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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-2011-0030**
CRL/110012

Name of Feed Additive: **Danisco Xylanase 40000 G/L**

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)
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EXECUTIVE SUMMARY

In the current application authorisation is sought under article 4(1) and 13(3) for *Danisco Xylanase 40000 G/L*, 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 reesei* (ATCC PTA 5588). The additive is intended to be marketed as different formulations: a solid (*Danisco Xylanase G*) and a liquid (*Danisco Xylanase L*) formulation with a target *endo-1,4-β-xylanase* activity of 40000 U/g.

The activity of *endo-1,4-β-xylanase* is expressed in xylanase units (U). According to the Applicant, one U unit is defined as the amount of enzyme which liberates 0.5 μmol of reducing sugar (expressed as xylose equivalents) from a cross-linked oat spelt xylan substrate per minute at pH 5.3 and 50 °C.

Specifically, authorisation is sought for the use of *Danisco Xylanase 40000 G/L* for laying hens and all poultry minor species. The *feed additive* is intended to be used in compound feed rich in non-starch polysaccharides (mainly arabinoxylans and beta-glucans). It is intended to be used in *premixtures* and/or complete *feedingstuffs*, with *endo-1,4-β-xylanase* activity ranging from 625 to 2500 U/kg in complete *feedingstuffs*.

For the determination of *endo-1,4-β-xylanase* in the *feed additive*, *premixtures* and *feedingstuffs*, the Applicant submitted colorimetric method, based on the quantification of water soluble dyed fragments produced by the action of *endo-1,4-β-xylanase* on commercially available azurine cross-linked arabinoxylan at pH 4.2 and 50 °C. The enzymatic activity of the sample is calculated using a reference enzyme standard with certified enzyme activity, available from the Applicant upon request. The validation and verification studies were performed using samples containing xylanase activities of 15500 U/g, 15500 U/kg and 1800 U/kg. Furthermore, the Applicant applied the abovementioned method to investigate the stability of feed samples containing 400 U/kg. The following method performance characteristics were reported:

- for the *feed additive*: - a relative standard deviation for *repeatability* (RSD_r) ranging from 6.6 to 9.5 %; - a relative standard deviation for *intermediate precision* (RSD_{ip}) ranging from 7.2 to 11 % and – a *recovery rate* (R_{Rec}) ranging from 103 to 112 %;
- for *premixtures*: - RSD_r ranging from 2.3 to 8.0 %; - RSD_{ip} ranging from 6.6 to 6.9 %; and - R_{Rec} ranging from 93 to 95 %;

- for *feedingstuffs*: - RSD_r ranging from 2.3 to 5.8 %; - RSD_{ip} ranging from 4.0 to 6.9 %; - R_{Rec} ranging from 93 to 97 %; and limits of detection (LOD) and quantification (LOQ) of 40 and 133 U/kg, respectively.

Based on the performance characteristics presented, the EURL recommends for official control the single-laboratory validated and further verified colorimetric method, based on the quantification of water soluble dyed fragments produced by the action of *endo-1,4- β -xylanase* on azurine cross-linked wheat arabinoxylan at pH 4.2 and 50 °C, 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

Danisco Xylanase 40000 G/L, *endo-1,4- β -xylanase*, 4a11, *Trichoderma reesei*, zootechnical additive, digestibility enhancers, laying hens, all poultry minor species.

1. BACKGROUND

In the current application authorisation is sought under article 4(1) (new use) and 13(3) (modification of an existing authorisation)¹ for *Danisco Xylanase 40000 G/L*, 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 Regulation (EC) No 9/2010 for chickens for fattening, laying hens, turkeys and ducks for fattening, and under the Commission Regulation (EC) No 528/2011 for weaned piglets and pigs for fattening. Specifically, authorisation is sought for the use of *Danisco Xylanase 40000 G/L* for laying hens and all poultry minor species.

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 reesei* (ATCC PTA 5588). The strain was deposited at the "American Type Culture Collection" (ATCC) in Manassas, VA, USA [3].

¹ The company is requesting a lower dose for laying hens.

The additive is intended to be marketed as different formulations [3]: a solid (*Danisco Xylanase G*) and a liquid (*Danisco Xylanase L*) formulation with a target *endo-1,4-β-xylanase* activity of 40000 U/g. Water and sorbital are the main carriers of the liquid formulation, while wheat flour is used for the solid formulation.

The activity of *endo-1,4-β-xylanase* is expressed in xylanase units (U). According to the Applicant, one U unit is defined as the amount of enzyme which liberates 0.5 μmol of reducing sugar (expressed as xylose equivalents) from a cross-linked oat spelt xylan substrate per minute at pH 5.3 and 50 °C [2].

The *feed additive* is intended to be used in compound feed rich in non-starch polysaccharides (mainly arabinoxylans and beta-glucans). It is intended to be used in *premixtures* and/or complete *feedingstuffs*, with *endo-1,4-β-xylanase* activity ranging from 625 to 2500 U/kg in complete *feedingstuffs* [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 *Danisco Xylanase 40000 G/L*, 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 [4].

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

For the determination of the activity of *endo-1,4-β-xylanase* in the *feed additive, premixtures* and *feedingstuffs*, the Applicant submitted a single-laboratory validated [5-8] and further

verified [9-11] colorimetric method, based on the quantification of water soluble dyed fragments produced by the action of *endo-1,4-β-xylanase* on commercially available azurine cross-linked arabinoxylan substrates (Xylazyme tablets). The enzymatic activity of the sample is calculated using a reference enzyme standard with certified enzyme activity, available from the Applicant upon request.

