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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**

Beltherm[®]

(FAD-2016-0010; CRL/150041)



**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**

Dossier related to: **FAD-2016-0010 - CRL/150041**

Name of Product: ***Beltherm*[®]**

Active Agent (s): **Endo 1,4-beta-xylanase**

Rapporteur Laboratory: **European Union Reference Laboratory for
Feed Additives (EURL-FA)
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Date: **11/08/2016**

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Date: **16/08//2016**

EXECUTIVE SUMMARY

In the current application authorisation is sought for *Beltherm*[®] *MP & ML* under article 4(1) (new feed additive) under the category/functional 4(a) "zootechnical additives"/"digestibility enhancers". Specifically, authorisation is sought for the use of the *feed additive* for chickens for fattening and reared for laying, turkeys for fattening, for breeding and reared for breeding and minor poultry species. According to the Applicant, *endo 1,4-beta-xylanase* is the active substance of *Beltherm*[®] produced by a genetically modified strain of the bacteria *Bacillus subtilis*. The Applicant expresses the *xylanase* enzymatic activity in ADXU units defined as "the amount of enzyme which liberates one micromole of reducing sugars (xylose equivalent) per minute from beechwood xylan at pH 6.0 and 70 °C". The product is intended to be marketed as a granulated and liquid formulations (MP and ML) having a guaranteed minimum *xylanase* activity of 500 ADXU/g. The carrier in the solid formulation is wheat flour, while glycerol in acetate buffer is used for the liquid formulation. The *feed additive* is intended to be included through *premixtures* or directly in *feedingstuffs* to obtain a minimum activity of 100 ADXU/kg of *feedingstuffs* for all the target species.

For the quantification of the *xylanase* activity in the *feed additive* the Applicant proposed a single-laboratory validated and further verified colorimetric method based on the measurement of reducing sugars (xylose equivalents) released by the action of *xylanase* on 3% beechwood xylan substrate in the presence of 3,5-dinitrosalicylic acid (DNS) at pH 6.0 and 70°C (DNS method).

For the quantification of the *xylanase* activity in *premixtures* and *feedingstuffs*, the Applicant proposed another single-laboratory validated and further verified colorimetric method, based on the quantification of water soluble dyed fragments produced by the action of *xylanase* on commercially available azurine cross-linked arabinoxylan substrates.

Based on the satisfactory performance characteristics, the EURL recommends for official control the proposed single-laboratory validated and further verified colorimetric methods for the quantification of the *xylanase* activity in the *feed additive* (DNS method) *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-beta-xylanase, *Beltherm*[®], "zootechnical additives"/"digestibility enhancers", chickens for fattening and reared for laying, turkeys for fattening, for breeding and reared for breeding and minor poultry species

1. BACKGROUND

In the current application authorisation is sought for *Beltherm*[®] *MP* & *ML* 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 and reared for laying, turkeys for fattening, for breeding and reared for breeding and minor poultry species [1][2].

According to the Applicant, *endo 1,4-beta-xylanase* is the active substance of *Beltherm*[®] produced by a genetically modified strain of the bacteria *Bacillus subtilis* [2][3][4]. The Applicant expresses the *xylanase* enzymatic activity in ADXU units defined as "the amount of enzyme which liberates one micromole of reducing sugars (xylose equivalent) per minute from beechwood xylan at pH 6.0 and 70 °C" [4].

The product is intended to be marketed as a granulated or liquid formulations (*MP* or *ML*) having a guaranteed minimum *xylanase* activity of 500 ADXU/g and containing wheat flour (*Beltherm*[®] *MP*) or glycerol in acetate buffer (*Beltherm*[®] *ML*) as carriers [4][5].

The *feed additive* is intended to be included through *premixtures* or directly in *feedingstuffs* to obtain a minimum activity of 100 ADXU/kg of *feedingstuffs* for all the target species [2][6].

Note: The EURL already evaluated the analytical methods for the quantification of *endo 1,4-beta-xylanase* in the frame of previous dossiers [7].

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761, 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 *Beltherm*[®] 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, and mycotoxins) are available from the respective European Union Reference Laboratories [8].

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* [9], *premixtures* and *feedingstuffs* [10] the Applicant submitted two single-laboratory validated [11] and further verified [12] methods based on colorimetry.

For the quantification of *xylanase* in the *feed additive*, the Applicant proposes a method based on the measurement of reducing sugars (xylose equivalents) released by the action of *xylanase* on 3% beechwood xylan substrate in the presence of 3,5-dinitrosalicylic acid (DNS) [9].

The *feed additive* sample (1 g) is extracted in 100 ml of 0.02 M citrate buffer (pH = 6.0). In parallel, a reference sample consisting of a reference enzyme standard with a certified enzymatic activity, is submitted to the same analytical procedure as the *feed additive*. After appropriate dilution, 0.7 ml of substrate solution containing 3% beechwood xylan is added to 0.1ml of the diluted extract and the samples are incubated at 70°C for 15 minutes. The reaction is then stopped by adding 1.0 ml DNS solution. All the samples are then kept for 15 minutes at 95°C followed by another 5 minutes at 25°C. The absorbance is finally measured at 570 nm and quantified against a xylose standard curve [9].

