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European Union Reference Laboratory for 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

Bergazym P100
(FAD-2014-0029; CRL/140018)

European Union Reference Laboratory
Feed Additives

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Dossier related to:	**FAD-2014-0029 - CRL/140018**
Name of Feed Additive:	***Bergazym P100***
Active Agent (s):	**Endo 1,4-β-xylanase**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **Geel, Belgium**
Report prepared by:	**María José González de la Huebra**
Report checked by: Date:	**Piotr Robouch (EURL-FA)** **23/09/2015**
Report approved by: Date:	**Christoph von Holst** **24/09/2015**

EXECUTIVE SUMMARY

In the current application authorisation is sought for *Bergazym P100* under article 4(1) under the category/functional 4(a) "zootechnical additives"/"digestibility enhancers" according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought for the use of the *feed additive* for chickens for fattening, weaned pigs and pigs for fattening

According to the Applicant, *endo-1,4-β-xylanase* produced by *Trichoderma reesei* (MUCL 49755) is the active substance of *Bergazym P100*. The Applicant expresses the *xylanase* enzymatic activity in endopentosanase units (EPU), defined as "the amount of enzyme which releases 0.0083 µmol of reducing sugars (xylose equivalent) per minute from oat spelt xylan at pH 4.7 and 50 °C".

The *feed additive* is intended to be included through *premixtures* or directly in *feedingstuffs* to obtain a minimum activity of 1500 EPU/kg in *feedingstuffs* for all the target species.

For the quantification of *xylanase* activity in the *feed additive*, *premixtures* and *feedingstuffs* the Applicant submitted a single-laboratory validated and further verified method based on the quantification of water soluble dyed fragments produced by the action of *xylanase* on a commercially available azurine cross-linked arabinoxylan substrate. External calibration is conducted using a reference standard with a known enzyme activity expressed in EPU. Based on the satisfactory performance characteristics the EURL recommends for official control the proposed single-laboratory validated and further verified colorimetric method for the quantification of the *xylanase* activity in the *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.

KEYWORDS

Endo-1,4-β-xylanase, *Bergazym P100*, "zootechnical additives"/"digestibility enhancers", chickens fattening, weaned pigs and pigs for fattening

1. BACKGROUND

In the current application authorisation is sought for *Bergazym P100* under article 4(1) (new feed additive) under the category/functional 4(a) "zootechnical additives"/"digestibility enhancers" according to the classification system of Annex I of Regulation (EC) No 1831/2003 [1][2]. Specifically, authorisation is sought for the use of the *feed additive* for chickens for fattening, weaned pigs and pigs for fattening [1][2].

According to the Applicant, *endo-1,4-β-xylanase* produced by *Trichoderma reesei* (MUCL 49755) is the active substance of *Bergazym P100* [2][3]. The Applicant expresses the *xylanase* enzymatic activity in endopentosanase units (EPU), defined as "the amount of enzyme which releases 0.0083 µmol of reducing sugars (xylose equivalent) per minute from oat spelt xylan at pH 4.7 and 50 °C" [4][5].

The product is intended to be marketed as a solid formulation with wheat meal and starch as carriers and having a guaranteed minimum *xylanase* activity of 15000 EPU/g [2][3].

The *feed additive* is intended to be included through *premixtures* or directly in *feedingstuffs* to obtain a minimum activity of 1500 EPU/kg of *feedingstuffs* for all the target species[2][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 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 *Bergazym P100* 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 additive

When required by EU legislation, analytical methods for official control of undesirable substances in the additive (e.g. arsenic, cadmium, lead, mercury, mycotoxins and dioxins) are available from the respective European Union Reference Laboratories [6].

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

For the quantification of *xylanase* activity in the *feed additive* [4], *premixtures* [4] and *feedingstuffs* [5] the Applicant submitted a single-laboratory validated [7] and further verified [8] method based on colorimetry.

The proposed method is based on the quantification of the water soluble dyed fragments produced by the action of *xylanase* on a commercially available azurine cross-linked arabinoxylan substrate (Xylazyme tablets) [4] [5].

For the *feed additive* and *premixtures* one 1 g aliquot is extracted with 100 ml of 0.1M acetate buffer (pH 4.7) and stirred for 20 minutes. Then the obtained supernatant is appropriately diluted with acetate buffer. A Xylazyme tablet is then added to an aliquot (0.5 ml) of the supernatant and then incubated at 50^{o}C during 30 minutes.

For the *feedingstuffs* two aliquots of 10 g are extracted with 100 ml of 0.1M acetate buffer (pH 4.7) and stirred for 30 minutes. The obtained solutions are further centrifuged during 15 minutes. A Xylazyme tablet is then added to an aliquot (0.5 ml) of the supernatants and then incubated at 50^{o}C during 150 minutes.

