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JOINT RESEARCH CENTRE
Institute for Reference Materials and Measurements
Community Reference Laboratory for Feed Additives

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CRL Evaluation Report on the Analytical Methods submitted in connection with the Application for Authorisation as a Feed Additive according to Regulation (EC) No 1831/2003

Dossier related to:	**FAD-2009-0062** **CRL/ 090040** **FAD-2010-0011** **CRL/ 100018**
Name of Additive:	**AveMix ® XG 10**
Active Substance (s):	**Endo-1,4-β-xylanase (E.C. 3.2.1.8)** **Endo-1,3(4)-β-glucanase (EC 3.2.1.6)**
Rapporteur Laboratory:	**Community Reference Laboratory for Feed Additives (CRL-FA),** **Geel, Belgium**
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EXECUTIVE SUMMARY

In the current application authorisation is sought for the feed additive *AveMix®XG 10* under the category zootechnical additives, functional group '4a': digestibility enhancers, according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought for the use of *AveMix®XG 10* for weaned piglets (FAD-2009-0062) and chickens for fattening (FAD-2010-0011). *AveMix®XG 10* contains two active agents: *endo-1,4-β-xylanase* (EC 3.2.1.8) and *endo-1,3(4)-β-glucanase* (EC 3.2.1.6), produced by the *Trichoderma longibrachiatum* strains MUCL 49755 and MUCL 49754, respectively. The feed additive is intended to be marketed as solid (*AveMix®XG 10*) and liquid (*AveMix®XG 10 L*) formulations, with soybean meal and sorbitol + water as carrier materials, respectively.

The enzymatic activities are expressed in *xylanase* unit (XU) and *betaglucanase* unit (BGU). According to the Applicant:

- one XU unit is the amount of enzyme which releases 1 µmol of reducing sugar (xylose equivalent) per minute from xylan of oat spelt at pH = 4.8 and 50°C;
- one BGU unit is the amount of enzyme which releases 1 µmol of reducing sugar (cellobiose equivalent) per minute from betaglucan of barley at pH = 5.0 and 50°C.

Both formulations have a guaranteed minimum activity of 40000 XU/g *endo-1,4-β-xylanase* and 9000 BGU/g *endo-1,3(4)-β-glucanase*. The minimum target activities in complete *feedingstuffs* are: - 4000 XU/kg *endo-1,4-β-xylanase* and 900 BGU/kg *endo-1,3(4)-β-glucanase* for weaned piglets; - 2000 XU/kg *endo-1,4-β-xylanase* and 450 BGU/kg *endo-1,3(4)-β-glucanase* for chickens for fattening.

For the determination of the activity of *endo-1,4-β-xylanase* in the *feed additives* and *premixtures* the Applicant proposes a single-laboratory validated and further verified colorimetric method based on the formation of reducing sugars reacting with 3,5-dinitrosalicilic acid (DNS) at pH = 4.8 and 50°C. For the determination of the activity of *endo-1,4-β-xylanase* in *feedingstuffs* the Applicant proposes a single-laboratory validated and further verified method based on the measurement of the rate of release of water soluble dyed fragments by *endo-1,4-β-xylanase* from the dye cross-linked wheat arabinoxylan at pH = 4.3 and 50°C. The activity of the sample is calibrated against reference enzyme standards with known activity determined at the definition conditions of the activity unit (pH 4.8 and 50°C). The method was single-laboratory validated and further verified and the following performance characteristics were reported for *feed additive* and *feedingstuffs*:

- a relative standard deviation for *repeatability* (RSD_r) ranging from 3.7 to 9.4 %;
- a relative standard deviation for *intermediate precision* (RSD_{int}) ranging from 3.9 to 9.3 %;

- a *recovery rate* (R_{Rec}) ranging from 101 to 107 %; and
- a limit of detection (LOD) and a limit of quantification (LOQ) of 939 and 1878 XU/kg *feedingstuffs*, respectively.

