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Community Reference Laboratory for Feed Additives

CRL Feed Additives

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

Dossier related to:	FAD-2009-0007
	CRL/080041
Name of product :	PepSoyGen-C
Active Agent (s):	*Bacillus subtilis GR-101*
	Aspergillus oryzae GB-107
Rapporteur Laboratory:	Community Reference Laboratory for
	Feed Additives (CRL-FA)
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Date:	16/04/2010
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Date:	23/04/2010

EXECUTIVE SUMMARY

In the current application authorisation is sought for the probiotic PepSoyGen-C, which consists of two active agents *Bacillus subtilis GR-101* and *Aspergillus oryzae GB-107*, under the category 'zootechnical additives', functional group 'other zootechnical additives' according to Annex I of Regulation (EC) No 1831/2003. Specifically, authorization is sought for the use of *Bacillus subtilis GR-101* and *Aspergillus oryzae GB-107* for piglets, chicken for fattening, calves for fattening and for rearing, fish (salmonidae and other fish), lambs and goats for rearing and fattening and for dogs. The product is to be used to treat feed materials to diminish anti-nutritional factors and will enter animals in live form. The proposed conditions of use do not include minimum or maximum concentrations of the feed additive. According to the applicant the *product* as such will not be placed on the market, but feed materials previously treated with the product.

For the enumeration of *Bacillus subtilis GR-101* in *premixtures* and *feedingstuffs* the CEN method (EN 15784) has been validated at a range between 10^5 and 10^9 CFU/g. The performance characteristics of the 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 $\log_{10}$ CFU/g and (2) a standard deviation for *reproducibility* (s_R) of 0.32 $\log_{10}$ CFU/g.
- for the *feedingstuffs*: (1) s_r = 0.07 $\log_{10}$ CFU/g and (2) s_R = 0.35 $\log_{10}$ CFU/g and
- a limit of detection (LOD) of $1x10^5$ CFU/kg in *feedingstuffs*.

However, since this specific application does not include target levels of *Bacillus subtilis GR-101* in *feedingstuffs*, the CRL cannot evaluate the suitability of this CEN standard.

For the enumeration of *Aspergillus oryzae GB-107* the applicant proposes internationally recognised US FDA/CFSAN BAM spread plate method for enumeration of yeasts, moulds and mycotoxins. The applicant considers that further validation or verification is not necessary since this is an official US method. However another international standard exists for the enumeration of yeasts and moulds (ISO 21527–1). No performance characteristics of this spread plate method were provided except the LOD of $1x10^5$ CFU/kg in feed. As this method was not tested on the product, the CRL cannot evaluate the suitability of this ISO method for official control. Molecular methods were used by the applicant for identification of active agents. The CRL recommends for official control for *Bacillus subtilis GR-101*, Pulsed Field Gel Electrophoresis (PFGE), a generally recognised standard methodology for

bacterial identification. For *Aspergillus oryzae GB-107* the CRL recommends for official control Polymerase Chain Reaction (PCR), generally recognised standard methodology for identification of yeasts and moulds.

Further testing or validation is not considered necessary.

KEYWORDS

Bacillus subtilis GR-101, Aspergillus oryzae GB-107, zootechnical additives, piglets, chicken for fattening, calves for fattening, calves for rearing, fish (salmonidae and other fish), lambs and goats for rearing and fattening, dogs.

1. BACKGROUND

PepSoyGen-C is a product composed of two active agents *Bacillus subtilis GR-101* and *Aspergillus oryzae GB-107* for which authorisation is sought under the Article 4(1) under the category of 'zootechnical additives' functional group 'other zootechnical additives' according to Annex I of Regulation (EC) No 1831/2003 [1]. Strains are deposited at the 'Korean Culture Center of Microorganisms (KCCM)' within the 'Korean Research Institute of Bioscience and Biotechnology (KRIBB)' in Daejeon, Korea [2]. Specifically, authorization is sought for the use of *Bacillus subtilis GR-101* and *Aspergillus oryzae GB-107* for piglets, chicken for fattening, calves for fattening, calves for rearing, fish (salmonidae and other fish), lambs and goats for rearing and fattening and for dogs. The *product* is to be used to treat feed materials to diminish anti-nutritional factors and will enter animals in live form. The product itself will not be brought on the market [3]. The applicant did not include in the proposed register entry the quantitative composition of the *product*, e.g. expressed as CFU/g of each active agent. Furthermore the conditions of use did not contain proposed levels of the active agents in *feedingstuffs* [3].

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 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 PepSoyGen-C dossier 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 and characterization of microbial strains *Bacillus subtilis GR-101* and *Aspergillus oryzae GB-107* the applicant used PCR (Polymerase Chain Reaction) and rDNA gene sequence analysis [4, 5]. These methods are suitable for the purpose of analysis.

The CRL recommends for official control for *Bacillus subtilis GR-101*, Pulsed Field Gel Electrophoresis (PFGE), a generally recognised standard methodology for genetic identification [6].

For official control for *Aspergillus oryzae GB-101*, the CRL recommends PCR typing, a generally recognised method for identification of yeasts and moulds [6]

Qualitative and quantitative composition of any impurities in the additive

The applicant analysed the *feed additive* for microbial contaminants (such as *Staphylococcus*, *Escherichia coli, Salmonella* and *Clostridium perfringens)* by using appropriate AOAC official methods [7]. For undesirable substances (i.e. cadmium, mercury, lead) internationally recognised standard methods are available at the respective Community Reference Laboratories, in accordance with Commission Regulation (EC) No 776/2006.

