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European Union Reference Laboratory for Feed Additives

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EURL Evaluation Report on the Analytical Methods submitted in connection with the Application for the Authorisation of Feed Additives according to Regulation (EC) No 1831/2003

Dossier related to:	**FAD-2008-0004**
	CRL/090022
Name of Feed Additive:	**Feedlyve AXC**
Active Substance(s):	**Endo-1,4-β-xylanase (E.C. 3.2.1.8)**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (CRL-FA)** **Geel, Belgium**
Report prepared by:	**Dijana Mitić (EURL-FA)**
Report revised by: Date:	**Piotr Robouch (EURL-FA)** **16/02/2012**
Report approved by: Date:	**Christoph von Holst** **16/02/2012**

EXECUTIVE SUMMARY

In the current application authorisation is sought under article 4(1) for *Feedlyve AXC,* under the category/functional group 4(a) "zootechnical additives" / "digestibility enhancers", according to the classification system of Annex I of Regulation (EC) No 1831/2003. According to the Applicant, the *feed additive* contains *endo-1,4-β-xylanase* (EC 3.2.1.8) as the active agent, produced by the strain *Trichoderma koningii* (MUCL 39203). The additive is intended to be marketed as several formulations:

- liquid *Feedlyve AXC 200L* and *Feedlyve AXC 1500L,* with a guaranteed minimum *endo-1,4-β-xylanase* activity of 200 and 1500 AXC/mL, respectively; and
- solid/powder *Feedlyve AXC 400P, Feedlyve AXC 1500P, Feedlyve AXC 6000P* and *Feedlyve AXC 12000P*, with a guaranteed minimum *endo-1,4-β-xylanase* activity of 400, 1500, 6000 and 12000 AXC/g, respectively.

The activity of *endo-1,4-β-xylanase* is expressed in xylanase units (AXC). According to the Applicant, one AXC unit is the quantity of enzyme which liberates 17.2 μmoles of reducing sugars (maltose equivalents) per minute from oat xylan at pH 4.7 and 30 ^{o}C.

Specifically, authorisation is sought for the use of *Feedlyve AXC* for turkeys for fattening. The *feed additive* is intended to be used in *premixtures* and/or complete *feedingstuffs,* with a minimum *endo-1,4-β-xylanase* activity in complete *feedingstuffs* of 75 AXC/kg.

For the quantification of *endo-1,4-β-xylanase* in the *feed additive* and *feedingstuffs,* the Applicant submitted similar single-laboratory validated and further verified colorimetric methods, based on the enzymatic hydrolysis of oat spelt xylan at pH 4.7 and 30 °C and the quantification of dyed oligomers released from the substrate Remazol-Brilliant-Blue-R xylan. The following performance characteristics were reported: - a relative standard deviation for *repeatability* (RSD_r) ranging from 1.3 to 14 %; - a relative standard deviation for *intermediate precision* (RSD_{ip}) ranging from 5.2 to 14 %; – a recovery rate (R_{Rec}) ranging from 91 to 115 % and a limit of quantification (LOQ) of 35 AXC/kg *feedingstuffs*.

No experimental data were submitted by the Applicant for the determination of *endo-1,4-β-xylanase* in *premixtures*. However, premixture samples could be diluted with blank feedingstuffs material (such as heat-treated wheat flour) and analysed as *feedingstuffs* using the method mentioned above.

Based on the performance characteristics presented, the EURL recommends for official control the single-laboratory validated and further verified colorimetric methods based on the enzymatic hydrolysis of oat spelt xylan at pH 4.7 and 30 °C and the quantification of dyed

oligomers released from the substrate Remazol-Brilliant-Blue-R xylan to determine the *endo-1,4-β-xylanase* in *feed additive*, *premixtures* and *feedingstuffs*, within the concentration range covered by the experimental data.

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

Feedlyve AXC, *endo-1,4-β-xylanase*, E1641, *Trichoderma koningii*, zootechnical additive, digestibility enhancers, turkeys for fattening.

