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European Union Reference Laboratory for Feed Additives

JRC F.5/CvH/MGH/AS/Ares

Evaluation Report on the Analytical Methods submitted in connection with the Application for Authorisation of a Feed Additive according to Regulation (EC) No 1831/2003

Preparation of bacillus licheniformis ENV01/DSM 32457

(FAD-2018-0064; CRL/180041)

European Union Reference Laboratory
Feed Additives

Evaluation Report on the Analytical Methods submitted in connection with the Application for Authorisation of a Feed Additive according to Regulation (EC) No 1831/2003

Dossier related to:	**FAD-2018-0064 - CRL/180041**
Name of Product :	***Preparation of Bacillus licheniformis ENV01/DSM 3245***
Active Agent (s):	**Bacillus licheniformis**
Rapporteur Laboratory:	**Centre wallon de Recherches agronomiques (CRA-W), Gembloux, Belgium**
Report prepared by:	**Véronique Ninane**
Report checked by: Date:	**María José González de la Huebra** **22/02/2019**
Report approved by: Date:	**Christoph von Holst** **25/02/2019**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 4(1) for a preparation of *Bacillus licheniformis* ENV01/DSM 32457 *(Optizime)* under the category / functional group 1(k) 'technological additives' / 'silage additives', according to Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought for the use of the *feed additive* in *silage* for all animal species.

According to the Applicant, the *feed additive* contains as active substance viable spores of the non-genetically modified strain *Bacillus licheniformis* ENV01/DSM 32457. The *feed additive* is to be marketed as a powder preparation containing a minimum *Bacillus licheniformis* ENV01/DSM 32457 content of $2.5x10^{10}$ Colony Forming Unit (CFU)/g. The *feed additive* is intended to be added, after its reconstitution in water, to *silage* at a minimum dose of $5x10^{7}$ CFU/kg of *silage*.

For the identification of *Bacillus licheniformis* ENV01/DSM 32457, the EURL recommends for official control Pulsed Field Gel Electrophoresis (PFGE), a generally recognised methodology for genetic identification of bacterial strains.

For the enumeration of *Bacillus licheniformis* ENV01/DSM 32457 in the *feed additive*, the Applicant submitted the ring-trial validated spread plate CEN method EN 15784. Based on the performance characteristics available, the EURL recommends this method for official control.

The Applicant did not provide any experimental method or data for the quantification of *Bacillus licheniformis* ENV01/DSM 32457 in *silage.* Since the unambiguous determination of the content of *Bacillus licheniformis* ENV01/DSM 32457 initially added to *silage* is not achievable by analysis, the EURL cannot evaluate nor recommend any method for official control to quantify the active substance in *silage*.

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, as last amended by Regulation (EU) 2015/1761) is not considered necessary.

KEYWORDS

Bacillus licheniformis ENV01/DSM 32457, technological additives, silage additives, all animal species

1. BACKGROUND

In the current application authorisation is sought under Article 4(1) for a preparation of *Bacillus licheniformis* ENV01/DSM 32457 *(Optimize)* under the category / functional group 1(k) 'technological additives' / 'silage additives', according to Annex I of Regulation (EC) No 1831/2003 [1]. Authorisation is sought for the use of the *feed additive* in *silage* for all animal species [2].

According to the Applicant, the *feed additive* contains as active substance viable spores of the non-genetically modified strain *Bacillus licheniformis* ENV01/DSM 32457 [3]. The strain is deposited at the Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) under the deposit number DSM 32457 [4].

The *feed additive* is to be marketed as a powder preparation containing a minimum *Bacillus licheniformis* ENV01/DSM 32457 content of $2.5x10^{10}$ Colony Forming Unit (CFU)/g [5].

The *feed additive* is intended to be added, after its reconstitution in water, to *silage* at a minimum dose of $5x10^{7}$ CFU/kg of *silage* [6].

Note: The EURL previously evaluated and recommended the analytical methods for the determination of *Bacillu*s spp. in the frame of several dossiers [7].

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761, on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the duties and the tasks of the European Union Reference Laboratory concerning applications for authorisations of feed additives, the EURL is requested to submit a full evaluation report to the European Food Safety Authority for each application or group of applications. For this particular dossier, the methods of analysis submitted in connection with *Bacillus licheniformis* ENV01/DSM 32457 and their suitability to be used for official controls in the frame of the authorisation were evaluated.

3. EVALUATION

Description of the analytical methods for the determination of the active substance in the feed additive, premixtures, feedingstuffs and when appropriate water (section 2.6.1 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

For the enumeration of *Bacillus licheniformis* ENV01/DSM 32457 in *feed additive* the Applicant submitted the ring-trial validated spread plate CEN method EN 15784 [2,10] that was already evaluated by the EURL in the frame of previous *Bacillus* spp. dossiers [7].

Twenty grams of the *feed additive* are suspended in a phosphate buffered saline. From this, one new dilution is prepared and heat-treated at 80 °C for 10 minutes. Decimal dilutions are prepared from the heat-treated suspension, spread plated on tryptone soya agar and incubated at 37 °C for 16-24 h aerobically. The performance characteristics reported from the validation study after logarithmic transformation of the CFU values [8] are:

- a repeatability standard deviation (S_r) ranging from 0.07 to 0.09 $\log_{10}$ CFU/g;

- a reproducibility standard deviation (S_R) ranging from 0.32 to 0.35 $\log_{10}$ CFU/g;

In addition, the EURL calculated a limit of quantification (LOQ) of $3x10^4$ CFU/g following the recommendations of the ISO 7218 standard [9].

