EUROPEAN COMMISSION

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

JRC.DDG.D.6/CvH/GS/mds/Ares (2010)312960

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

Dossier related to:	**FAD-2009-0038** **CRL/080026**
Product name:	**Danisco Xylanase**
Active Substance(s):	***Endo-1,4-β-xylanase*** **(EC 3.2.1.8)**
Rapporteur Laboratory:	**Community Reference Laboratory for Feed Additives (CRL-FA)** **Geel, Belgium**
Report prepared by:	**Sulhattin Yasar, Piotr Robouch (CRL-FA)**
Report checked by: Date:	**Giuseppe Simone (CRL-FA)** **07/06/2010**
Report approved by: Date:	**Christoph von Holst (CRL-FA)** **07/06/2010**

EXECUTIVE SUMMARY

In the current application authorisation is sought for *endo-1,4-β-xylanase* under the category 'zootechnical additives', group 4(a), according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought for the preparation of *endo-1,4-β-xylanase* to be marketed as a powder (*Danisco Xylanase G*) and as liquid formulation (*Danisco Xylanase L*). The product is intended to be used as a digestibility enhancer for weaned piglets and pigs for fattening.

The active agent of the preparation of *Danisco Xylanase* is an enzyme of *endo-1,4-β-xylanase* (EC 3.2.1.8) produced by a strain of *Trichoderma reesei* (ATCC PTA 5588). The enzymatic activity is expressed in "U" units. One U is defined as the amount of *endo-1,4-β-xylanase* enzyme that releases 0.5 μmol of reducing sugar (xylose equivalents) from a cross-linked oat spelt xylan substrate per minute at pH 5.3 and 50°C. Both liquid and powder products have a target activity of 40000 U/g. *Danisco Xylanase G* is intended to be mixed into *premixtures* and/or *feedingstuffs*, whereas *Danisco Xylanase L* is sprayed directly onto feed to obtain an enzyme activity level of 2000 to 4000 U/kg in *feedingstuffs*.

For the determination of the activity of *endo-1,4-β-xylanase* in the *feed additive*, *premixtures* and *feedingstuffs*, the applicant proposes a 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 wheat arabinoxylan substrates. Enzymatic activity of the sample is calculated using a reference enzyme standard. The applicant methods are single-laboratory validated methods which are verified by a second independent laboratory.

For the determination of the activity of *endo-1,4-β-xylanase* in the *feed additive*, the applicant proposes a method which measures the enzyme-catalysed formation of water soluble dyed fragments released from an azurine cross-linked wheat arabinoxylan substrate. The rate of release is measured on a spectrophotometer at 590 nm and quantified against a reference enzyme standard, available from the applicant upon request. The analysis is carried out at pH 4.2 and 50 °C. The following method performance characteristics were derived from the validation and verification studies: - a relative standard deviation for *repeatability* (RSD_r) ranging from 6.6 to 9.5%, - a relative standard deviation for *intermediate precision* (RSD_{int}) ranging from 7.2 to 11%, and - a *recovery rate* (R_{Rec}) ranging from 103 to 112%.

For the determination of the activity of *endo-1,4-β-xylanase* in *premixtures*, the applicant proposes a method based on the same principle as described below. The premixture samples were diluted with cereal *feedingstuffs* and treated as the samples of *feedingstuffs*. The

following method performance characteristics were derived from the validation and verification studies: - RSD_r ranging from 2.3 to 8.0%, - RSD_{int} ranging from 6.6 to 8.0% and - R_{Rec} ranging from 93 to 95%.

For the determination of the activity of *endo-1,4-β-xylanase* in ***feedingstuffs***, the applicant proposes a method, based on the same principle as described above, measuring enzymatic activity on an azurine cross-linked wheat arabinoxylan substrate at pH 4.2 and 50°C. Calibration is performed on standards prepared from identical blank feed samples fortified with exact amounts of the reference enzyme, available from the applicant. The following method performance characteristics were derived from the validation and verification studies: - RSD_r ranging from 2.3 to 5.8%, - RSD_{int} ranging from 4.0 to 6.9%, - R_{Rec} ranging from 97 to 100%, and - a limit of detection (LOD) and of quantification (LOQ) of 40 and 133 U/kg *feedingstuffs*, respectively. When identical blank feed samples are not available, standard addition technique should be used.

Based on the satisfactory performance characteristics mentioned above, the CRL recommends for official control the methods submitted by the Applicant for the determination of *endo-1,4-beta-xylanase (Danisco Xylanase)* in the *feed additive*, *premixtures* and *feedingstuffs*.

Further testing or validation is not considered necessary.

KEYWORDS

Endo-1,4 beta-xylanase, Zootechnical additive, digestibility enhancer, weaned piglet, pigs for fattening

1. BACKGROUND

Danisco Xylanase is a product for which authorisation as feed additive is sought under the category 'zootechnical additives', functional groups 'digestibility enhancers', according to Annex I of Regulation (EC) No 1831/2003 [1]. The product contains *endo-1,4-β-xylanase* as the active agent [2], produced by a strain *Trichoderma reesei* (ATCC PTA 5588), deposited at the American Type Culture Collection (ATCC) in Manassas, VA, USA [3]. The activity of *endo-1,4-β-xylanase* is expressed in "U" units. According to the applicant, one U is defined as the amount of enzyme releasing 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 [4]. The product is intended to be marketed in two forms: 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.