1. BACKGROUND

In the current application authorisation is sought under article 4(1) (new use) for *Feedlyve AXC,* under the category/functional group 4(a) "zootechnical additives"/"digestibility enhancers" [1], according to the classification system of Annex I of Regulation (EC) No 1831/2003. The *feed additive* is already authorised under the Commission Regulations (EC) No 828/2007 for chickens for fattening. According to the Applicant, the *feed additive* contains *endo-1,4-β-xylanase* (EC 3.2.1.8) as the active agent [1,2], produced by the strain *Trichoderma koningii*, formerly known as *longibrachiatum,* (MUCL 39203) [13]. The strain was deposited at the "Mycothéque de l'Université Catholique de Louvain" (MUCL), part of Belgian Collection of Microorganisms (BCCM) [2,3]. The additive is intended to be marketed as different formulations [2]:

- liquid *Feedlyve AXC 200L* and *Feedlyve AXC 1500L,* with a guaranteed minimum *endo-1,4-β-xylanase* activity of 200 and 1500 AXC/mL, respectively; and
- solid/powder *Feedlyve AXC 400P, Feedlyve AXC 1500P, Feedlyve AXC 6000P* and *Feedlyve AXC 12000P*, with a guaranteed minimum *endo-1,4-β-xylanase* activity of 400, 1500, 6000 and 12000 AXC/g, respectively.

Water, sorbitol, potassium sorbate and monopotassium phosphate are used as carriers of the liquid formulations, while wheat flour and tricalcium phosphate are used for the solid formulations.

The activity of *endo-1,4-β-xylanase* is expressed in xylanase units (AXC). According to the Applicant, one AXC unit is the quantity of enzyme which liberates 17.2 µmoles of reducing sugars (maltose equivalents) per minute from oat xylan at pH 4.7 and 30 ^{o}C [4].

Specifically, authorisation is sought for the use of *Feedlyve AXC* for turkeys for fattening. The *feed additive* is intended to be used in *premixtures* and/or complete *feedingstuffs,* with a minimum *endo-1,4-β-xylanase* activity of 75 AXC/kg in complete *feedingstuffs* [1,4].

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 *Feedlyve AXC*, and their suitability to be used for official controls in the frame of the authorisation, were evaluated.

3. EVALUATION

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 [5].

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

For the quantification of *endo-1,4-β-xylanase* in the *feed additive,* the Applicant submitted a colorimetric method based on the enzymatic hydrolysis of oat spelt xylan at pH 4.7 and 30 °C and the quantification of dyed oligomers released from the substrate Remazol-Brilliant-Blue-R xylan [6]. The enzyme samples are first diluted in 0.4 M acetate buffer (pH 4.7). The substrate (0.5 mL) is placed in tubes and equilibrated in water bath at 30 °C for 5 minutes. Then 0.2 mL of the diluted enzyme samples is added. The solution is incubated at 30 °C for 20 minutes. The reaction is stopped by adding 2 mL of 95.5% ethanol (warmed to 30 °C). Solution is well mixed and returned to water bath for additional 10 minutes to allow

precipitation. Afterwards the solution is mixed again and the tubes are centrifuged at 3000 rpm for 10 minutes at 20 °C. The absorbances of the sample solutions are measured spectrophotometrically at 590 nm, using water as the blank. The quantification is performed using an *endo-1,4-β-xylanase* standard curve, based on reference enzyme provided by the Applicant.

The Applicant provided together with the validation report [7] additional experimental data obtained by a second expert laboratory [8, 9] to prove the "transferability" of his analytical method; this set of data is considered as a "verification" study. Table 1 summarises the performance characteristics reported in the frame of the validation and verification studies.

For the determination of the *endo-1,4-β-xylanase* activity in *feedingstuffs*, the Applicant submitted a colorimetric method [10], similar to the one described above. *Feedingstuffs* samples (100 g) are suspended in 500 mL of acetate buffer (pH 4.7), stirred for 30 minutes and centrifuged at 3000 rpm for 10 minutes. The supernatant is filtered and pH is checked and corrected if necessary. The appropriate volume of substrate solution is placed in tubes and equilibrated in water bath at 30 °C for 5 minutes. Then appropriate volume of the diluted enzyme samples is added. The solution is incubated at 30 °C for 200 minutes (100 minutes for samples with 200-800 AXC/kg). The reaction is stopped by adding adequate volume of precipitant solution. Tubes are well mixed and returned to water bath for additional 10 minutes to allow precipitation. Afterwards the tubes are centrifuged at 3000 rpm for 10 minutes at 20 °C. The absorbances of the sample solutions are measured spectrophotometrically at 590 nm, using precipitant solution as the blank. The quantification is performed using a calibration curve obtained with enzyme standard diluted in feed.