Based on the performance characteristics presented, the EURL recommends for official control the ring-trial validated 15784 method for the enumeration of *Bacillus licheniformis* ENV01/DSM 32457 in *feed additive.*

The Applicant did not provide any experimental method or data for the quantification of *Bacillus licheniformis* ENV01/DSM 32457 in *silage.* Furthermore, the unambiguous determination of the content of *Bacillus licheniformis* ENV01/DSM 32457 initially added to *silage* is not achievable by analysis. Therefore, the EURL cannot evaluate nor recommend any method for official control to quantify *Bacillus licheniformis* ENV01/DSM 32457 in *silage*.

Methods of analysis for the determination of the residues of the additive in food (section 2.6.2 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

An evaluation of corresponding methods of analysis is not relevant for the present application.

Identification/Characterisation of the feed additive (section 2.6.3 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

For the identification of the strain *Bacillus licheniformis* ENV01/DSM 32457, the Applicant applied multi-locus sequence typing (MLST) [3]. The EURL recommends instead for official control Pulsed-Field Gel Electrophoresis (PFGE), a generally recognised methodology for genetic identification of bacterial strains [10]. A PFGE method for microbial identification of authorised probiotics at strain level in feedingstuffs [10] is currently being evaluated by the CEN Technical Committee 327 to become an European Standard.

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, as last amended by Regulation (EU) 2015/1761) is not considered necessary.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation, the EURL recommends for official control Pulsed Field Gel Electrophoresis (PFGE) for the identification of *Bacillus licheniformis* ENV01/DSM 32457 and the ring-trial validated spread plate method EN 15784 for the enumeration of this strain in the *feed additive*.

The Applicant did not provide any experimental method or data for the determination of *Bacillus licheniformis* ENV01/DSM 32457 in *silage*. Furthermore, the unambiguous determination of the content of *Bacillus licheniformis* ENV01/DSM 32457 initially added to *silage* is not achievable by analysis. Therefore, the EURL cannot evaluate nor recommend any method for official control to determine *Bacillus licheniformis* ENV01/DSM 32457 in *silage.*

Recommended text for the register entry (analytical method)

- Identification: Pulsed Field Gel Electrophoresis (PFGE)

- Enumeration in the *feed additive*: Spread plate method on tryptone soya agar (EN 15784)

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Bacillus licheniformis* ENV01/DSM 32457 have been sent to the European Union Reference Laboratory for Feed Additives. The dossier has been made available to the EURL by EFSA.

6. REFERENCES

[1] *Application, Reference SANTE E5: F.A. 1831/0065-2018

[2] *Application, Proposal for Register Entry, Annex A

[3] *Technical dossier, Section II: 2.2 Characterisation of the Active Substance

[4] *Technical dossier, Section II: Annex II.2.2.1.1

[5] *Technical dossier, Section II: 2.1. Identity of the additive

[6] *Technical dossier, Section II: 2.5 Conditions of use of the additive

[7] EURL Evaluation Reports:
https://ec.europa.eu/jrc/sites/jrcsh/files/FinRep-FAD-2009-0007.pdf
https://ec.europa.eu/jrc/sites/jrcsh/files/FinRep-FAD-2009-0013.pdf
https://ec.europa.eu/jrc/sites/jrcsh/files/FinRep-FAD-2009-0023.pdf
https://ec.europa.eu/jrc/sites/jrcsh/files/FinRep-FAD-2009-0041.pdf
https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2015-0006-bacillus_subtilis.pdf
https://ec.europa.eu/jrc/sites/jrcsh/files/finrep_fad_2015_0008_enviva_pro202gt.pdf
https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2016-0070-baci_subtilis.pdf
https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2017-0060-cinergy_life.pdf

[8] EN 15784:2009 - Animal feeding stuffs - Isolation and enumeration of presumptive Bacillus spp

[9] EN ISO 7218:2007 - Microbiology of food and animal feeding stuffs – General requirements and guidance for microbiological examinations

[10] European Community Project SMT4-CT98-2235."Methods for the Official Control of Probiotics Used as Feed Additives", Report 20873/1 EN (2002) ISBN 92-894-6250-7 (Vol. I) and Report 20873/3 EN (2002) ISBN 92-894-6252-3 (Vol. III)

*Refers to Dossier no: FAD-2018-0064

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation is the Centre wallon de Recherches agronomiques (CRA-W), Gembloux, Belgium.. This report is in accordance with the opinion of the consortium of National Reference Laboratories as referred to in Article 6(2) of Commission Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761.

8. ACKNOWLEDGEMENTS

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- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Centro di referenza nazionale per la sorveglienza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia, Pesca, Alimentació i Medi Natural. Generalitat de Catalunya, Cabrils (ES)
- Laboratoire de Rennes (SCL L35), Service Commun des Laboratoires DGCCRF et DGDDI, Rennes (FR)