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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

Bacillus subtilis DSM 32324

(FAD-2017-0058; CRL/170037)

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-2017-0058 – CRL/170037**
Name of Product:	***Bacillus subtilis DSM 32324***
Active Agent (s):	**Bacillus subtilis DSM 32324**
Rapporteur Laboratory:	**Centre Wallon de Recherches Agronomiques (CRA-W), Gembloux, Belgium**
Report prepared by:	**Véronique Ninane**
Report checked by: Date:	**Stefano Bellorini (EURL-FA)** **28/03/2018**
Report approved by: Date:	**Christoph von Holst** **28/03/2018**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 4(1) for *Bacillus subtilis DSM 32324* under the category / functional group 1(n) 'technological additives' / 'hygiene condition enhancers', according to Annex I of Regulation (EC) No 1831/2003. Authorisation is sought for the use of the *feed additive* for all animal species.

According to the Applicant, the *feed additive* contains as *active substance* viable spores of the non-genetically modified strain *Bacillus subtilis* DSM 32324. The *feed additive* is to be marketed as a powder containing a minimum *Bacillus subtilis* DSM 32324 content of $1.3x10^{10}$ Colony Forming Unit (CFU)/g. The *feed additive* is intended to be used directly in *feedingstuffs* or through *premixtures* at a minimum dose of $1.0x10^{8}$ CFU/kg of complete *feedingstuffs*.

For the identification of *Bacillus subtilis* DSM 32324, 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 subtilis* DSM 32324 in the *feed additive*, *premixtures* and *feedingstuffs* 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.

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.

KEYWORDS

Bacillus subtilis DSM 32324, technological additives, hygiene condition enhancers, all animal species.

1. BACKGROUND

In the current application authorisation is sought under Article 4(1) for *Bacillus subtilis DSM 32324* under the category / functional group 1(n) 'technological additives' / 'hygiene condition enhancers', according to Annex I of Regulation (EC) No 1831/2003 [1].

Authorisation is sought for the use of the *feed additive* for all animal species [1,2].

According to the Applicant, the *feed additive* contains as *active substance* viable spores of the non-genetically modified strain *Bacillus subtilis* DSM 32324 [3]. The strain is deposited at the Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany) under the deposit number DSM 32324 [3,4].

The *feed additive* is to be marketed as a powder containing a minimum *Bacillus subtilis* DSM 32324 content of $1.3x10^{10}$ Colony Forming Unit (CFU)/g [5].

The *feed additive* is intended to be used directly in *feedingstuffs* or through *premixtures* at a minimum dose of $1.0x10^{8}$ CFU/kg of complete *feedingstuffs* [6].

Note: The EURL previously evaluated the analytical methods for the determination of *Bacillus subtilis* in the frame of several dossiers (e.g. FADs 2009-0007; 2009-0013; 2009-0023; 2015-0006; 2016-0070) [7].

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005, as last amended by Regulation (EC) No 885/2009, 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 subtilis DSM 32324* and their suitability to be used for official controls in the frame of the authorisation were evaluated.

3. EVALUATION

Identification /Characterisation of the feed additive

For the identification of *Bacillus subtilis* DSM 32324, the Applicant applied multi-locus sequence analysis, pulsed-field gel electrophoresis (PFGE) and DNA fingerprinting [3].

The EURL recommends for official control the PFGE, a generally recognised methodology for genetic identification [8]. This methodology for microbial identification is currently being evaluated by the CEN Technical Committee 327 to become European Standard.

Qualitative and quantitative composition of impurities in the additive

The Applicant analysed the *feed additive* for microbial contaminants (e.g. yeast and mould *Escherichia coli, Bacillus cereus,* coliforms and *Salmonella*) using the methods mentioned in the technical dossier [5]. As for the determination of other undesirable substances in the *feed additive* (e.g. mycotoxins), analytical methods for official control are available from the respective European Union Reference Laboratories [9].

Description of the analytical methods for the determination of the active substances in feed additive, premixtures and feedingstuffs

For the enumeration of *Bacillus subtilis* DSM 32324 in the *feed additive*, *premixtures* and *feedingstuffs* the Applicant submitted the ring-trial validated spread plate CEN method EN 15784 [10] that was already evaluated by the EURL in the frame of previous *Bacillus subtilis* dossiers [7].

20 g of the *feed additive* (or 50 g of *premixtures* or *feedingstuffs*) are suspended in a phosphate buffered saline (or in 0.2 % sodium hydroxide solution for *premixtures* or *feedingstuffs*). 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 [10] 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; and
- a *limit of quantification* (LOQ) of $2x10^4$ CFU/g.

Based on the performance characteristics presented, the EURL recommends for official control the ring-trial validated method EN-15784 for the enumeration of *Bacillus subtilis* DSM 32324 in the *feed additive*, *premixtures* and *feedingstuffs.*

Note: The EN 15784 method is not applicable to mineral feeds composed mainly of minerals and containing at least 40 % crude ash. For these matrices laboratories may consider using the ring-trial validated VDLUFA method 28.2.2 instead [11].

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.

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 subtilis* DSM 32324 and the ring-trial validated spread plate method EN 15784 for enumeration of this strain in the *feed additive*, *premixtures* and *feedingstuffs*.

Note: The method EN 15784 is not applicable to mineral feeds composed mainly of minerals and containing at least 40 % crude ash. For these matrices laboratories may consider using the ring-trial validated VDLUFA method 28.2.2 instead.

Recommended text for the register entry (analytical method)

- Identification: Pulsed Field Gel Electrophoresis (PFGE)
- Enumeration in the *feed additive*, *premixtures* and *feedingstuffs*: 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 subtilis DSM 32324* 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. 1831/0045-2017

[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.2.1.2a

[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-2015-0006-bacillus_subtilis.pdf
https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2016-0070-baci_subtilis.pdf

[8] 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)

[9] Commission Regulation (EC) No 776/2006 amending Annex VII to Regulation (EC) No 882/2004 of the European Parliament and of the Council as regards to Community Reference Laboratories

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

[11] VDLUFA method –Enumeration of Bacillus licheniformis and Bacillus subtilis (VDLUFA Methodenbuch Bd.III, 28.2.2)

*Refers to Dossier no: FAD-2017-0058

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation was 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 (EC) No 885/2009.

8. ACKNOWLEDGEMENTS

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- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)