Danisco Xylanase is intended to be mixed into *premixtures* and/or complete *feedingstuffs* to obtain enzyme activity levels of 2000 to 4000 U/kg in complete *feedingstuffs,* for weaned piglets and pigs for fattening [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 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 for each application or for each group of applications. For this particular dossier, the methods of analysis, submitted in connection with *Danisco Xylanase*, and their suitability to be used for official controls in the frame of authorisation, were evaluated.

3. EVALUATION

Identification/Characterisation of the feed additive

Quantitative 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 [5]

Description of the analytical methods for the determination of the active agent in the 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 proposed colorimetric methods [6,7] based on the quantification of water soluble dyed fragments produced by the action of *endo-1,4 β-xylanase* on commercially available cross-linked xylan substrates. The enzymatic activity of the sample is calculated using a reference enzyme standard. These methods were validated [8,9] and were further verified by a second independent laboratory [10-12].

Two portions of 1.0 g of dry *feed additive* or two portions of 0.5 g of liquid *feed additive* are extracted in 100 ml of acetate buffer and incubated with azurine cross-linked wheat arabinoxylan. The analysis is carried out at pH 4.2 and 50^{o}C. The rate of dye release is

measured on a spectrophotometer at 590 nm and quantified against a reference enzyme standard [6], available from the applicant upon request. The following method performance characteristics were derived from the validation [8] and verification [10] studies: - a relative standard deviation for *repeatability* (RSD_r) ranging from 6.6 to 9.5%, - a relative standard deviation for *intermediate precision* (RSD_{int}) ranging from 7.2 to 11.1% and - a *recovery rate* (R_{Rec}) ranging from 103 to 112%.

When analysing *premixtures,* the samples of premixture were diluted with cereal feedingstuffs and treated as the samples of feedingstuffs by applying the corresponding feed method [7], as described below. The following method performance characteristics were derived from the validation [9] and verification [11] studies: - RSD_r ranging from 2.3 to 8.0%, - RSD_{int} ranging from 6.6 to 8.0% and - R_{Rec} ranging from 93 to 95%.

For the quantification of the activity of *endo-1,4-β-xylanase* in *feedingstuffs,* the applicant proposes a method, based on the same principle as described above, measuring enzymatic activity on azurine cross-linked wheat arabinoxylan at pH 4.2 and 50°C for 60 min [7]. Calibration is performed on standards prepared from identical blank feed samples fortified with exact amounts of the reference enzyme, available from the applicant. The following method performance characteristics were derived from the validation [9] and verification [12] studies: - RSD_r ranging from 2.3 to 5.8%, - RSD_{int} ranging from 4.0 to 6.9%, - R_{Rec} ranging from 97 to 100%, and - a limit of detection (LOD) and of quantification (LOQ) of 40 and 133 U/kg *feedingstuffs*, respectively. When identical blank feed samples are not available, standard addition technique should be used.

Based on the satisfactory performance characteristics mentioned above, the CRL recommends for official control the analytical methods submitted by the applicant for the determination of *endo-1,4-beta-xylanase* in the *feed additive*, *premixtures* and *feedingstuffs*

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of *Danisco Xylanase* authorisation, the CRL recommends for official control the methods submitted by the applicant to determine the activity of *endo-1,4-beta-xylanase* in the *feed additive*, *premixtures* and *feedingstuffs*. The reference enzyme standard is available from the applicant.

Further testing or validation of the methods to be performed through the consortium of National Reference Laboratories in accordance with article 10 of Commission Regulation (EC) No 378/2005 is not considered necessary.

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

Characterisation of the active substances in the *feed additive*, *premixtures* and *feedingstuffs*:

– Colorimetric method measuring water soluble dye released by action of *endo-1,4-β-xylanase* from azurine cross-linked wheat arabinoxylan substrates.

5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

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

6. REFERENCES

[1] *Application - Reference SANCO/D/2 Forw. Appl. 1831/042-2009.
[2] *Technical Dossier - Section II.2.1.3.
[3] *Technical Dossier – Section II – Annex_II_B 13.
[4] *Application - Annex III, Proposal of Register entry.
[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 Community reference laboratories, Official Journal of the European Union L 136.
[6] *Technical Dossier – Section II - Annex_II_B28
[7] *Technical Dossier – Section II - Annex_II_B29
[8] *Technical Dossier – Section II - Annex_II_B30
[9] *Technical Dossier – Section II - Annex_II_B31
[10] *Technical Dossier – Section II - Annex_II_B32
[11] *Technical Dossier – Section II - Annex_II_B33
[12] *Technical Dossier – Section II - Annex_II_B34

* Refers to Dossier No. FAD-2009-0038

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

The following National Reference Laboratories contributed to this report:

- Plantedirektoratet, Laboratorium for Foder og Gødning, Lyngby (DK)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Thüringer Landesanstalt für Landwirtschaft (TLL), Abteilung Untersuchungswesen, Jena (DE)
- Foderavdelningen, Statens Veterinärmedicinska Anstalt (SVA), Uppsala (SE)
- Skúšobné laboratórium – Oddelenie analýzy krmív, Ústredný kontrolný a skúšobný ústav poľnohospodársky, Bratislava (SLK)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)