For the determination of the activity of *endo-1,3(4)-β-glucanase* in the *feed additives* and *premixtures* the Applicant proposes a single-laboratory validated and further verified colorimetric method based on the formation of reducing sugars reacting with DNS at pH = 5.0 and 50°C. For the determination of the activity of *endo-1,3(4)-β-glucanase* in the *feedingstuffs* the Applicant proposes a single-laboratory validated and further verified method based on the measurement of the rate of release of water soluble dyed fragments by *endo-1,3(4)-β-glucanase* from the dye cross-linked barley glucan at pH = 4.6 and 40°C. The activity of the sample is calibrated against reference enzyme standards with known activity determined at the definition conditions of the activity unit (pH 5.0 and 50°C). The method was single-laboratory validated and further verified and the following performance characteristics were reported for *feed additive* and *feedingstuffs*:

- RSD_r ranging from 1.6 to 8.2 %;
- RSD_{int} ranging from 3.8 to 7.9 %;
- R_{Rec} ranging from 103 to 109 %; and
- LOD = 90 BGU/kg and LOQ = 180 BGU/kg *feedingstuffs.*

No experimental data were submitted by the Applicant for the determination of *endo-1,4-β-xylanase* and *endo-1,3(4)-β-glucanase* in *premixtures*. Premixture samples could be diluted with blank feedingstuffs material, to be analysed using methods for *feedingstuffs* mentioned above. However, in the absence of experimental evidence the CRL could not evaluate nor recommend any method for the determination of *endo-1,4-β-xylanase* and *endo-1,3(4)-β-glucanase* in *premixtures*.

Based on the acceptable performance characteristics reported, the CRL recommends for official control the single-laboratory validated and further verified methods submitted by the Applicant to determine *endo-1,4-β-xylanase* and *endo-1,3(4)-β-glucanase* activities in *feed additives* 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

AveMix®XG 10, *endo-1,4-β-xylanase*, *endo-1,3(4)-β-glucanase, Trichoderma longibrachiatum, Trichoderma reesei*, digestibility enhancer, weaned piglets, chickens for fattening

1. BACKGROUND

In the current application authorisation is sought under articles 4(1) (new use) for FAD-2009-0062 and 13(3) (modification of existing authorisation) for FAD-2010-0011 for *AveMix®XG 10* under the category zootechnical additives, functional group '4a': digestibility enhancers, according to the classification system of Annex I of Regulation (EC) No 1831/2003 [1, 2]. The *feed additive* is already authorized under Comission Regulation (EC) No 1091/2009. Specifically, authorisation is sought for the use of *AveMix®XG 10* for weaned piglets (FAD-2009-0062) and chickens for fattening (FAD-2010-0011).

AveMix®XG 10 contains two active agents: *endo-1,4-β-xylanase* (EC 3.2.1.8) and *endo-1,3(4)-β-glucanase* (EC 3.2.1.6), produced by the *Trichoderma longibrachiatum* (formerly known as *Trichoderma reesei*) strains MUCL 49755 and MUCL 49754 [3, 4], respectively. Both strains are deposited at the Belgian Coordinated Collections of Microorganisms (BCCM) [5, 6].

The activity of *endo-1,4-β-xylanase* is expressed as *xylanase* unit (XU) and the activity of *endo-1,3(4)-β-glucanase* is expressed as *betaglucanase* unit (BGU). According to the Applicant [5, 6]:

- one XU unit is the amount of enzyme which releases 1 µmol of reducing sugar (xylose equivalent) per minute from xylan of oat spelt at pH = 4.8 and 50°C;

- one BGU unit is the amount of enzyme which releases 1 µmol of reducing sugar (cellobiose equivalent) per minute from betaglucan of barley at pH = 5.0 and 50°C.

The feed additive is intended to be marketed as solid (*AveMix®XG 10*) and liquid (*AveMix®XG 10 L*) formulations. The solid form is a brownish powder with soybean meal as a carrier while the liquid form is a brown liquid with sorbitol and water as carriers [5, 6]. Both formulations have a minimum activity of 40000 XU/g *endo-1,4-β-xylanase* and 9000 BGU/g *endo-1,3(4)-β-glucanase* [3, 4]. The minimum target activities in complete *feedingstuffs* are:

- 4000 XU/kg *endo-1,4-β-xylanase* and 900 BGU/kg *endo-1,3(4)-β-glucanase* for weaned piglets [3]; and

- 2000 XU/kg *endo-1,4-β-xylanase* and 450 BGU/kg *endo-1,3(4)-β-glucanase* for chickens for fattening [4].

