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Community Reference Laboratory for Feed Additives

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CRL Evaluation Report on the Analytical Methods submitted in connection with Section II, 2.5 (Control Methods) of the Application for Authorisation as a Feed Additive according to Regulation (EC) No 1831/2003

Dossier related to:	EFSA-Q-2007-190 FAD-2007-0043
Name of Additive:	Ecobiol®
Active Agent(s):	*Bacillus amyloliquefaciens* CECT 5940
Rapporteur Laboratory:	Community Reference Laboratory for Feed Additives (CRL-FA)
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Report revised by: Date:	Christoph von Holst 10/07/2008
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EXECUTIVE SUMMARY

In the current application authorisation is sought for the product Ecobiol® under the category 'zootechnical additives', functional group 'gut flora stabilisers' according to Annex I of Regulation (EC) No 1831/2003. The active agent is *Bacillus amyloliquefaciens* CECT 5940. The preparations Ecobiol® and Ecobiol® plus contain a minimum total concentration of 1 x 10^9 or 1 x 10^{10} colony forming units (c.f.u.) per gram premixture of *Bacillus amyloliquefaciens* CECT 5940, respectively. Specifically, authorisation is sought to use the preparations Ecobiol® and Ecobiol® for chickens for fattening. The conditions of use are proposed with a recommended final dosage of 1 x 10^9 c.f.u./kg complete feedingstuffs.

For the quantification of the active agents (*Bacillus amyloliquefaciens* CECT 5940) of Ecobiol® and Ecobiol® plus in the *product*, *premixtures* and *feedingstuffs* the applicant uses trypticase soya agar (TSA). This is appropriate for the intended purpose.

For the quantitative determination of the colony forming units of the active agents for official controls in the *product*, *premixtures* and *feedingstuffs*, a spread plate enumeration method is recommended which has been ring-trial validated using premixtures and feedingstuff samples (J. AOAC Int. 2003. 86, 568-575). The method is similar to that used by the applicant using tryptone soya agar (TSA). The performance characteristics of the method are standard deviations for repeatability (s_r) and reproducibility (s_R) of around 0.07 – 0.28 $\log_{10}$ and 0.32 – 0.58 $\log_{10}$ calculated from the base 10 logarithms of the measured c.f.u./g in feedingstuffs, respectively. The limit of quantification (LOQ) for the method is 10 x 10^6 c.f.u./kg sample which is well below the minimum anticipated target level of application in feedingstuffs.

The identity of the bacterial strains, *Bacillus amyloliquefaciens* CECT 5940, was analysed by polymerase chain (PCR) methodology. For official controls in the frame of the authorisation pulsed field gel electrophoresis (PFGE), a generally recognised standard methodology for microbial identification, is recommended.

On the basis of the supplied documentation, no supplementary experimental work (testing or method validation) is required.

KEYWORDS

Ecobiol®, product, *Bacillus amyloliquefaciens* CECT 5940, zootechnical, chickens

1. BACKGROUND

Bacillus amyloliquefaciens CECT 5940 is the active agent incorporated in the preparations Ecobiol® and Ecobiol® plus for which authorisation is sought under the category 'zootechnical products', functional group 'gut flora stabilisers' according to Annex I of Regulation (EC) No 1831/2003. *Bacillus amyloliquefaciens* CECT 5940 is present in the preparations Ecobiol® and Ecobiol® plus at a concentration of 1 x 10^9 and 1 x 10^{10} c.f.u. per gram, respectively [1]. The strain is deposited at the Spanish Type Culture Collection (CECT), University of Valencia, Spain [2]. The intended use of the current application (EFSA-Q-2007-190) is for chickens for fattening at a recommended dosage of 1 x 10^9 c.f.u./kg complete feedingstuffs [1].

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 authorisations of feed products, 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 Ecobiol® and Ecobiol® plus dossier (EFSA-Q-2007-190) and their suitability to be used for official controls in the frame of the authorisation, were evaluated.

