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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**

Cibenza[®] EP150
(FAD-2013-0017; CRL/120039)



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Dossier related to: **FAD-2013-0017 - CRL/120039**

Name of Product: ***Cibenza[®] EP150***

Active Substances: **Protease EC 3.4.21.19
Bacillus licheniformis PWD-1 (ATCC 53757)**

Rapporteur Laboratory: **European Union Reference Laboratory for
Feed Additives (EURL-FA)
Geel, Belgium**

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Date: **12/02/2015**

Report approved by: **Christoph von Holst**
Date: **19/02/2015**

EXECUTIVE SUMMARY

In the current application authorisation is sought under article 4(1) for *Cibenza*® EP150 under the category/functional group 1(d) "zootechnical additives"/"other zootechnical additives", according to the classification system of Annex I of Regulation (EC) No 1831/2003. The authorisation is sought for the use of the *feed additive* for chickens for fattening, chickens reared for laying, and minor avian species (ducks, geese, pigeons and other game birds) for meat production. *Cibenza*® EP150 is a dry preparation containing two active substances: a *protease* (EC 3.4.21.19) and viable spores of non-genetically modified *Bacillus licheniformis* (strain PWD-1, ATCC 53757). The Applicant stated the following minimum activity/concentration in the product: 6×10^5 U/g for *protease* and 1×10^9 Colony Forming Units (CFU)/g for *Bacillus licheniformis*. The Applicant defined the *protease* activity units (U) as the amount of protease that liberates 1 micromole of para-nitroaniline (pNA) from the Succinyl-Ala-Ala-Pro-Phe-pNA (C₃₀H₃₆N₆O₉) substrate per minute at pH 8.0 and 37°C. *Cibenza*® EP150 is intended to be used in *premixtures* and *feedingstuffs*, with the minimum activity/concentration in *feedingstuffs* of: 3×10^5 U/kg for *protease* and 5×10^8 CFU/kg for *Bacillus licheniformis*, respectively.

For the identification and characterisation of the strain *Bacillus licheniformis* ATCC 53757 the Applicant studied biochemical parameters and applied DNA sequencing, together with Pulsed Field Gel Electrophoresis (PFGE). The EURL recommends for official control PFGE, the generally recognised standard methodology for microbial identification that is currently being evaluated by the CEN Technical Committee 327 to become European Standards.

For the enumeration of spores of *Bacillus licheniformis* ATCC 53757 in *feed additive*, *premixtures* and *feedingstuffs*, the Applicant submitted the ring-trial validated CEN spread plate method (EN 15784). However, this method is not applicable to *mineral feeds* composed mainly of minerals and containing at least 40 % crude ash. During the review process one of the National Reference Laboratories identified the ring-trial validated VDLUFA method 28.2.2 for the enumeration in mineral feed. Based on the performance characteristics available, the EURL recommends for official control the EN 15784 and VDLUFA methods.

For the quantification of the *protease* activity in the *feed additive*, *premixtures* and *feedingstuffs* the Applicant submitted a single-laboratory validated and further verified colorimetric methods based on the quantification of para-nitroaniline (pNA) released by the action of *protease* on the synthetic peptide substrate (Succinyl-Ala-Ala-Pro-Phe-pNA). Based on the satisfactory experimental evidence provided the EURL recommends for official control the colorimetric methods submitted.

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

Cibenza[®] EP150, *Protease*, *Bacillus licheniformis*, zootechnical additives, performance enhancer, chickens for fattening, chickens reared for laying, minor avian species (ducks, geese, pigeons and other game birds) for meat production

1. BACKGROUND

In the current application authorisation is sought under article 4(1) (new additive) for *Cibenza*[®] EP150 under the category/functional group 1(d) "zootechnical additives"/"other zootechnical additives", according to the classification system of Annex I of Regulation (EC) No 1831/2003 [1]. The authorisation is sought for the use of the *feed additive* for chickens for fattening, chickens reared for laying, and minor avian species (ducks, geese, pigeons and other game birds) for meat production [2].

Cibenza[®] EP150 is a dry preparation containing two active substances: a *protease* (EC 3.4.21.19) and viable spores of the producer strain, a non-genetically modified *Bacillus licheniformis* (strain PWD-1, ATCC 53757) [3]. The Applicant stated the following minimum activity/concentration in the product: 6×10^5 U/g for *protease* and 1×10^9 Colony Forming Units (CFU)/g for *Bacillus licheniformis* [3].