1. 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 tasks of the Community Reference Laboratory concerning applications for authorisations of feed additives, the CRL is requested to submit a full evaluation report to the European Food Safety Authority (EFSA) for each application or group of applications. For this particular dossier, the methods of analysis submitted in connection with *AveMix®XG 10* and their suitability to be used for official controls in the frame of the authorisation, were evaluated.

2. EVALUATION

Identification/Characterization 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 and heavy metals - cadmium, mercury and lead) are available at the respective Community Reference Laboratories [7].

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

Endo-1,4-β-xylanase

For the determination of the activity of *endo-1,4-β-xylanase* in the *feed additive* and *premixtures* the Applicant proposes a colorimetric method based on the formation of reducing sugar reacted with 3,5-dinitrosalicilic acid (DNS), where the colour change is proportional to xylose equivalents measured at 550 nm [8, 9]. The sample solution is prepared by extracting 1 g of sample with 100 ml of 0.05 M citrate buffer (pH = 4.8) and diluting the sample (1/4000). A sample-tube containing the sample solution and the substrate solution (2% xylan) together with a sample-blank tube containing only the substrate solution (2% xylan) are incubated at 50^{o}C for 15 min. A blank-tube containing buffer and three standard-tubes containing the buffer and a xylose solution with increasing concentrations (0.5 g xylose in citrate buffer) are prepared without incubation. A DNS solution is added to all tubes, and homogenously mixed with a vortex. The sample solution is added to the sample-blank tube after mixing. Finally, a standard solution (300 mg glucose/L) is added to all tubes. The tubes are then boiled for 10 min, cooled and centrifuged at 4500 rpm for 10 min. The colour change produced is proportional to the amount of reduced sugar released and to the activity of *endo-1,4-β-*

xylanase present in the sample. The optical density is measured on a spectrophotometer at 550 nm and quantified against a reference enzyme standard available upon request from the Applicant. The method was single-laboratory validated [10] and further verified [11] and the reported performance characteristics for *feed additive* are presented in Table 1.

No experimental data were submitted by the Applicant for the determination of *endo-1,4-β-xylanase* in *premixtures*. Premixture samples could be diluted with blank feedingstuffs material, to be analysed using the method for *feedingstuffs* mentioned below. However, in the absence of experimental evidence the CRL could not evaluate nor recommend any method for the determination of *endo-1,4-β-xylanase* in *premixtures*.

For the determination of the activity of *endo-1,4-β-xylanase* in the *feedingstuffs* the Applicant proposes a single-laboratory validated and further verified method based on the Megazyme method [12, 13]. The method is based on measurement of the rate of release of water soluble dyed fragments by *endo-1,4-β-xylanase* from the dye cross-linked wheat arabinoxylan in a form of "Xylazyme AX tablet". The enzyme is extracted from the feed with 0.1 M acetic acid (pH = 4.3) at 22°C. Appropriate volume of a xylanase standard solution - with a *xylanase* activity of 62876 XU/ml - is added to the three samples tubes containing the feed extract. Sample tubes together with a blank tube containing only the buffer are then incubated at room temperature for 3 min. One Xylazyme AX tablet (cross linked wheat arabinoxylan) is added to each tube and further incubated at 50°C for 30 min. The reaction is stopped by the addition of a TRIZMA 2% solution, and the absorbance of the formed colour is measured at 590 nm. The activity of the sample is calibrated against reference enzyme standards with known activity determined at the definition conditions of the activity unit (pH 4.8 and 50°C). This standard addition method was single-laboratory validated [12] and further verified [14, 15] and the reported performance characteristics are presented in Table 1. Furthermore the Applicant reported a limit of detection (LOD) and a limit of quantification (LOQ) [14, 15]:

- LOD = 939 XU/kg and

- LOQ = 1878 XU/kg *feedingstuffs,* thus allowing the quantification of the minimum target activity in complete *feedingstuffs.*

Based on the acceptable performance characteristics reported, the CRL recommends for official control the single-laboratory validated and further verified methods submitted by the Applicant for the determination of *endo-1,4-β-xylanase* activity in the *feed additive* and *feedingstuffs*.

