.
[17] Technical dossier/Supplementary information February 2015/Annex ii3b and further clarifications June 2017.
[18] Technical dossier/Clarifications June 2017.
[19] Technical dossier/Supplementary information February 2015 Annex iib-4 and Supplementary information May 2017 Annex Q1c.
[20] Technical dossier/Supplementary information February 2015/Annex iib-5a and Supplementary information May 2017/Annex_Q1c_ATCC Identification DS114.
[21] Technical dossier/Supplementary information May 2017/Annex Q1d ATCC10001_Mol charact.
[22] Technical dossier/Supplementary information May 2017/Annex Q1d_ATCC deposition certificate.
[23] Technical dossier/Supplementary information May 2017/Annex Q1d Identification.
[24] Technical dossier/Supplementary information February 2015/Annex ib-2, ib-3, iib-6 and iiib.
[25] Technical dossier/Supplementary information February 2015/Annex ivb-5.
[26] Technical dossier/Section II/Annex 2.2.46 and supplementary information February 2015/Annex iib-7 and Supplementary information May 2017/Annex Q1e _certificate of deposition_120604-294.
[27] Technical dossier/Section II/Annex 2.2.46.
[28] Technical dossier/Section II/Annex II.2.2.9 and Supplementary information February 2015 annex iib-8 and supplementary information May 2017/Annex Q1d_xylanase_deposition_mucl39203.
[29] Technical dossier/Section II/Annex II.2.2.46 and Supplementary information February 2015/Annex iib-7 and Supplementary information May 2017 Annex Q1e certificate of deposition_614.94.
[30] Technical dossier/Supplementary information February 2018.
[31] Technical dossier/Supplementary information May 2017/Annex Q1d glucanase deposition mucl39199.

3.1.2. Manufacturing process

Details of the production method used for each enzyme were provided together with details of medium ingredients and processing aids and, in several cases, included HACCP plans.[32] Although the process conditions differ, essentially each enzyme is produced individually by fermentation as a pure culture in batch, batch fed or continuous culture in sterilised fermentation vessels using sterilised fermentation ingredients (temperature sterilisation or microfiltration). After fermentation, the enzyme is harvested and concentrated using different methodologies. The enzyme preparation is then prepared. Depending on the process, preservatives and carrier material may be added. The data reported under the characterisation refers to the enzyme products obtained after this step. In case of product D, the characterisation was done on the enzyme obtained from strain CBS 585.94. The applicant stated that no antimicrobials were used during the manufacturing processes.

3.1.3. Characterisation of the additives

The minimum declared content for each enzyme preparation is shown in Table 2. Data on the batch to batch variation was provided for the different products.[33] In general, the minimum declared activity was confirmed by the analysis of the batches, except in product K. It is noted the high overdosage present in some of the products, especially products C and D; however, the enzyme activity measured with the company's method showed activities in line with the specifications applied for that method.

Table 2: Minimum declared activity and analysed activity of production batches

Ref.	Physical form	DNS units/g product Specified
A	Granule	129,800
B	Powder	101,050
C	Powder	1,200
D	Granule	55,600
E	Powder	235,850
F	Powder	2,750
G	Powder	6,000
H	Powder	25,650
J	Powder	51,600
K	Powder	70,000
L	Powder	10,000

DNS: Dinitrosalicylic acid; for details on the definition see Annex A.
(a): Technical dossier/Section II.

Data on the particle size distribution and dusting potential was provided for all products.[34] Particle size distribution (laser diffraction) showed that products A and D do not have respirable particles, the other products show high amounts of respirable particles, and in all cases, the amount of particles < 50 μm is higher than 1.5%. The dusting potential was measured using the Stauber Heubach method and was expressed as g/m^3. Results showed in most of the cases a low to moderate dusting potential (< 0.5 g/m^3) with the exception of the xylanase (J and K) products which showed a high-dusting potential (> 2 g/m^3).

