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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-2010-0007 CRL/ 090020
Name of Product:	Danisco Glycosidase TPT and Danisco Glycosidase L
Active Agent (s):	Endo-1,4-beta-xylanase (E.C. 3.2.1.8) Endo-1,3(4)-beta-glucanase (EC 3.2.1.6)
Rapporteur Laboratory:	Community Reference Laboratory for Feed Additives (CRL-FA), Geel, Belgium
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Report approved by: Date:	Christoph von Holst (CRL-FA) 28/06/2010

EXECUTIVE SUMMARY

In the current application authorisation is sought under article 4(1) for *Danisco Glycosidase TPT* and *Danisco Glycosidase L* under the category/functional group '4a': digestibility enhancers, according to Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought for the use of *Danisco Glycosidase TPT* and *L* for poultry, weaned piglets and pigs for fattening. The product consist of *endo-1,4-β-xylanase* (EC 3.2.1.8) and *endo-1,3(4)-β-glucanase* (EC 3.2.1.6) as the active agents produced by the strains *Trichoderma reesei* (ATCC PTA 5588) and *Trichoderma reesei* (ATCC SD-2106), respectively.

The activity of *endo-1,4-β-xylanase* is expressed as *xylanase* unit (U). According to the applicant, one U is the amount of enzyme which releases 0.48 µmol of reducing sugar (xylose equivalent) per minute from a wheat arabino xylan at pH 4.2 and 50°C. The activity of *endo-1,3(4)-β-glucanase* is expressed as *betaglucanase* unit (U), where one U is the amount of enzyme which releases 2.4 µmol of reducing sugar (glucose equivalent) per minute from a barley glucan at pH 5.0 and 50^{o}C.

The product is intended to be marketed as solid (*Danisco Glycosidase TPT*) and liquid (*Danisco Glycosidase L*) formulations. Both formulations have a guaranteed minimum activity of 12200 U/g of *endo-1,4-β-xylanase* and of 1520 U/g of *endo-1,3(4)-β-glucanase*. *Danisco Glycosidase* is intended to be mixed into *premixtures* and/or complete *feedingstuffs*: with activity :

- ranging from 610 to 2440 U *endo-1,4-β-xylanase*/kg and 76 to 304 U *endo-1,3(4)-β-glucanase*/kg in *feedingstuffs,* for weaned piglets, pigs for fattening and all poultry except turkeys for fattening and turkeys reared for breeding and
- ranging from 1220 to 2440 U *endo-1,4-β-xylanase*/kg and 152 to 304 U *endo-1,3(4)-β-glucanase*/kg in *feedingstuffs,* for turkeys for fattening and turkeys reared for breeding.

For the determination of the activity of *endo-1,4-β-xylanase* in the *feed additive*, *premixtures* and *feedingstuffs*, the applicant proposes colorimetric methods 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. These methods were single laboratory validated and further verified by a second independent laboratory. The following method performance characteristics were derived from the validation and verification studies:

- 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_{int}) 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_{int} ranging from 6.6 to 6.9 %, and - R_{Rec} ranging from 93 to 95 %, and

- for *feedingstuffs*: - RSD_r ranging from 2.3 to 5.8 %, - RSD_{int} ranging from 4.0 to 6.9 %, - R_{Rec} ranging from 93 to 97 %, and a limit of quantification (LOQ) of 79 U/kg well below the minimum activity proposed by the applicant.

For the determination of the activity of *endo-1,3(4)-β-glucanase* in the *feed additive*, *premixtures* and *feedingstuffs*, the applicant proposes colorimetric methods based on the quantification of water soluble dyed fragments produced by the action of *endo-1,3(4)-β-glucanase* on commercially available cross-linked barley betaglucan substrates. The enzymatic activity of the sample is calculated using a reference enzyme standard. These methods were single laboratory validated and further verified by a second independent laboratory. The following method performance characteristics were derived from the validation and verification studies:

- for a *feed additive*: - RSD_r ranging from 2.5 to 3.2 %, - RSD_{int} ranging from 3.0 to 11.9 %, and - R_{Rec} ranging from 88 to 109 %,

- for *premixtures*: - RSD_r ranging from 4.3 to 8.5 %, - RSD_{int} ranging from 6.8 to 14 %, and - R_{Rec} ranging from 92 to 97 %, and

- for *feedingstuffs*: - RSD_r ranging from 7.3 to 8.5 %, - RSD_{int} ranging from 8.5 to 14.0 %, - R_{Rec} ranging from 92 to 94 %, and an acceptable LOQ = 51 U/kg.