Table 1: Method performance characteristics for the determination of *endo-1,4-β-xylanase* in *feed additive* (FA) and *feedingstuffs* (FS).
The FS samples used contained (a) 2000 XU/kg and (b) 4000 XU/kg *feedingstuffs*

	RSD_r (%)		RSD_{int} (%)		R_{Rec} (%)	
	Validation	Verification	Validation	Verification	Validation	Verification
FA	3.7 [10]	5.3 [11]	5 [10]	5.1 [11]	104.5 [10]	101 [11]
FS (a)	3.8 [12]	9.4 [14]	3.9 [12]	9.3 [14]	103.5 [12]	104 [14]
FS (b)	3.8 [12]	6.9 [15]	3.9 [12]	6.7 [15]	103.5 [12]	107 [15]

RSD_r,RSD_{int} - relative standard deviation for *repeatability* and *intermediate precision*,
R_{Rec} – recovery rate

Endo-1,3(4)-β-*glucanase*

For the determination of the activity of *endo-1,3(4)-β-glucanase* in the *feed additive* and *premixtures* the Applicant proposes a colorimetric method based on the formation of reducing sugars reacted with DNS, where the colour change is proportional to cellobiose equivalent measured at 550 nm [9, 16]. The sample solution is prepared by diluting the sample (1/500) in 0.1 M acetic acid-sodium acetate buffer (pH = 5.0). A sample-tube containing the sample solution and the substrate solution (1% beta-glucan) together with a sample-blank tube containing only the substrate solution (1% beta-glucan) are incubated at 50°C for 10 min. A blank-tube containing the buffer and a standard-tube containing the buffer and a cellobiose solution (1 g cellobiose in 50 ml acetate buffer) are prepared without incubation. DNS solution is added to all tubes and homogenously mixed with vortex. The sample solution is added to the sample-blank tube after mixing. Finally, a standard solution (300 mg glucose/L) is added to all tubes. The tubes are then boiled for 10 min and cooled in cold water. The colour change produced is proportional to the amount of reduced sugar released and to the activity of *endo-1,3(4)-β-glucanase* present in the sample. The optical density is measured on a spectrophotometer at 550 nm and quantified against a reference enzyme standard available upon request from the Applicant. The method was single-laboratory validated [17] and further verified [18] and the reported performance characteristics for *feed additive* are presented in Table 2.

No experimental data were submitted by the Applicant for the determination of *endo-1,3(4)-β-glucanase* in *premixtures*. Premixture samples could be diluted with blank feedingstuffs material, to be analysed using the method for *feedingstuffs* mentioned below. However, in the

absence of experimental evidence the CRL could not evaluate nor recommend any method for the determination of *endo-1,3(4)-β-glucanase* in *premixtures*.

For the determination of the activity of *endo-1,3(4)-β-glucanase* in the *feedingstuffs* the Applicant proposes a single-laboratory validated and further verified method based on the Megazyme method [19, 20]. The method is based on the measurement of the rate of release of water soluble dyed fragments by *endo-1,3(4)-β-glucanase* from Azo barley glucan from Megazyme. Appropriate volume of *AveMix®XG 10* standard solution, stabilised in buffer and 0.1% bovine serum albumine (BSA), with a maximum *betaglucanase* activity of 10640 BGU/g is added to feed samples. The samples are extracted with 40 mM acetate buffer (pH = 4.6), filtered and diluted 3 times with the same buffer. The feed extracts and a blank sample containing buffer are then incubated with substrate (Azo barley glucan) at 40°C for 210 min. The reaction is stopped by adding a stopping reagent (industrial methylated spirits/methoxyethanol) and centrifuged. The formed colour is measured at 590 nm using a spectrophotometer. The activity of the sample is calibrated against reference enzyme standards with known activity determined at the definition conditions of the activity unit (pH 5.0 and 50°C).This standard addition method was single-laboratory validated [19] and further verified [21, 22] and the reported performance characteristics are presented in Table 2. Furthermore the Applicant reported an LOD and an LOQ [21, 22]:

- LOD = 90 BGU/kg and LOQ = 180 BGU/kg *feedingstuffs.*

Based on the acceptable performance characteristics reported, the CRL recommends for official control the single-laboratory validated and further verified methods submitted by the Applicant for the determination of *endo-1,3(4)-β-glucanase* activity in the *feed additive* and *feedingstuffs*.