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

For the enumeration of *Bacillus subtilis GR-101* in *premixtures* and *feedingstuffs* the applicant uses an enumeration spread plate method [8]. This method for the enumeration of *Bacillus subtilis* spores in the *premixtures* and *feedingstuffs* applies a heat treatment of the initial sample at 80°C for 10 min to reduce vegetative background flora. Subsequently, appropriate

dilutions are spread on non-selective tryptone soy agar (TSA) plates and plates are incubated at 37°C for 18 – 24 h. This method is similar to the spread plate CEN method for the enumeration of *Bacillus* spp (EN 15784) [9]. This method was ring-trial validated using the *premixtures* and *feedingstuffs* samples containing *Bacillus subtilis* spores, at a range between 10^5 and 10^9 CFU/g. The performance characteristics of the CEN method reported after logarithmic transformation of measured values (CFU) are:

- For the premixtures:
    - a standard deviation for repeatability (s_r) of 0.09 $\log_{10}$ CFU/g and
    - a standard deviation for reproducibility (s_R) of 0.32 $\log_{10}$ CFU/g.
- For the feedingstuffs:
    - a standard deviation for repeatability (s_r) of 0.07 $\log_{10}$ CFU/g and
    - a standard deviation for reproducibility (s_R) of 0.35 $\log_{10}$ CFU/g.
- and a limit of detection (LOD) of $1x10^5$ CFU/kg in *feedingstuffs*.

However, since this specific application does not include target levels of *Bacillus subtilis GR-101* in *feedingstuffs*, the CRL cannot evaluate the suitability of this CEN standard for official control.

For the enumeration of *Aspergillus oryzae GB-107* the applicant proposes internationally recognised US FDA/CFSAN BAM spread plate method for enumeration of yeasts, moulds and mycotoxins. The applicant uses potato dextrose agar (PDA) for the enumeration of viable *Aspergillus oryzae*. The sample is suspended and diluted in peptone water and the appropriate dilutions are spread on PDA agar plates. The agar plates are incubated at 25°C for 4 - 5 days before counting [10]. The applicant did not provide any validation and verification data, considering FDA method as an 'official' method. However another internationally recognised method for the enumeration of viable yeasts and moulds in products intended for human consumption or feeding of animals exists (ISO 21527-1). This method is a spread plate method using dichloran – rose Bengal chloramphenicol agar (DRBC). The sample is suspended and diluted in peptone water and the appropriate dilutions are spread on DRBC agar plates. The agar plates are incubated at 25°C for 2 - 5 days before counting [11]. The ISO method was not ring trial validated and provided no method performance characteristics, except for an LOD of $1x10^5$ CFU/kg in *feedingstuffs*. Since no target levels of *Aspergillus*

oryzae GB-107 are given and no validation or verification data are available, the CRL is not able to evaluate the suitability of the FDA or ISO method for official control use when determining *Aspergillus oryzae GB-107* in *premixtures* and *feedingstuffs*.

4. CONCLUSIONS AND RECOMMENDATIONS

No methods for enumeration of *Bacillus subtilis GR-101* and *Aspergillus oryzae GB-107* in *premixtures* and *feedingstuffs* can be recommended by the CRL.

For the analysis of the identity of the bacterial strain *Bacillus subtilis GR-101* the CRL recommends Pulsed Field Gel Electrophoresis (PFGE), for which the CEN technical Committee 327 is currently developing a European Standard for this methodology.

For the analysis of the identity of *Aspergillus oryzae GB-107* the CRL recommends Polymerase Chain Reaction (PCR) typing, a generally recognised standard methodology for identification of yeasts and moulds.

Further testing or validation is not considered necessary.

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

- Identification:

  Bacillus subtilis GR-101: Pulsed Field Gel Electrophoresis (PFGE),

  Aspergillus oryzae GB-107: Polymerase Chain Reaction (PCR) typing

5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of PepSoyGen-C 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.1831/006-2009

[2] *Technical dossier, section II/ 2.2.1.2. Micro-organisms

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

[4] *Annex 2.1.3.b Scientific Data Requirements-B_subtilis

[5] *Annex 2.1.3.a Scientific Data Requirements-A_oryzae

[6] European Community Project SMT4-CT98-2235.'Methods for the Official Control of Probiotics Used as Feed Additives, Volume 1. 2002. Report 20873-1. Office for official Publications of the European Communities. ISBN 92-894-6250-7 (Vol. I)

[7] *Technical dossier, section II/ 2.1.4 Purity

[8] *Technical dossier, section II/2.6.1.2 Enumeration method of viable *Bacillus subtilis*

[9] EN 15789 'Animal feeding stuffs – Isolation and enumeration of *Bacillus* spp'

[10] *Annex 2.6.1. Yeast, moulds and mycotoxins. US FDA/CFSAN BAM

[11] ISO 21527-1 Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of yeasts and moulds - Part 1: Colony count technique in products with water activity greater than 0,95

*Refers to Dossier no: FAD-2009-0007

7. RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was Community Reference Laboratory for Feed Additives, IRMM, Geel, 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.

8. ACKNOWLEDGEMENTS

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

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- Laboratoire de Rennes, SCL L35, Service Commun des Laboratoires, Rennes (FR)

- Instytut Zootechniki w Krakowie, Krajowe Laboratorium Pasz, Lublin (PL)