For the determination of the activity of *endo-1,4-β-xylanase* in *feed additive* [5], the samples are prepared by extracting two portions of 1 g of solid additive (0.5 g for liquid) in 100 mL of 0.2M acetate buffer (pH 4.2), diluted and equilibrated at 50 °C for 5 minutes. After adding the Xylazyme tablet the samples are incubated at 50 °C for 10 minutes. The reaction is stopped by adding a stop solution (2% Tris). The rate of dye release is measured on a spectrophotometer at 590 nm and quantified against a reference enzyme standard, available from the Applicant upon request. The method performance characteristics derived from the validation [7] and verification [9] studies are presented in Table 1.

For the determination of the activity of *endo-1,4-β-xylanase* in *feedingstuffs* [6], the samples (5 g) are extracted in 50 mL of 0.2M acetate buffer (pH 4.2) after stirring on magnetic stirrer for 10 minutes. The extract is then filtered, acetate buffer is added and the samples are equilibrated at 50 °C for 2 minutes. After adding the Xylazyme tablet the samples are incubated at 50 °C for 60 minutes. The reaction is stopped by adding a stop solution (2% Tris). The rate of dye release is measured on a spectrophotometer at 590 nm. Calibration is performed on standards prepared from identical blank feed samples fortified with exact amounts of the reference enzyme, available from the Applicant upon request. The method performance characteristics derived from the validation [8] and verification [11] studies are presented in Table 1.

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

	Concentration (U/kg)	RSD _r (%)		RSD _{ip} (%)		R _{Rec} (%)	
		Validation	Verification	Validation	Verification	Validation	Verification
FA	≥15500000	9.5 [7]	6.6 [9]	11.1 [7]	7.2 [9]	103 [7]	112 [9]
PM	15500	2.3 [8]	8.0 [10]	6.9 [8]	6.6 [10]	93 - 95 [8]	94 [10]
FS	1800	2.3 [8]	5.8 [11]	6.9 [8]	4.0 [11]	93 - 95 [8]	97 [11]

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

R_{Rec}: a recovery rate

Additionally, the Applicant applied the abovementioned method to investigate the stability of feed samples containing 400 U/kg [12]. The Applicant reported limits of detection (LOD) and quantification (LOQ) of 40 and 133 U/kg *feedingstuffs*, respectively [8]. When identical blank feed samples are not available, standard addition technique should be used.

For the determination of the activity of *endo-1,4-β-xylanase* in *premixtures* [6], the samples are diluted with heat treated wheat flour and analysed as *feedingstuffs* samples. The method performance characteristics derived from the validation [8] and verification [10] studies are presented in Table 1.

The xylanase measurement results submitted in the Dossier are traceable to the xylanase unit (U), through the reference enzyme standard - provided by the Applicant - with a certified enzymatic activity determined under the standard conditions (pH 5.3). This reference enzyme standard used for calibration ensures the measurement traceability, even though the determination of the xylanase activity in the *feed additive*, *premixtures* and *feedingstuffs* was carried out at different pH of 4.2.

Based on the performance characteristics presented, the EURL recommends for official control the single-laboratory validated and further verified colorimetric method, based on the quantification of water soluble dyed fragments produced by the action of *endo-1,4-β-xylanase* on azurine cross-linked wheat arabinoxylan at pH 4.2 and 50 °C, to determine the activity of *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.

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 quantification of water soluble dyed fragments produced by the action of *endo-1,4-β-xylanase* on azurine cross-linked wheat arabinoxylan at pH 4.2 and 50 °C, to determine the activity of *endo-1,4-β-xylanase* in *feed additive*, *premixtures* and *feedingstuffs*.

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 water soluble dyed fragments produced by the action of *endo-1,4-β-xylanase* on azurine cross-linked wheat arabinoxylan at pH 4.2 and 50 °C.

One xylanase unit (U) is the amount of enzyme which liberates 0.5 μmol of reducing sugar (expressed as xylose equivalents) from a cross-linked oat spelt xylan substrate per minute at pH 5.3 and 50 °C.

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Danisco Xylanase 40000 G/L* 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. 1831/0107-2011
 - [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] 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
 - [5] *Technical dossier, Section II, Annex_II_B28_Y5 Xyl Prod MoA
 - [6] *Technical dossier, Section II, Annex_II_B29_Y5 Xyl Feed&Premix MoA
 - [7] *Technical dossier, Section II, Annex_II_B30_Y5 Xyl Prod MoA validation
 - [8] *Technical dossier, Section II, Annex_II_B31_Y5 Xyl Feed&Premix validation
 - [9] *Technical dossier, Section II, Annex_II_B32_Y5 Xyl Prod MoA verification
 - [10] *Technical dossier, Section II, Annex_II_B33_Y5 Xyl Premix verification
 - [11] *Technical dossier, Section II, Annex_II_B34_Y5 Xyl Feed verification
 - [12] *Technical dossier, Section II, Annex_II_B25_Danisco Xylanase low dose feed
- *Refers to Dossier No. FAD-2011-0030

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:

- Schwerpunktlabor Futtermittel des Bayerischen Landesamtes für Gesundheit und Lebensmittelsicherheit (LGL), Oberschleißheim (DE)
- Plantedirektoratet, Laboratorium for Foder og Gødning, Lyngby (DK)
- Państwowy Instytut Weterynaryjny, Puławy (PL)
- Skúšobné laboratórium – Oddelenie analýzy krmív, Ústredný kontrolný a skúšobný ústav poľnohospodársky, Bratislava (SK)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)