Table 1. Performance characteristics of analytical method for the quantification of *xylanase* activity in the *feed additive* (FA), *premixtures* (PM) and *feedingstuffs* (FS), as recalculated by the EURL [13][14] from the validation (Val) and verification (Ver) data.

Matrices	Mean concentration ADXU /g	RSD _r (%)		RSD _{ip} (%)		R _{Rec} (%)	
		Val [11]	Ver [12]	Val [11]	Ver [12]	Val [11]	Ver [12]
FA	550-1500	0.4-4.1*	2.3-3.8*	1.1-4.9*	3.0-5.7*	92-104	104-111
PM	20	3.9*	-	3.9*	-	99	-
FS	0.2-0.4	2.7-5.2*	6.2-7.3*	4.8-8.5*	6.2-8.6*	88-96	91-96

RSD_r and RSD_{ip}: relative standard deviation for *repeatability* and *intermediate precision*; R_{Rec}: *recovery rate* (%)

*Recalculated by EURL from [11][12]

For the quantification of the *xylanase* activity in *premixtures* and *feedingstuffs* the Applicant submitted another single-laboratory validated and further verified colorimetric method, based on the quantification of water soluble dyed fragments produced by the action of *xylanase* on commercially available azurine cross-linked arabinoxylan substrates (Xylazyme AX tablets) [10].

The *feedingstuffs* samples (100 g) are extracted in 1000 ml of 25 mM phosphate buffer (pH 6.0) for 60 minutes and one aliquot of 50 ml is further centrifuged during 5 minutes. A Xylazyme tablet is then added to an aliquot (0.5 ml) of the supernatant and then incubated at 90 °C for 30 minutes. The reaction is stopped by adding 5 ml of a stop solution (Trizma base 2% at pH 9). Samples are then vigorously mixed on a vortex mixer, let to stabilize for 5 min and mixed again. Finally the solution is filtered through a paper filter and the absorbance measured at 595 nm. The quantification is performed using a feed calibration curve obtained with a reference enzyme [10].

Table 1 presents the performance characteristics provided by the Applicant and recalculated by the EURL, based on the experimental data presented in the frame of the validation [11] and verification [12] studies. Additionally the Applicant reported a limit of quantification (LOQ) in *feedingstuffs* of 24 ADXU/kg.

Based on the performance characteristics available, the EURL recommends for official control the two single-laboratory validated and further verified colorimetric methods 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.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation the EURL recommends for official control the two single-laboratory validated and further verified colorimetric methods 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*,

- colorimetric method measuring reducing sugars released by action of *xylanase* on beechwood xylan substrate in the presence of 3,5-dinitrosalicylic acid (DNS)

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

- colorimetric method measuring water soluble dye released by action of *xylanase* from azurine cross-linked arabinoxylan substrates

One *xylanase* unit (ADXU) corresponds to the amount of enzyme which liberates one micromole of reducing sugars (xylose equivalents) from beechwood xylan per minute at pH 6.0 and 70°C.

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Beltherm*[®] 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. 1831/0010-2016
 - [2] *Application, Proposal for Register Entry – Annex A
 - [3] *Technical dossier, Section II: Annex II.01
 - [4] *Technical dossier, Section II, 2.1.3 Qualitative and quantitative composition
 - [5] *Technical dossier, Section II, 2.1.4 Purity
 - [6] *Technical dossier, Section II, 2.1.5 Conditions of use of the additive
 - [7] EURL Evaluation Reports
<https://ec.europa.eu/jrc/sites/default/files/finrep-fad-2013-0047-ronozymewx.pdf>
<https://ec.europa.eu/jrc/sites/default/files/finirep-fad-2014-0029-bergazymp100.pdf>
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<https://ec.europa.eu/jrc/sites/default/files/finrep-fad-2010-0285-belfeed.pdf>
 - [8] 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
 - [9] *Technical dossier, Section II: Annex II.04, Method Xylanase activity in *Beltherm* & External Laboratory report Additives
 - [10] *Technical dossier, Section II: Annex II.04, Method Xylanase activity in feedingstuff & External Laboratory report Feedstuff
 - [11] *Technical dossier, Section II: Annex II.04, In-house validation report Additive, In-house validation report Premix & In-house validation report feedingstuff
 - [12] *Technical dossier, Section II: Annex II.04, EURL Verification form *Beltherm* & EURL Verification form *Beltherm* in feedingstuff
 - [13] *Supplementary information, EURL_beltherm_val.pdf
 - [14] *Supplementary information, EURL_beltherm_ver.pdf
- *Refers to Dossier no: FAD-2016-0010

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 (EU) 2015/1761.

8. ACKNOWLEDGEMENTS

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- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Fødevarestyrelsens Laboratorie Ringsted (DK)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
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