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Feed Additives

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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

Preparation of endo-1,4-beta-xylanase (EC 3.2.1.8)

(FAD-2021-0068; CRL/120027
(FAD-2021-0080; CRL/120028)

European Union Reference Laboratory
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Dossier related to:	**FAD-2021-0068 - CRL/210027** **FAD-2021-0080 – CRL/210028**
Name of Product:	***Preparation of endo-1,4-beta-xylanase***
Active Agent (s):	**Endo-1,4-beta-xylanase (EC 3.2.1.8)**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
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Report checked by: Date:	**Stefano Bellorini** **25/05/2023**
Report approved by: Date:	**Christoph von Holst** **30/05/2023**

EXECUTIVE SUMMARY

In the current applications, authorisations are sought under Article 4 for a *preparation of endo-1,4-beta-xylanase (EC 3.2.1.8)* for piglets, other minor growing porcine species, all avian species, ornamental, exotic and game birds, under the category/functional group 4 (a) "zootechnical additives"/"digestibility enhancers" according to Annex I of Regulation (EC) No 1831/2003.

According to the Applicant, the active substance of the *feed additive* is *endo-1,4-beta-xylanase*, produced by fermentation of the genetically modified yeast *Komagaetella phaffii* CGMCC 7.371. The activity of *endo-1,4-beta-xylanase* is expressed in *xylanase* units (U), where one unit is the amount of enzyme required to release one micromole of reducing sugar equivalents from arabinoxylan per minute at 37 °C and pH 6.5.

The *feed additive* is intended to be marketed as powder (VTR-xylanase powder) and liquid (VTR-xylanase liquid) formulations, having a guaranteed minimum *endo-1,4-beta-xylanase* activities of 100000 and 30000 U / g respectively and it is intended to be incorporated directly into complete *compound feed* or through *premixtures* to obtain a minimum *xylanase* activity of 1000 or 2000 U / kg *compound feed* depending on the target species.

For the determination of *endo-1,4-beta-xylanase* (*xylanase*) in the *feed additive*, *premixtures* and *compound feed* the Applicant submitted a single-laboratory validated and further verified colorimetric method, based on the quantification of water soluble dyed fragments produced by the action of *xylanase* on a commercially available (Xylazyme AX®, Megazyme) azurine cross-linked wheat arabinoxylan substrate.

Based on the experimental evidences and on the acceptable performance characteristics provided by the Applicant, the EURL recommends for official control the colorimetric (Megazyme) method for the determination of *1,4-beta-xylanase* in the *feed additive*, *premixtures* and *compound feed*.

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, as last amended by Regulation (EU) 2015/1761) is not considered necessary.

KEYWORDS

Preparation of endo-1,4 beta-xylanase (EC 3.2.1.8)*; Komagaetella phaffii* CGMC 7.371*;* "zootechnical additives"/"digestibility enhancers"*;* piglets, other minor growing porcine species all avian species, ornamental, exotic and game birds.

1. BACKGROUND

In the current applications, authorisations are sought under Article 4(1) (authorisation of a new feed additive) for a *preparation of endo-1,4-beta-xylanase (EC 3.2.1.8)* for piglets, (weaned and sucking piglets), other minor growing porcine species [1, 2], all avian species, ornamental, exotic and game birds [3,4], under the category/functional group 4 (a) "zootechnical additives"/"digestibility enhancers" according to Annex I of Regulation (EC) No 1831/2003.

According to the Applicant, the active substance of the *feed additive* is *endo-1,4-beta-xylanase*, produced by fermentation of the genetically modified yeast *Komagaetella phaffii* CGMCC 7.371 [5].

The *feed additive* is intended to be marketed as powder (VTR-xylanase powder) and liquid (VTR-xylanase liquid) formulations, having a guaranteed minimum *endo-1,4-beta-xylanase* activities of 100000 and 30000 U / g respectively [5]. The activity of *endo-1,4-beta-xylanase* is expressed in *xylanase* units (U), where, according to the Applicant, one unit is the amount of enzyme required to release one micromole of reducing sugar equivalents from arabinoxylan per minute at 37 °C and pH 6.5 [6].

The *feed additive* is intended to be incorporated directly into complete *compound feed* or through *premixtures* to obtain a minimum *xylanase* activity of 2000 U / kg for laying hens and the target porcine species [6-7] and 1000 U / kg *compound feed* for chickens for fattening and other avian species [7].

Note: The EURL has evaluated analytical methods for the determination of other *endo-1,4-beta-xylanase* (EC 3.2.1.8) additives in the frame of previous dossiers [8].

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 *preparation of endo-1,4-beta-xylanase (EC 3.2.1.8)* and their suitability to be used for official controls in the frame of the authorisation were evaluated.

3. EVALUATION

Description of the analytical methods for the determination of the active substance in the feed additive, premixtures, feedingstuffs and when appropriate water (section 2.6.1 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

For the determination of *endo-1,4-beta-xylanase* (*xylanase*) in the *feed additive*, *premixtures* and *compound feed* the Applicant submitted a colorimetric method, based on the quantification of water soluble dyed fragments produced by the action of *xylanase* on a commercially available (Xylazyme AX®, Megazyme) azurine cross-linked wheat arabinoxylan substrate [9].