Based on the satisfactory performance characteristics mentioned above, the CRL recommends for official control the single laboratory validated and further verified analytical methods submitted by the applicant for the determination of the activity of *endo-1,4-β-xylanase* and *endo-1,3(4)-β-glucanase* in the *feed additive*, *premixtures* 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

Danisco Glycosidase TPT and *L*, endo-1,4-β-*xylanase*, endo-1,3(4*)*-β-*glucanase, Trichoderma reesei,* digestibility enhancers, poultry, turkeys for fattening, turkeys reared for breeding, weaned piglets, pigs for fattening

1. BACKGROUND

In the current application authorisation is sought under article 4(1) for *Danisco Glycosidase TPT* and *Danisco Glycosidase L* under the category/functional group '4a': digestibility enhancers, according to Annex I of Regulation (EC) No 1831/2003 [1]. Specifically, authorisation is sought for the use of *Danisco Glycosidase TPT* and *L* for poultry, weaned piglets and pigs for fattening. *Danisco Glycosidase TPT* and *L* consist of *endo-1,4-β-xylanase* (EC 3.2.1.8) and *endo-1,3(4)-β-glucanase* (EC 3.2.1.6) as the active agents [2] produced by the strains *Trichoderma reesei* (ATCC PTA 5588) and *Trichoderma reesei* (ATCC SD-2106), respectively. Both strains have been deposited at the American Type Culture Collection (ATCC) in Virginia, USA [3]. The activity of *endo-1,4-β-xylanase* is expressed as *xylanase* unit (U). According to the applicant, one U is the amount of enzyme which releases 0.48 µmol of reducing sugar (xylose equivalent) per minute from a wheat arabino xylan at pH 4.2 and 50°C. The activity of *endo-1,3(4)-β-glucanase* is expressed as *betaglucanase* unit (U), where one U is the amount of enzyme which releases 2.4 µmol of reducing sugar (glucose equivalent) per minute from a barley glucan at 50^{o}C, pH 5.0 [2]. The product is intended to be marketed as solid (*Danisco Glycosidase TPT*) and liquid (*Danisco Glycosidase L*) formulations. The main carrier of the typical solid formulations is sodium sulphate. Both formulations have a guaranteed minimum *endo-1,4-β-xylanase* activity of 12200 U/g and an *endo-1,3(4)-β-glucanase* activity of 1520 U/g [2]. *Danisco Glycosidase* is intended to be mixed into *premixtures* and/or complete *feedingstuffs* with activity :

- ranging from 610 to 2440 U *endo-1,4-β-xylanase*/kg and 76 to 304 U *endo-1,3(4)-β-glucanase*/kg in *feedingstuffs,* for weaned piglets, pigs for fattening and all poultry except turkeys for fattening and turkeys reared for breeding, and
- ranging from 1220 to 2440 U *endo-1,4-β-xylanase*/kg and from 152 to 304 U *endo-1,3(4)-β-glucanase*/kg in *feedingstuffs,* for turkeys for fattening and turkeys reared for breeding [2].

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 (EFSA) for each application or group of applications. For this dossier, the methods of analysis submitted in connection with *Danisco Glycosidase TPT* and *L*, and their suitability to be used for official controls in the frame of the authorisation, were evaluated.

3. EVALUATION

Identification /Characterisation 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, cadmium, lead, mercury, aflatoxin B1 and dioxins) are available from the respective Community Reference Laboratories [4].

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

Endo-1,4-β-xylanase

For the determination of the activity of *endo-1,4-β-xylanase* in the *feed additive*, *premixtures* and *feedingstuffs*, the applicant proposes colorimetric methods [5,6] 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 from Megazyme. The enzymatic activity of the sample is calculated using reference enzyme standards with certified enzyme activity available from the applicant. These methods were validated [7,8] and further verified by a second independent laboratory [9-11].

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 azurine cross-linked wheat arabinoxylan substrate. When analysing the *feed additive*, the samples are prepared by extracting two portions of 1.0 g or 0.5 g of either dry or liquid additive in 100 ml of acetate buffer and further incubated. The rate of dye release is measured on a spectrophotometer at 590 nm and quantified against a calibration standard curve. The analysis is carried out at pH 4.2 and 50°C for 10 min [5]. 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 *premixtures*, the applicant proposes a method, which is based on the same principle than the method for the analysis of the feed additive. The samples of premixture are diluted with heat treated wheat flour and treated as the samples of feedingstuffs by applying the corresponding feed method [6], as described below. The method performance characteristics derived from the validation [8] and verification [11] studies are presented in Table 1.

For the determination of the activity of *endo-1,4-β-xylanase* in *feedingstuffs*, the applicant proposes a method, measuring enzymatic activity on cross-linked wheat arabinoxylan at pH 4.2 and 50°C for 60 min [6]. Calibration is performed on standards prepared from identical blank feed samples fortified with exact amounts of the calibrator solution. The method performance characteristics derived from the validation [8] and verification [10] studies are presented in Table 1. When identical blank feed samples are not available, the CRL recommends the "standard addition" technique to be used. However, experimental data to support this approach has not been provided.

Based on the satisfactory performance characteristics presented in Table 1, 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*.