The Applicant defines the *protease* activity units (U) as the amount of protease that liberates 1 micromole of para-nitroaniline (pNA) from the Succinyl-Ala-Ala-Pro-Phe-pNA (C₃₀H₃₆N₆O₉) substrate per minute at pH 8.0 and 37°C [3].

Cibenza[®] EP150 is intended to be used in *premixtures* and *feedingstuffs*, with the minimum activity/concentration in *feedingstuffs* of: 3×10^5 U/kg for *protease* and 5×10^8 CFU/kg for *Bacillus licheniformis*, respectively [2].

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 *Cibenza® EP150*, 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 impurities in the feed additive

When required by EU legislation, analytical methods for official control of undesirable substances in the additive such as heavy metals (arsenic, cadmium, lead and mercury), dioxins, microbiological agents and mycotoxins are available from the respective European Union Reference Laboratories [4].

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

For the identification and the characterisation of the strain *Bacillus licheniformis* ATCC 53757 the Applicant studied biochemical parameters and applied DNA sequencing, together with Pulsed Field Gel Electrophoresis (PFGE) [5]. Although the first procedure is suitable for the purpose, the EURL recommends for official control PFGE, the generally recognised standard methodology for microbial identification that is currently being evaluated by the CEN Technical Committee 327 to become European Standards [6].

For the enumeration of spores of *Bacillus licheniformis* ATCC 53757 in *feed additive, premixtures* and *feedingstuffs*, the Applicant submitted the ring-trial validated CEN spread plate method (EN 15784) [3]. The performance characteristics of the EN 15784 method reported after logarithmic transformation (CFU) are [7]:

- a standard deviation for repeatability (s_r) of $0.09 \log_{10}$ CFU/g
- a standard deviation for reproducibility (s_R) of $0.32 \log_{10}$ CFU/g.

However, this method is not applicable to *mineral feeds* composed mainly of minerals and containing at least 40 % crude ash. During the review process one of the National Reference Laboratories identified the ring-trial validated VDLUFA method 28.2.2 [17] for which a relative standard deviation for *repeatability* (RSD_r) of 9.4 % and a relative standard deviation for *reproducibility* (RSD_R) of 26.9 % were obtained in *mineral feed* samples containing 3.2×10^{10} CFU *Bacillus licheniformis* /kg.

Based on these performance characteristics available, the EURL recommends for official control the EN 15784 method for the enumeration of *Bacillus licheniformis* ATCC 53757 in the *feed additive, premixtures* and *feedingstuffs* (excluding mineral feed); and the VDLUFA method for the enumeration of of *Bacillus licheniformis* ATCC 53757 in *mineral feed*.

For the quantification of the *protease* activity in the *feed additive*, *premixtures* and *feedingstuffs* the Applicant submitted a single-laboratory validated and further verified colorimetric methods based on the quantification of para-nitroaniline (pNA) released by the action of *protease* on the synthetic peptide substrate (Succinyl-Ala-Ala-Pro-Phe-pNA) [8,9,10]. The test sample of *feed additive* or *premixtures* is extracted in reaction buffer (pH 8.0) and an aliquot of the solution is incubated with the synthetic peptide substrate solution at 37°C for 10 min. The reaction is stopped by adding 10% v/v acetic acid. The pNA content is then determined by colorimetry at 410 nm and quantified against the pNA standard curve. As for *feedingstuffs* samples they are first extracted with feed extraction buffer (pH 10.0), and an aliquot of the extract is then added to the reaction buffer (pH 10.0) and incubated with the synthetic peptide substrate solution at 37°C for 1 hour. The reaction is stopped by adding 10% v/v acetic acid. The quantification is performed using a calibration curve obtained with known amounts of *Cibenza® EP150 (feed additive)* diluted in feed.

The performance characteristics obtained in the validation [8-10] and verification [11,12] studies and summarised in EURL Verification forms [13-16] are presented in Table 1. Furthermore, the Applicant determined the following limits of quantification (LOQ) [10]: 6×10^4 and 1.3×10^5 U/kg *feedingstuffs* for mash and pellet, respectively.

Based on the satisfactory experimental evidence available the EURL recommends for official control the colorimetric methods submitted by the Applicant for the quantification of *protease* in *Cibenza® EP150* in *feed additive*, *premixtures* and *feedingstuffs*.

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.

Table 1: Performance characteristics of analytical methods for the determination of *protease* in *feed additive* (FA), *premixtures* (PM) and *feedingstuffs* (FS).