The additives are routinely monitored for microbial contamination. Limits are set for coliforms, yeasts and filamentous fungi ($< 10^3$ colony-forming unit (CFU)/g additive), *Escherichia coli* (< 10 CFU/g additive) and *Salmonella* spp. (absence in 25 g additive). Data from at least three batches of each product confirmed compliance with these limits for coliforms, *Salmonella*, *E. coli,* yeast and filamentous fungi.[35] For all products, the counts of filamentous fungi were < 10 CFU/g. These data support the absence of the production strain for the fungal-derived enzymes. For the bacterial-derived enzymes, the

[32] Technical dossier/Section II/Annexes 2.31 to 2.3.9 and Supplementary information January 2015/Annex Qiiia.
[33] Technical dossier/Section II/Annexes 2.1.6 to 2.1.17 and Supplementary information May 2017/Annex Q7a to c.
[34] Technical dossier/Section II/annexes II.2.1.55 to II.2.1.63 and Supplementary information February 2015/Annex iia.
[35] Technical dossier/Section II/Annexes II.2.1.18 to II.2.1.41 and Supplementary information February 2015/Annex Qiva and Supplementary information May 2017/Annexes Q11.

production strain was not detected for product A and B, but for product C, no data was provided. However, the manufacturing process should ensure that the strain is not present.[36]

Specifications are set for lead (< 5 mg/kg), cadmium (< 0.5 mg/kg), mercury (< 0.5 mg/kg) and arsenic (< 3 mg/kg). Analysis of at least three batches of each enzyme preparation showed compliance with these specifications,[37] with the exception for product F for which only lead data were made available and for C and G for which the limit of detection of arsenic and cadmium was 5 mg/kg.

Specifications are also set for aflatoxin B1 (< 1 μg/kg), data on at least three batches of each enzyme showing compliance was provided.[38] The data provided also included the other mycotoxins.

For the products produced by filamentous fungi, data on the capacity of the strain to produce secondary metabolites were provided for the product F, and showed that the strain does not produce them in laboratory conditions.[39] For the rest of the products except product J, the data provided were on the presence of secondary metabolites in the enzyme products (three batches in each case). In all the cases, the substances investigated were not detected (limit of detection (LOD) = 50 ng/g enzyme preparation).

The antimicrobial activity was investigated in the enzyme products in three batches of each enzyme and was absent.[40]

3.1.4. Stability

The shelf-life of the enzyme preparations was measured using the in-house method of each supplier using the sealed, moisture tight packaging in which the additives are normally distributed.[41] Storage temperatures variously considered were 5°C (products A, D and F), 10°C (products A, D and E), 18–25°C (all products, except E) or 40°C (A, C and D). In 8 of 11 cases, only a single batch of the enzyme was studied. The enzymes had a shelf-life, indicated by high enzyme recovery values, of at least 6 months at room temperature (range 6–18 months).

Since the application of the enzymes can be through an aqueous suspension/solution, data on the short-term stability of the enzymes in water was provided for the different enzyme preparations.[42] For products A, B, D, E and F, the stability of the enzymes was tested by preparing suspensions of the enzyme in water (0.1 mg enzyme/mL). Two subsamples were kept either at 5°C or at 25°C for 48 h. At 0, 24 and 48 h, the suspensions were homogenised and analysed for enzyme activity. Enzyme activity losses at 5°C ranged from 0.2% to 8.5% and at 25°C ranged from 1.8% to 20.8%. For products C, G, J and L, the stability of the enzymes was tested by preparing suspensions in water (10 mg/mL). Samples were kept at 20–22°C and analysed for enzyme activity at 0, 24 and 48 h, enzyme activity losses after 48 h were minimal (below 10%). For products H and K, the enzymes were mixed with water and were kept at 15°C for 72 h. No losses on the enzyme activity were reported.