For the *feed additive*. *premixtures* and *feedingstuffs* the reaction is stopped, after the incubation time, by adding 2 ml of a stop solution (TRIS solution 3%). Samples are vigorously mixed, let cool down to room temperature for 30 min and mixed again. Finally the solutions are filtered through a paper filter and the absorbance measured against a blank at 590 nm. External calibration is performed with standards prepared using a reference standard with a known enzyme activity expressed in EPU [4]. The calibrants are submitted in parallel to the same analytical procedure than the respective sample's supernatants (including the appropriate incubation times i.e. 30 min for the *feed additive* and *premixtures* or 150 min for *feedingstuffs*). Table 1 presents the performance characteristics recalculated by the EURL [9][10] based on experimental data obtained by the Applicant in the frame of the validation [7] and verification [8] studies. Additionally the Applicant reported a limit of quantification (LOQ) in *feedingstuffs* of 697 EPU/kg.

Contrary to the *feed additive* and *feedingstuffs* no experimental data and/or performance characteristics for *premixtures* were provided by the Applicant. However as *premixtures* are included within the scope of the method provided for the *feed additive* the EURL considers this method and/or performance characteristics also suitable for *premixtures.*

Table 1. Performance characteristics of analytical method for the quantification of *xylanase* activity in the *feed additive* (FA), and *feedingstuffs* (FS), as recalculated by the EURL [9][10] from the validation (Val) and verification (Ver) data.

Matrices	Mean concentration EPU/g	RSDr (%)		RSDip (%)		R_{Rec} (%)	
		Val [9]	Ver [10]	Val [9]	Ver [10]	Val [7]	Ver [8]
FA	13869-15000	4.4	4.7	4.4	5.8	100	106
FS	1.836 -1.770	10	7.9	10	8.7*	100	96

RSD_r:and RSD_{ip}: relative standard deviation for *repeatability* and *intermediate precision*; R_{Rec}: *recovery* rate (%)
*Calculated after removal of an outlier

Based on the performance characteristics available, the EURL recommends for official control the single-laboratory validated and further verified colorimetric method (with different incubation times for the *feed additive* and *premixtures* than for *feedingstuffs*) for the quantification of *xylanase* activity in the *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.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation the EURL recommends for official control the single-laboratory validated and further verified colorimetric method for the quantification of *xylanase* activity in the *feed additive*, *premixtures* and *feedingstuffs.*

Recommended text for the register entry (analytical method)

For the quantification of *xylanase* activity in the *feed additive*, *premixtures* and *feedingstuffs*,

- colorimetric method measuring water soluble dye released by action of *endo-1,4-β-xylanase* from azurine cross-linked wheat arabinoxylan substrates.

One endopentosanase unit (EPU) corresponds to the amount of enzyme which liberates 0.0083 µmol of reducing sugars (xylose equivalents) from oat spelt xylan per minute at pH 4.7 and 50°C.

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Bergazym P100* 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 SANCO/G1: Forw. Appl. 1831/0046-2014

[2] *Application, Proposal for Register Entry – Annex A

[3] *Technical dossier, Section II, Identity, characterisation and conditions of use of the additive; methods of analysis

[4] *Technical dossier, Section II: Annexes 2.6.1.a

[5] *Technical dossier, Section II: Annexes 2.6.1.b

[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 to Community Reference Laboratories

[7] *Technical dossier, Section II: Annexes 2.6.1.c & 2.6.1.d

[8] *Technical dossier, Section II: Annexes 2.6.1.e & 2.6.1.f

[9] *Technical dossier, Supplementary Information, EURL -ANOVA- calculation-BergazymP100- validation.pdf

[10] *Technical dossier, Supplementary Information, EURL-ANOVA-calculation-BergazymP100-verification.pdf

*Refers to Dossier no: FAD-2014-0029

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation was 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

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- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Fødevarestyrelsen, Laboratorierne, Ringsted og Aarhus[1] (DK)
- Univerza v Ljubljani, Veterinarska fakulteta. Nacionalni veterinarski inštitut, Enota za patologijo prehrane in higieno okolja, Ljubljana (SI)
- Laboratorio Arbitral Agroalimentario, Ministerio de Agricultura, Alimentación y Medio Ambiente, Madrid[2] (ES)

[1] Name and address according to Regulation (EC) No 885/2009: Plantedirektoratet, Laboratorium for Foder og Gødning, Lyngby

– Państwowy Instytut Weterynaryjny, Puławy (PL)

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

[2] Name and address according to Regulation (EC) No 885/2009: Laboratorio Arbitral Agroalimentario, Ministerio de Agricultura, Pesca y Alimentación, Madrid