3. EVALUATION

The numbering system under this point refers to the 'Guidelines for the assessment of products in feedingstuffs, part II: Enzymes and Micro-organisms' (2.5 Control methods), in the following referred to as 'the Guidelines'.

Description of some of the methods listed under item 2.5.1. of the Guidelines

Qualitative and quantitative composition of the product

The applicant uses trypticase soya agar to quantify colony forming units of the strain *Bacillus amyloliquefaciens* CECT 5940 in the product [3]. The proposed method is suitable for the intended purpose and similar to the method recommended for official controls in the frame of the authorisation which was validated in a full collaborative study [4]. The applicant confirms that the ring trial validated method [4] is currently used to test Ecobiol® [3]. The genetic

identity of the strains was examined using polymerase chain reaction (PCR) methodology [5]. Pulsed-field gel electrophoresis (PFGE) is a generally recognised standard methodology for microbial identification and is suggested for official controls in the frame of the authorisation.

Description of qualitative and quantitative methods for routine control of the active agent in premixtures and feedingstuffs (cf. requirements of Guidelines section 2.5.2)

For the enumeration of the strain of the active agent, *Bacillus amyloliquefaciens* CECT 5940, in *premixtures* and *feedingstuffs*, the same method as above is recommended by the applicant [3] and the CRL-FA for official controls [4]. The fully ring trial validated method includes a preparation of an initial sample suspension using 20 g for preparations and 50 g for feedingstuff samples in 0.2 % KOH solution. Decimal dilutions are prepared in peptone salt diluent and subjected to a heat treatment at 80 ºC for 10 min. Subsequently, appropriate dilutions are spread on tryptone soya agar and plates are incubated at 37 °C for 24 – 48 h. The method's performance characteristics are standard deviations for repeatability (s_r) and reproducibility (s_R) of around 0.07 – 0.28 $\log_{10}$ and 0.32 – 0.58 $\log_{10}$ calculated from the base 10 logarithms of the measured c.f.u./g in feedingstuffs, respectively. The limit of quantification (LOQ) for the method is 10 x 10^6 c.f.u./kg sample which is well below the minimum anticipated target level of application in feedingstuffs.

Pulsed-field gel electrophoresis (PFGE) is considered as a suitable method for official controls in the frame of the authorisation to identify *Bacillus amyloliquefaciens* CECT 5940.

4. CONCLUSIONS AND RECOMMENDATIONS

The applicant uses appropriate conventional methods to enumerate the active agent. A ring-trial validated method using tryptone soya agar (TSA) and a heat treatment of the sample dilutions is recommended for official controls in the frame of the authorisation [4]. Pulsed-field gel electrophoresis (PFGE) is widely used by reference laboratories to identify bacterial isolates and it is therefore recommended as a suitable methodology in this context for official controls.

On the basis of the supplied documentation, no supplementary experimental work (testing or method validation) is required.

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

Enumeration: Spread plate method using tryptone soya agar following a heat treatment

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 preparations Ecobiol® and Ecobiol® plus for chickens for fattening have been sent to the Community Reference Laboratory for Feed Additives. The dossier has been made available to the CRL by EFSA.

6. REFERENCES

[1] Proposal of Register entry, Annex III

[2] Technical dossier, section II, 2.2.2. Biological origin; Evidence of deposition

[3] Technical dossier, section II, 2.5.2. Description of the qualitative and quantitative methods; Microbiological determinations

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

[5] Technical dossier, section II, 2.2.2. Biological origin; Identification of the strain

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation was the Community Reference Laboratory for Feed Additives (CRL-FA), Geel, Belgium. The initial evaluation report was made available for commenting to the consortium of National Reference Laboratories.

8. ACKNOWLEDGEMENTS

The following National Reference Laboratories contributed to the report:

- NRL – RP Praha, Praha, Czech Republic
- Thüringer Landesanstalt für Landwirtschaft, Jena, Germany
- French NRL-FA, Rennes, France