3.1.5. Conditions of use

The enzymes are intended for use with all forages and for all animal species.[43,44] The proposed enzyme units per kg of fresh matter are provided in Table 3.[45]

[36] Technical dossier/Section II/Annex 2.2.24 and Supplementary information May 2017/Annexes Q11 and Q9 and Q10.
[37] Technical dossier/Section II/Annexes II.2.1.42 to 2.1.53 and Supplementary information May 2017 Q9 and Q10.
[38] Technical dossier/Section II/Annex 2.1.30 to 2.1.41 and Supplementary information May 2017/Annex Q9.
[39] Technical dossier/Supplementary information May 2017/Annex Q3_secondary metabolites.
[40] Technical dossier/Section II/Annexes 2.2.24 to 2.2.34, 2.1.31 and 2.1.34 and Supplementary information May 2017 Annex Q8.
[41] Technical dossier/Section II/Annexes 2.4.1 to 2.4.10 and Supplementary information May 2017/Annex Q4.
[42] Technical dossier/Supplementary information February 2015/Annex Qva.
[43] In an expert report it was indicated that product H and K are to be used in easy to ensile substrates. Technical dossier/ Supplementary information May 2017/Annex Q14_Efficacy_Report.
[44] According to the applicant the application of these enzymes is done with 'bacteria directly to silage (granular application) or of dissolving dried bacterial cells in water with enzymes and spraying onto silage (liquid application)'.
[45] Supplementary information May 2017.

Table 3: Recommended enzyme activity per kg of fresh matter for the different enzymes

Product	DNS enzyme units[(a)]/kg forage fresh matter
A	40.0
B	10.0
C	3.7
D	17.0
E	23.0
F	7.0
G	0.5
H	3.0
J	3.2
K	15.0
L	3.4

(a): DNS dinitrosalicylic acid, for details see Annex A.

3.2. Safety

3.2.1. Safety for the target species, consumer and environment

The enzymes per se as feed additives are of no concern for the consumers of products derived from animals receiving such enzymes. Regarding the target species and environment, the recommended inclusion level of the enzymes under assessment is very low (below 5 mg/kg forage fresh matter); moreover, the enzymes under assessment occur as normal constituents of silage, and therefore, the FEEDAP Panel considers that there are no concerns as regards to the active substance for the target species and the environment.

However, the FEEDAP Panel recognises that this conclusion applies to the active substance, but in order to extend it to the additive(s), there is the need to consider the presence of other substances that could be of concern (e.g. toxins produced by the production strain or contaminants).

The production strains for products A, B and C belong to a species (*B. amyloliquefaciens*) that is considered by EFSA to be suitable for the Qualified Presumption of Safety (QPS) approach to safety assessment (EFSA, 2007; EFSA BIOHAZ Panel, 2017). This approach requires the identity of the strain to be conclusively established and evidence that the strain does not show resistance to antimicrobials relevant for humans and animals and the absence of cytotoxic activity. In the view of the FEEDAP Panel, the identity of the strains has been established and the qualifications were met, and consequently, products A, B and C are regarded as safe.

For the products derived from fungal strains, the strains or resulting products[46] were tested for the presence of secondary metabolites which could be of toxicological concern (See Section 3.1.3). These were found to be below the limits of detection (D, E, G, H, K and L) or the strain was shown not capable of their production (product F). Taking into account the limits of detection and the inclusion levels of the enzyme products in the silage, the Panel considers that the products are considered safe. The Panel notes that, for product D, the conclusion would apply only in the case that the production strain was *A. oryzae* CBS 585.94. However, from the data in the dossier, it is not possible to ascertain that this is the production strain from which the product is actually made.

Finally, the data provided regarding the purity of the products showed values for chemical contaminants (heavy metals and mycotoxins) that would not be of concern, especially considering the inclusion level of the products.

Considering all the above, the FEEDAP Panel concludes that the additives under assessment pose no concerns to the target animals, consumers of food products derived from animals fed the silage obtained when using these additives or to the environment.

