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Community Reference Laboratory – Feed Additives

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CRL Evaluation Report on the Analytical Methods submitted in connection with Section II, 2.6 (Control Methods) of the Application for Authorisation as a Feed Additive

according to Regulation (EC) No 1831/2003

Dossier related to:	EFSA-Q-2008-473
	FAD-2008-0039
	CRL/080010
Name of Additive:	*Bacillus subtilis ATCC PTA-6737*
Active Agent(s):	*Bacillus subtilis ATCC PTA-6737*
Rapporteur Laboratory:	Thüringer Landesanstalt für Landwirtschaft (TLL), Abteilung Untersuchungswesen, Jena
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Date:	02/04/2009
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Date:	14/04/2009

EXECUTIVE SUMMARY

In the current application authorisation is sought for the microbial feed additive *Bacillus subtilis ATCC PTA-6737* (*Bacillus subtilis PB6*) under the category 'zootechnical additives', functional group 'gut flora stabilisers' according to Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought for the use of *Bacillus subtilis PB6* as a gut flora stabiliser for chickens for fattening. The *feed additive* has a minimum of $1x10^{10}$ colony-forming units (CFU) per gram of viable spores of *Bacillus subtilis ATCC PTA-6737* as *active agent* in maltodextrin carrier. The *feed additive* is intended to be mixed into complete *feedingstuffs* at a final concentration ranging from $1x10^7$ to $5x10^7$ CFU/kg of *feedingstuffs*.

For the enumeration of *Bacillus subtilis ATCC PTA-6737* in the *feed additive, premixtures and feedingstuffs*, the applicant proposes the draft CEN method - prEN 15784:2008 E – an internationally recognised spread plate method. This method was ring-trial validated using the *premixtures* and *feedingstuffs* samples containing *Bacillus subtilis* spores. The performance characteristics of the draft CEN method reported after logarithmic transformation of measured values (CFU) are:

- For the *premixtures*: (1) a standard deviation for repeatability (s_r) of 0.09 $\log_{10}$ and (2) a standard deviation for between-laboratory reproducibility (s_R) of 0.32 $\log_{10}$.

- For the *feedingstuffs*: (1) a s_r of 0.07 $\log_{10}$ and (2) a s_R of 0.35 $\log_{10}$.

The applicant used the above mentioned spread plate method to analyse the various matrices containing *Bacillus subtilis ATCC PTA-6737* spores and reported the following results: (a) $1x10^9$ to $1.5x10^{11}$ CFU/g of *feed additive*; (b) $1x10^7$ to $1.5x10^9$ CFU/kg for *premixtures* and (c) $1x10^7$ to $1.5x10^8$ CFU/kg for *feedingstuffs.* The results obtained for *feed additive* and *premixtures* are considered acceptable; this method is therefore recommended for official controls for the *feed additives* and *premixtures* in the frame of the authorisation.

As regards *feedingstuffs*, the CRL notes that the limit of quantification reported by the applicant upon request (LOQ = $1x10^7$ CFU/kg *feedingstuffs*) is identical to the minimum dose proposed and is below the LOQ reported in the draft CEN method ($2x10^7$ CFU/kg). On the basis of the available information, the draft CEN method is recommended for official control of the *feedingstuffs* containing *Bacillus subtilis PB6* at the dosages above the LOQ reported

by CEN. Below $2x10^7$ CFU/kg the CRL is not able to conclude on the suitability of this method for official control purposes.

Molecular methods were used by the applicant for identification of the active agent. For official controls pulsed field gel electrophoresis (PFGE), a generally recognised standard methodology for microbial identification, is recommended.

Further testing or validation is not considered necessary.

KEYWORDS

Bacillus subtilis ATCC PTA-6737, zootechnical, gut flora stabiliser, chickens for fattening

1. BACKGROUND

"Bacillus subtilis PB6" is a *feed additive* for which authorisation is sought under the category 'zootechnical additives', functional group 'gut flora stabilisers' according to Annex I of Regulation (EC) No 1831/2003 [1]. "*Bacillus subtilis PB6"* is provided in form of a free flowing, dry powder containing at least $1.0x10^{10}$ CFU/g spores of *Bacillus subtilis ATCC PTA-6737* as active agent [2]. The strain is deposited as *Bacillus subtilis* at the American Type Culture Collection as number ATCC PTA-6737 [3]. The intended use of the current application is for chickens for fattening. The recommended dose ranges from $1x10^7$ to $5x10^7$ CFU/kg *feedingstuffs* [2].

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005 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 tasks of the Community Reference Laboratory concerning applications for authorizations of *feed additives*, the CRL is requested to submit a full evaluation report to the European Food Safety Authority (EFSA) for each application. For this particular dossier, the methods of analysis submitted in connection with the *Bacillus subtilis PB6* dossier (EFSA-Q-2008-473) 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

Qualitative and quantitative composition of the additive

For identification of the strain *Bacillus subtilis ATCC PTA-6737* molecular methods such as 16S rDNA gene sequence analysis and nucleotide sequency were used. These methods are suitable for the purpose of analysis [4]. For official controls pulsed field gel electrophoresis (PFGE), a generally recognised standard methodology for microbial identification, is recommended.

Qualitative and quantitative composition of any impurities in the additive

The applicant analyses the *feed additive* for microbial contaminants such as coliform bacteria, *Escherichia coli, Salmonella, Staphylococcus aureus,* yeasts and moulds by using appropriate AOAC official methods [5]. For undesirable substances (i.e. lead, arsenic, mercury, cadmium, aflatoxins, ochratoxin) internationally recognised standard methods are available at the respective Community Reference Laboratories [6].