	Activity U/g	RSD _r (%)		RSD _{ip} (%)		R _{rec} (%)	
		Validation	Verification	Validation	Verification	Validation	Verification
FA	689275	1.3-2.0 [8,13]	1.0-2.7 [11,13]	1.9 [8,13]	2.3 [11,13]	94.8-97.0 [8,13]	99.3-101.9 [11,13]
PM	65070	10.3 [9,14]	7.4 [12,14]	14.3 [9,14]	12.2 [12,14]	103 [9,14]	73 [12,14]
FS mash	300	1.5-5.3 [10,15]	1.3-4.7 [11,15]	6.8 [10,15]	3.4 [11,15]	110.5-125.8 [10,15]	111.1-112.6 [11,15]
FS pellet	300	1.9-4.2 [10,16]	4.1-5.5 [11,16]	3.1 [10,16]	6.7 [11,16]	93.9-96.4 [10,16]	86.4-95.1 [11,16]

RSD_r and RSD_{ip}: relative standard deviation for *repeatability* and *intermediate precision*; R_{rec}: recovery rate

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 the *Bacillus licheniformis* ATCC 53757;
- the ring-trial validated CEN spread plate method (EN 15784) for the enumeration of the *Bacillus licheniformis* ATCC 53757 in the *feed additive, premixture* and *feedingstuffs* (excluding mineral feed); or the VDLUFA method 28.2.2 for the enumeration in mineral feed; and
- the single laboratory validated and further verified colorimetric methods submitted by the Applicant for the quantification of *protease* the *feed additive, premixtures* and *feedingstuffs*.

Recommended text for the register entry (analytical method)

Identification and enumeration of *Bacillus licheniformis* ATCC 53757 in the *feed additive, premixtures* and *feedingstuffs*

- Identification: Pulsed Field Gel Electrophoresis (PFGE)
- Enumeration: Spread plate method using tryptone soya agar - EN 15784

Quantification of *protease* in the *feed additive, premixtures* and *feedingstuffs*:

- Colorimetric method measuring para-nitroaniline (pNA) released by the enzymatic reaction of *protease* on Suc-Ala-Ala-Pro-Phe-pNA substrate at 37 °C

One *protease* activity units (U) is the amount of protease that liberates 1 micromole of para-nitroaniline (pNA) from the Succinyl-Ala-Ala-Pro-Phe-pNA (C₃₀H₃₆N₆O₉) substrate per minute at pH 8.0 and 37°C.

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference sample of Cibenza® EP150 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] *Reference SANCO/D/2 Forw. Appl. 1831/0001-2013
 - [2] *Application, Proposal for Register Entry
 - [3] *Technical dossier, Section II: Identity, characterisation and conditions of use of the additive; methods of analysis
 - [4] 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
 - [5] *Technical dossier, Section II – Annex_II_2_1_2_2 "Studies on the Bacillus licheniformis PWD-1 Strain"
 - [6] 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)
 - [7] EN 15784: "Animal feeding stuffs – Isolation and enumeration of presumptive Bacillus spp."
 - [8] *Technical dossier, Supplementary information, Section II – Annex 6.1.1.2
 - [9] *Technical dossier, Section II – Annex 6.1.2
 - [10] *Technical dossier, Supplementary information – Annex 6.1.3 updated
 - [11] *Technical dossier, Supplementary information – Annex 6.1.4.2
 - [12] *Technical dossier, Section II – Annex 6.1.4
 - [13] *Technical dossier, Supplementary information – Annex 6.1.5.2
 - [14] *Technical dossier, Section II – Annex 6.1.6
 - [15] *Technical dossier, Supplementary information – Annex 6.1.7 updated
 - [16] *Technical dossier, Supplementary information – Annex 6.1.8 updated
 - [17] VDLUFA method – 28.2.2 Determination of Baccillus licheniformis and Baccillus subtilis (Method book III, 8 Suppl. 2012, VDLUFA , Darmstadt
- * Refers to Dossier no: FAD-2013-0017

7. RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was the European Union 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, as last amended by Regulation (EC) No 885/2009.

8. ACKNOWLEDGEMENTS

The following National Reference Laboratories contributed to this report:

- Plantedirektoratet, Laboratorium for Foder og Gødning, Lyngby Fødevarestyrelsen, Ringsted¹ DK
- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Państwowy Instytut Weterynaryjny, Puławy (PL)
- Centre wallon de Recherches agronomiques (CRA-W), Gembloux (BE)
- Thüringer Landesanstalt für Landwirtschaft (TLL), Abteilung Untersuchungswesen, Jena (DE)
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- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)

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