3.2.2. Users

No data were submitted on skin/eye irritation or skin sensitisation. Therefore, no conclusions can be drawn on the skin and eye irritancy or skin sensitisation potential of the additives under

[46] For product D the analysis were done on the enzyme obtained from *A. oryzae* CBS 585.94.

assessment. Given the proteinaceous nature of the active agents, the additives under assessment should be considered to be potential respiratory sensitisers.

3.3. Efficacy[47]

The applicant provided *in vitro* studies in which it was studied the capacity of the enzymes to release sugars from the non-starch polysaccharides present in different forage substrates. These data/studies were not considered in the assessment because they did not provide evidence of the efficacy related to the ensiling process.[48,49]

The applicant also provided *in vitro* studies investigating the ensiling capacity of the enzymes under assessment. Three different protocols were followed.

Considering the effects on dry matter preservation, pH and lactic acid content, the Panel concludes that the enzyme products amylase from *B. amyloliquefaciens* DSM 9553 (product A), from *B. amyloliquefaciens* NCIMB 30251 (in product B), from *A. oryzae* CBS 585.94 (product D) and from *A. oryzae* ATTC SD-5374 (product E) and cellulase from *T. longibrachiatum* ATCC PTA-100001 (product F) have the potential to improve the characteristics of the silage material prepared from easy, moderate and difficult to ensile material.

The Panel concludes that the xylanase from *T. longibrachiatum* CBS 614.94 (product K) has a potential to increase the preservation of easy to ensile material when used in combination with *P. pentosaceus* NCIMB 30238 and *L. plantarum* NCIMB 30237, with or without product H. The comparison of the results obtained when adding K and the microbial incoulants compared to the microbial inoculants alone, showed an added effect of the enzyme. However, the Panel cannot conclude on the efficacy of the enzyme K when used alone.

The Panel cannot conclude on the efficacy for amylase from *B. amyloliquefaciens* NCIMB 30251 (in product C), cellulase from *T. reesei* ATCC PTA-10001 (product G), cellulase from *A. niger* CBS 120604 (product H), xylanase from *T. koningii* MUCL 39203 (product J) and glucanase from *A. tubingensis* MUCL 39199 (product L) because the study designs do not allow a conclusion on the efficacy of these enzymes.

Conclusions

The additives under assessment are considered safe for the target species, consumer of food products from animals fed treated silage and for the environment. The Panel notes that, for product D, the conclusion would apply only in the case that the production strain was *A. oryzae* CBS 585.94.

In the absence of data, no conclusion can be drawn on the skin and eye irritancy of the additive or skin sensitisation potential of the products under evaluation. Owing to the proteinaceous nature of the active substances, these products should be considered to have the potential to be a respiratory sensitiser.

Amylase from *B. amyloliquefaciens* DSM 9553 (product A), from *B. amyloliquefaciens* NCIMB 30251 (product B), from *A. oryzae* (product D) and from *A. oryzae* (ATTC SD-5374 (product E) and cellulase from *T. longibrachiatum* ATCC PTA-100001 (product F) have the potential to improve the characteristics of the silage material prepared from easy, moderate and difficult to ensile material. The xylanase from *T. longibrachiatum* CBS 614.94 (product K) has a potential to increase the preservation of easy to ensile material when used in combination with *P. pentosaceus* NCIMB 30238 and *L. plantarum* NCIMB 30237, with or without product H. The comparison of the results obtained when adding K compared to the microbial inoculant alone showed an added effect of the enzyme. However, the Panel cannot conclude on the efficacy of the enzyme K when used alone. The Panel cannot conclude on the efficacy for amylase from *B. amyloliquefaciens* NCIMB 30251 (in product C), cellulase from *T. reesei* ATCC PTA-10001 (product G), cellulase from *A. niger* CBS 120604 (product H), xylanase from *T. koningii* MUCL 39203 (product J) and glucanase from *A. tubingensis* MUCL 39199 (product L).

Documentation provided to EFSA

1) Enzymes as silage additive. November 2010. Submitted by SILAC EEIG.
2) Enzymes as silage additive. Supplementary information. February 2015. Submitted by SILAC EEIG.
3) Enzymes as silage additive. Supplementary information. May 2017. Submitted by SILAC EEIG.