A sample of the *feed additive* or *compound feed* is suspended in phosphate buffer (pH 6.5) shaking it gently at room temperature for 15 min. The obtained extract is then centrifuged and appropriately diluted. An aliquot of the obtained supernatant is then placed into a test tube. One Xylazyme AX® tablet is added to the tube and incubated at 37 ^{o}C. The reaction is then stopped by adding TRIS buffer into the tube, mixing vigorously and letting rest at room temperature. Then the solution is shaken again and filtered through a syringe membrane filter. The content of *xylanase* is finally determined by colorimetry at 590 nm using a calibration curve prepared with a reference *xylanase* enzyme in the buffer solution (for the *feed additive*) or in a blank feed extract (for the *compound feed*). The *xylanase* enzyme of known activity is available from the Applicant upon request [10].

The Applicant, upon request of the EURL single-laboratory validated and further verified the proposed method for the *feed additive* formulation [11, 12] and for *compound feed* [13, 14]. A summary of the relevant performance characteristics as provided by the Applicant is shown in Table 1.

Furthermore, the Applicant reported a limit of quantification (LOQ) of 0.57 U / g *compound feed.*

For the determination of *endo-1,4-beta-xylanase (xylanase)* in *premixtures* the Applicant proposed a solid dilution of the *premixtures* with blank feed followed by the analysis of the diluted samples according to the method for *compound feed* described above [10]. Furthermore, the Applicant, in the frame of the stability studies [15], provided experimental evidences demonstrating the suitability of this approach.

Based on the performance characteristics available, the EURL recommends for official control the described above single-laboratory validated and further verified colorimetric methods for the quantification of *xylanase* activity in the *feed additive*, *premixtures* and *compound feed*.

Table 1: Performance characteristics provided by the Applicant in the frame of the validation and verification studies for the determination of *xylanase* activity in the *feed additive* (FA) [11-12] and *compound feed* (FS) [13-14].

Matrix	Activity (U/g)		RSD_r (%)		RSD_{ip} (%)		R_{Rec} (%)	
	Applicant	*Lab 2*	*Applicant*	*Lab 2*	*Applicant*	*Lab 2*	*Applicant*	*Lab 2*
FA	99544	107043	3.8	2.0	11.7	2.7	109	118
FS	2.2	2.1	8.0	5.5	8.4	6.4	111.7	105

RSD_r; RSD_{ip}: relative standard deviations for *repeatability* and for *intermediate precision*, respectively; R_{Rec}: *Recovery rate*

Methods of analysis for the determination of the residues of the additive in food (section 2.6.2 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

An evaluation of corresponding methods of analysis is not relevant for the present application.

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. as last amended by Regulation (EU) 2015/1761) is not considered necessary.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation the EURL recommends for official control the colorimetric (Megazyme) method for the determination of *1,4-beta-xylanase* in the *feed additive*, *premixtures* and *compound feed*.

Recommended text for the register entry (analytical method)

For the determination of *endo-1,4-beta-xylanase* in the *feed additive*, *premixtures* and *compound feed*:

- colorimetric method based the enzymatic reaction of *endo-1,4-beta-xylanase* on the azurine cross-linked wheat arabinoxylan substrate

One xylanase unit (U) is the amount of enzyme required to release one micromole of reducing sugar equivalents from arabinoxylan per minute at 37 °C and pH 6.5.

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *preparation of endo-1,4-beta-xylanase* 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 SANTE/E5: FORW. APPL. 1831-0052-2021

[2] [*]Annex I – Submission number 1612973700172-2822

[3] [+]Application, Reference SANTE/E5: FORW. APPL. 1831-0051-2021

[4] [*+]Annex I – Submission number 1612973712159-2824

[5] [*+]Technical dossier, Section II: 2.1.3 Qualitative and quantitative composition

[6] [*]Technical dossier, Section II: 2.1.2 Proposal for classification

[7] [+]Technical dossier, Section II: 2.1.2 Proposal for clasification

[8] EURL evaluation Reports:
https://joint-research-centre.ec.europa.eu/document/download/fce81a56-5c41-496b-8ad1-32e7bc2cecf0_en?filename=finrep_fad-2021-0033_b_xylamax.pdf
https://joint-research-centre.ec.europa.eu/document/download/3c717632-0196-4775-923d-8f622914eb60_en?filename=finrep_fad-2020-0110_xygest-ht.pdf
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https://joint-research-centre.ec.europa.eu/document/download/7e3b15c4-2f83-47f2-944d-5881a5c9a84c_en?filename=ammendment_finrep_fad-2016-0079_natugrain.pdf

[9] [*+]Technical dossier, Section II: 2.6.1. Methods of analysis for the active substance

[10] Suppl. information « Annex_1_SOP_VTR_Xylanase_March23.pdf»

[11] Suppl. information Annex_2_Biotask_Validation_VTR_Xylanase_March23. pdf»

[12] Suppl information « Annex_3_ECMA_Verification_VTR_Xylanase_March23 .pdf»

[13] Suppl. information « Annex_3_a_Validation_feed_report.pdf»

[14] Suppl. information « Annex_5_Verification_Feed.pdf»»

[15] [*+]Technical dossier, Section II: Annex_II_4_1_2_stability_feeds.pdf

[*]Refers to Dossier no: FAD-2021-0080

[+] Refers to Dossier no: FAD-2021-0068

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation is the European Union Reference Laboratory for Feed Additives, JRC, 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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- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia, Pesca, Alimentació i Medi Natural. Generalitat de Catalunya, Cabrils (ES)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Instytut Zootechniki - Państwowy Instytut Badawczy, Krajowe Laboratorium Pasz, Lublin (PL)
- Laboratoire de Rennes (SCL L35), Service Commun des Laboratoires DGCCRF et DGDDI, Rennes (FR)