Description of the analytical methods for the determination of active agent(s) in feed additive, premixtures and feedingstuffs

For the enumeration of *Bacillus subtilis ATCC PTA-6737* in the *feed additive, premixtures and feedingstuffs*, the applicant proposes the draft CEN method - prEN 15784:2008 E – an internationally recognised spread plate method [7].

This method was ring-trial validated using the *premixtures* and *feedingstuffs* samples containing *Bacillus subtilis* spores [8]. The performance characteristics of the draft CEN method reported after logarithmic transformation of measured values (CFU) are:

- For the *premixtures*: (1) a standard deviation for repeatability (s_r) of 0.09 $\log_{10}$ and (2) a standard deviation for between-laboratory reproducibility (s_R) of 0.32 $\log_{10}$.

- For the *feedingstuffs*: (1) a s_r of 0.07 $\log_{10}$ and (2) a s_R of 0.35 $\log_{10}$.

For the enumeration of spores of *Bacillus subtilis ATCC PTA-6737* in *feed additive, premixtures* and *feedingstuffs* the applicant uses the same surface plate count method [9]. This

method for the enumeration of *Bacillus subtilis* spores in the *premixtures* applies a heat treatment of the initial sample suspension at 80°C for 10 min to reduce the vegetative background flora. Subsequently, appropriate dilutions were spread on non-selective tryptone soya agar and the plates were incubated at 37°C for 16 – 24 h.

The applicant used the above mentioned spread plate method to analyse the various matrices containing *Bacillus subtilis ATCC PTA-6737* spores and reported the following results [9]: (a) $1x10^9$ to $1.5x10^{11}$ CFU/g of *feed additive*; (b) $1x10^7$ to $1.5x10^9$ CFU/kg for *premixtures* and (c) $1x10^7$ to $1.5x10^8$ CFU/kg for *feedingstuffs.* The results obtained for *feed additive* and *premixtures* are considered acceptable; this method is therefore recommended for official controls for the *feed additives* and *premixtures* in the frame of the authorisation.

As regards *feedingstuffs*, the CRL notes that the limit of quantification reported by the applicant [10], upon request (LOQ = $1x10^7$ CFU/kg *feedingstuffs*) is identical to the minimum dose proposed and is below the LOQ reported in the draft CEN method ($2x10^7$ CFU/kg). On the basis of the available information, the draft CEN method is recommended for official control of the *feedingstuffs* containing *Bacillus subtilis PB6* at the dosages above the LOQ reported by CEN. Below $2x10^7$ CFU/kg the CRL is not able to conclude on the suitability of this method for official control purposes.

4. CONCLUSIONS AND RECOMMENDATIONS

The applicant proposes the draft CEN method (prEN 15784:2008 E) for the enumeration of the active agent *Bacillus subtilis ATCC PTA-6737* in the *feed additive*, *premixtures* and *feedingstuffs*. This method is found suitable for official controls for the *feed additives* and *premixtures*. However, the LOQ reported by the applicant for *feedingstuffs* is identical to the minimum dose proposed. Therefore, the CRL is not able to conclude on the suitability of the draft CEN method for official control of the *feedingstuffs* containing *Bacillus subtilis PB6* at dosages below the LOQ ($2x10^7$ CFU/kg *feedingstuffs*) reported by CEN. Further testing or validation is not considered necessary.

Recommended text for the register entry, fourth column (Composition, chemical formula, description, analytical method)

- Enumeration: Spread plate method using tryptone soya agar with pre-heat treatment of feed samples.
- Identification: Pulsed-field gel electrophoresis (PFGE)

5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, samples of the additive Bacillus subtilis PB6 for chickens for fattening have been sent to the Community Reference Laboratory for Feed Additives Authorisation. The dossier has been made available to the CRL by EFSA.

6. REFERENCES

[1] *Application/Ref:SANCO/D/2:Forw.Appl.1831/026-2008

[2] * Application/ A Proposal of Register Entry_Annex III

[3] *Technical dossier/section II/Annex.II.9

[4] *Technical dossier/section II/ /Annex.II.8

[5] *Technical dossier/section II, 2.6.3. Methods of the analysis relating to the identity and characterisation of the additive.

[6] 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 Community reference laboratories, Official Journal of the European Union L 136..

[7] Draft CEN method (prEN 15784:2008 E): Animal feeding stuffs - Isolation and enumeration of presumptive Bacillus spp. (version April 2008).

[8] Leuschner R.G.K., Bew J., Cruz A. 2003. Enumeration of probiotic bacilli spores in animal feed: Interlaboratory study. J. AOAC Int. 86(3), 568-575

[9] *Technical dossier/section II/ /Annex.II.22

[10] *Supplementary information/LOQ comments (Nuyens)_05_03_09

*Refers to the dossier of FAD-2008-0039.

7. RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was the National Reference Laboratory for Feed Additive (NRL), Thüringer Landesanstalt für Landwirtschaft (TLL), Abteilung Untersuchungswesen, Jena, Germany

8. ACKNOWLEDGEMENTS

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– Univerza v Ljubljani, Veterinarska fakulteta. Nacionalni veterinarski inštitut, Enota za patologijo prehrane in higieno okolja, Ljubljana

– Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien

– Kmetijski inštitut Slovenije, Ljubljana

– Landwirtschaftliche Untersuchungs- und Forschungsanstalt (LUFA) Speyer

– Państwowy Instytut Weterynaryjny, Puławy

– Sächsische Landesanstalt für Landwirtschaft, Fachbereich 8 — Landwirtschaftliches Untersuchungswesen, Leipzig