[47] This Section has been amended following the provisions of Article 8(6) and Article 18 of Regulation (EC) No 1831/2003.
[48] Technical dossier/Section IV/Annexes IV.4.1.1 to IV.4.1.5.
[49] Technical dossier/Supplementary information February 2015/Annexes viia1 and viia2.

4) Enzymes as silage additive. Supplementary information. January 2018. Submitted by SILAC EEIG.
5) Evaluation report of the European Union Reference Laboratory for Feed Additives on the Methods(s) of Analysis for Enzymes as silage additives.
6) Comments from Member States.

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EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2012c. Guidance on studies concerning the safety of use of the additive for users/workers. EFSA Journal 2012;10(1):2539, 5 pp. https://doi.org/10.2903/j.efsa.2012.2539

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2012d. Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. EFSA Journal 2012;10(6):2740, 10 pp. https://doi.org/10.2903/j.efsa.2012.2740

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2014. Guidance on the assessment of the toxigenic potential of Bacillus species used in animal nutrition. EFSA Journal 2014;12(5):3665, 10 pp. https://doi.org/10.2903/j.efsa.2014.3665

Abbreviations

ATCC	American type culture collection
CFU	colony-forming unit
CMC	carboxymethyl cellulose
DNS	dinitrosalicylic acid
DSMZ	Deutsche Sammlung von Mikroorganisms and Zellkulturen
EURL	European Union Reference Laboratory
FEEDAP	The Panel on Additives and Products or Substances used in Animal Feed
LOD	limit of detection
MIC	Minimum Inhibitory Concentration
QPS	Qualified Presumption of Safety

Annex A – Executive Summary of the Evaluation Report of the European Union Reference Laboratory for Feed Additives on the Method(s) of Analysis for Enzymes as silage additives

In the current application, authorisation is sought under article 10(2) for *Enzymes as silage additives*, under the 'category'/'functional groups' 1(k) 'technological additives'/'silage additives' according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought for the use of the *feed additives* for all animal species.

This group application includes 11 enzymes, which are not intended to be added together in the silage. They are to be marketed separately in solid forms (granular or powder) containing 10–90% dextrin compounds (maltodextrin, amylodextrin or dextrin). According to the Applicant, *alpha-amylase; endo-1,4-beta-glucanase; endo-1,3(4)-beta-glucanase* and *endo-1,4-beta-xylanase* are active substances originating from different microorganisms.

The Applicant expressed the enzyme activities units as follows:

- One *alpha-amylase* DNS unit is the amount of reducing sugar released as maltose equivalents in μmol/g per min at pH 4.5 and 37°C from starch under specified conditions of the assay.
- One *beta-glucanase* DNS unit is the amount of reducing sugar released as glucose equivalents in μmol/g per min at pH 4.5 and 37°C from carboxymethyl cellulose (CMC) under specified conditions of the assay.
- One *xylanase* DNS unit is the amount of reducing sugar released as xylose equivalents in μmol/g per min at pH 4.5 and 37°C from birchwood xylan under specified conditions of the assay.

Each *feed additive is* intended to be incorporated into silage either directly in granular form or spraying a water solution onto silage.

For the quantification of the different enzyme activities in the *feed additive*, the Applicant provided harmonised single-laboratory validated and further verified colorimetric methods based on the quantification of coloured compounds produced by dinitrosalicylic acid (DNS). Based on the satisfactory performance characteristics available, the European Union Reference Laboratory (EURL) recommends for official control these colorimetric methods for the quantification of the *enzyme* activities in the individual *feed additives*.

As the accurate quantification of *enzymes* added to *silage* is not achievable experimentally, the EURL cannot evaluate nor recommend any method for official control.

Further testing or validation of the methods to be performed through the consortium of National Reference Laboratories as specified by Article 10 (Commission Regulation (EC) No 378/2005) is not considered necessary.