Table 1: Performance characteristics for *endo-1,4-β-xylanase*

	LOD (U/g)		LOQ (U/g)		RSD_r (%)		RSD_{int} (%)		R_{Rec} (%)	
	Validation (1)	Verification (2)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)
FA	4.7	10.3	15.6	34.6	9.5	6.6	11.1	7.2	103	112
PM	0.320	0.229	1.064	0.764	2.3	8.0	6.9	6.6	93-95	94
FS	0.040	0.024	0.133	0.079	2.3	5.8	6.9	4.0	93-95	97

FA - feed additive, PM - premixtures, FS – feedingstuffs

LOD - limit of detection, LOQ - limit of quantification, 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*, *premixtures* and *feedingstuffs*, the applicant proposes colorimetric methods [12,13] based on the quantification of water soluble dyed fragments produced by the action of *endo-1,3(4)-β-glucanase* on commercially available azurine cross-linked barley betaglucan substrates from Megazyme. The enzymatic activity of the sample is then calculated using reference enzyme standards with certified enzyme activity available from the applicant. These methods were validated [14,15] and further verified by a second independent laboratory [16-18].

For the determination of the activity of *endo-1,3(4)-β-glucanase* in the *feed additive,* the applicant proposes a method which measures the enzyme-catalysed formation of water soluble dyed fragments released from azurine cross-linked barley betaglucan substrates. When analysing the *feed additive*, the samples are prepared by extracting two portions of 1.0 g or 0.5 g of either dry or liquid additive in 100 ml of acetate buffer and further incubated. The rate of dye release is measured on a spectrophotometer at 590 nm and quantified against a calibration standard curve. The analysis is carried out at pH 5.0 and 50°C for 10 min [12]. The method performance characteristics derived from the validation [14] and verification [16] studies are presented in Table 2.

For the determination of the activity of *endo-1,3(4)-β-glucanase* in *premixtures,* the applicant proposes a method, which is based on the same principle than the method for the analysis of the feed additive. The samples of premixture are diluted with heat treated wheat flour and treated as the samples of feedingstuffs by applying the corresponding feed method [13], as described below. The method performance characteristics derived from the validation [15] and verification [18] studies are presented in Table 2.

For the determination of the activity of *endo-1,3(4)-β-glucanase* in *feedingstuffs,* the applicant proposes a method, measuring enzymatic activity on cross-linked barley betaglucan at pH 5.0 and 50°C for 30 min [13]. Calibration is performed on standards prepared from identical blank feed samples fortified with exact amounts of the betaglucanase calibrator solution. The method performance characteristics derived from the validation [15] and verification [17] studies are presented in Table 2. When identical blend feed samples are not available, the CRL recommends the "standard addition" technique to be used. However, experimental data to support this approach has not been provided.

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-1,3(4)-β-glucanase* in the *feed additive*, *premixtures* and *feedingstuffs.*

Table 2: Performance characteristics for *endo-1,3(4)-β-glucanase*

	LOD (U/g)		LOQ (U/g)		RSD_r (%)		RSD_{int} (%)		R_{Rec} (%)	
	Validation (1)	Verification (2)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)
FA	0.8	0.18	2.5	0.58	2.5	3.2	11.9	3.0	109	88
PM	0.12	0.36	0.408	1.208	8.5	4.3	14	6.8	92-93	97
FS	0.015	0.01	0.051	0.035	8.5	7.3	14	8.5	92-93	94

FA - feed additive, PM - premixtures, FS – feedingstuffs
LOD - limit of detection, LOQ - limit of quantification, 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 colorimetric methods submitted by the applicant to determine the activities of *endo-1,4-beta-xylanase* and *endo-1,3(4)-β-glucanase* in the *feed additive*, *premixtures* and *feedingstuffs*. The reference enzyme standards are 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 (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.
- Colorimetric method measuring water soluble dye released by action of *endo-1,3(4)-β-glucanase* from azurine cross-linked barley betaglucan substrates.

5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Danisco Glycosidase TPT* and *L* 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/Ref:SANCO/D/2: Forw. Appl. 1831/0007-2010

[2] *Application, Proposal for Register Entry, Annex A

[3] *Technical dossier, Section II: Identity, characterisation and conditions of use of the additive

[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_B41

[6] *Technical Dossier, Section II, Annex_II_B43

[7] *Technical Dossier, Section II, Annex_II_B45

[8] *Technical Dossier, Section II, Annex_II_B47

[9] *Technical Dossier, Section II, Annex_II_B49

[10] *Technical Dossier, Section II, Annex_II_B51

[11] *Technical Dossier, Section II, Annex_II_B53

[12] *Technical Dossier, Section II, Annex_II_B42

[13] *Technical Dossier, Section II, Annex_II_B44

[14] *Technical Dossier, Section II, Annex_II_B46

[15] *Technical Dossier, Section II, Annex_II_B48

[16] *Technical Dossier, Section II, Annex_II_B50

[17] *Technical Dossier, Section II, Annex_II_B52

[18] *Technical Dossier, Section II, Annex_II_B54

* Refers to Dossier No. FAD-2010-0007

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

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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- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
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