Table 2: Method performance characteristics for the determination of *endo-1,3(4)-β-glucanase* in *feed additive* (FA) and *feedingstuffs* (FS).
The FS samples used contained (a) 450 BGU/kg and (b) 900 BGU/kg *feedingstuffs*

	RSD_r (%)		RSD_{int} (%)		R_{Rec} (%)	
	Validation	Verification	Validation	Verification	Validation	Verification
FA	1.6 [17]	5.9 [18]	3.8 [17]	5.7 [18]	107 [17]	103 [18]
FS (a)	5.7 [19]	8.2 [21]	5.4 [19]	7.9 [21]	109 [19]	104 [21]
FS (b)	5.7 [19]	7.4 [22]	5.4 [19]	7.1 [22]	109 [19]	107 [22]

RSD_r,RSD_{int} - relative standard deviation for *repeatability* and *intermediate precision*,
R_{Rec} – recovery rate

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 CRL recommends for official control the single-laboratory validated and further verified methods submitted by the Applicant for the determination of *endo-1,4-β-xylanase* and *endo-1,3(4)-β-glucanase* in *feed additive* and *feedingstuffs.*

Recommended text for the register entry, fourth column (Composition, chemical formula, description, analytical method)

For the determination of *endo-1,4-β-xylanase* and *endo-1,3(4)-β-glucanase* in the *feed additive*:

- Colorimetric methods based on the formation of reducing sugars reacted with dinitrosalicilic acid (DNS).

For the determination of *endo-1,4-β-xylanase* and *endo-1,3(4)-β-glucanase* in *feedingstuffs*:

- Colorimetric method measuring water soluble dye released by action of *endo-1,4-β-xylanase* from dye cross-linked wheat arabinoxylan substrate;

- Colorimetric method measuring water soluble dye released by action of *endo-1,3(4)-β-glucanase* from dye cross-linked barley betaglucan substrate.

5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *AveMix®XG 10* have been sent to the Community Reference Laboratory for Feed Additives Authorisation. The dossier has been made available to the CRL by EFSA.

6. REFERENCES

[1] *Application/Ref:SANCO/D/2:Forw.Appl.1831/049-2009
[2] +Application/Ref:SANCO/D/2:Forw.Appl.1831/0011-2010
[3] *Application, Proposal for Register Entry, Annex A
[4] +Application, Proposal for Register Entry, Annex A
[5] *Technical dossier, Section II,1. Identity of the additive
[6] +Technical dossier, Section II,1. Identity of the additive
[7] 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
[8] *+Annex II.6.1.1. Xylanase assay
[9] *+Annex II.6.1.9. Miller, G.L. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. Analytical Chemistry.Vol. 31, No. 3 (1959)
[10] *+Annex II.6.1.10. Validation procedure xylanase assay
[11] *+Supplementary information: Verification_xyl_additive
[12] *+Annex II.6.1.3. Xylanase activity in feedingstuffs
[13] *+Annex II.6.1.12. Megazyme: Measurement of xylanase in animal feeds using Xylazyme AX tablets
[14] *+Supplementary information: Verification_Xyl_feed 50 ppm
[15] *+Supplementary information: Verification_Xyl_feed 100 ppm
[16] *+Annex II.6.1.2. β-glucanase assay
[17] *+Annex II.6.1.11. Validation procedure β-glucanase assay
[18] *+Supplementary information: Verification_gluc_additive
[19] *+Annex II.6.1.4. β-glucanase activity in feedingstuffs
[20] *+Annex II.6.1.13. Megazyme: Malt Beta-Glucanase assay procedure (Azo-Barley Glucan Method)
[21] *+Supplementary information: Verification_gluc_feed 50 ppm
[22] *+Supplementary information: Verification_gluc_feed 100 ppm

*Refers to Dossier no: FAD-2009-0062
+Refers to Dossier no: FAD-2010-0011

7. RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was Community 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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