The Applicant provided a validation [11] and a "transferability" (verification) [8, 9] reports. All the performance characteristics are summarised in Table 1. Furthermore, the lowest activity analysed was set by the Applicant as the limit of quantification to derive a LOQ of 35 AXC/kg *feedingstuffs* [12].

No experimental data were submitted by the Applicant for the determination of *endo-1,4-β-xylanase* in *premixtures*. However, premixture samples could be diluted with blank feedingstuffs material (or heat-treated wheat flour) to be analysed as a *feedingstuffs* sample.

Based on the performance characteristics presented, the EURL recommends for official control the single-laboratory validated and further verified colorimetric methods based on the enzymatic hydrolysis of oat spelt xylan at pH 4.7 and 30 °C and the quantification of dyed oligomers released from the substrate Remazol-Brilliant-Blue-R xylan, to determine the *endo-1,4-β-xylanase* in *feed additive*, *premixtures* and *feedingstuffs*, within the concentration range covered by the experimental data.

Table 1: Performance characteristics for the quantification of *endo-1,4-β-xylanase* in the *feed additive (FA)* and *feedingstuffs (FS)*

	Concentration Range (AXC/kg)	RSD_r (%)		RSD_{ip} (%)		R_{Rec} (%)	
		Validation	Verification	Validation	Verification	Validation	Verification
FA	194000-211000	6-7 [7]	12-13 [8]	9 [7]	12 [8]	104* [7]	96* [8]
FS	100-400	1.3-14 [11]	2.6-12 [8]	5.2-12 [11]	8.4-14[8]	91-97* [11]	115* [8]

RSD_r and RSD_{ip}: relative standard deviation for *repeatability* and *intermediate precision*, respectively.

R_{Rec}: a recovery rate

*- recalculated by EURL

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, submitted by the Applicant, based on the enzymatic hydrolysis of oat spelt xylan at pH 4.7 and 30 °C and the quantification of dyed oligomers released from the substrate Remazol-Brilliant-Blue-R xylan. to determine the *endo-1,4-β-xylanase* in *feed additive*, *premixtures* and *feedingstuffs*, within the concentration range covered by the experimental data.

Recommended text for the register entry (analytical method)

For the quantification of *endo-1,4-β-xylanase* in the *feed additive, premixtures* and *feedingstuffs:*

- colorimetric method based on the quantification of dyed oligomers produced by the action of *endo-1,4-β-xylanase* on Remazol-Brilliant-Blue-R xylan at pH 4.7 and 30 ^{o}C.

1 AXC unit is the quantity of enzyme which liberates 17.2 µmoles of reducing sugars (maltose equivalents) per minute from oat xylan at pH 4.7 and 30 ^{o}C.

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Feedlyve AXC* 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/D/2 Forw. Appl. 1831/007-2008

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

[3] *Technical dossier, Section II, Annex_II_2_2_B

[4] *Supplementary Information, E-mail from Lyven 22_12_2011

[5] 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

[6] *Supplementary Information, FEEDLYVE AXC - FAD-2008-0004 – Annexes: see Annex II.5.1.A bis

[7] *Technical dossier, Section II, Annex II_5_1_G

[8] *Technical dossier, Section II, Annex II_5_1_H

[9] *Supplementary Information, FEEDLYVE AXC - FAD-2008-0004 – Annexes: see Annex II_5_1_I

[10] *Supplementary Information, FEEDLYVE AXC - FAD-2008-0004 – Annexes: see Annex II_5_2_A bis

[11] *Technical dossier, Section II, Annex II_5_2_B

[12] *Supplementary Information, Answers to CRL questions

[13] *Supplementary Information, Annex_II_2_2G_Strain Deposit Certificate

*Refers to Dossier No. FAD-2008-0004

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

The following National Reference Laboratories contributed to this report:

- Plantedirektoratet, Laboratorium for Foder og Gødning, Lyngby (DK)
- Schwerpunktlabor Futtermittel des Bayerischen Landesamtes für Gesundheit und Lebensmittelsicherheit (LGL), Oberschleißheim (DE)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)