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

JRC.DG.D.6/CvH/CMP/mds/Ares(2010)499548

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-2009-0014 CRL/090004
Product name:	*Biogalactosidase BL (AlphaGal BL)*
Active Substance(s):	Alpha-galactosidase (EC 3.2.1.22) Endo-1,4-beta-glucanase (EC 3.2.1.4)
Rapporteur Laboratory:	National Research Institute of Animal Production, National Laboratory for Feedingstuffs (NRIAP-NLF), Lublin, Poland
Report prepared by:	Waldemar Korol (NRIAP-NLF)
Report checked by: Date:	Carlos Magno Pinto/Piotr Robouch 09/08/2010
Report approved by: Date:	Christoph von Holst 09/08/2010

EXECUTIVE SUMMARY

In the current application authorisation is sought for *Biogalactosidase BL* (AlphaGal BL) under the category "zootechnical additives", functional groups 4(a), according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought to use *Biogalactosidase BL* as a digestibility enhancer for chicken for fattening. The additive is intended to be marketed as a solid formulation.

Biogalactosidase BL consists of two active agents: (i) *α-galactosidase* (EC 3.2.1.22) produced by *Sacharomyces cerevisiae* (CBS 615.94) and (ii) *endo-1,4-β-glucanase* (EC 3.2.1.4) produced by *Aspergillus niger* (CBS 120604). The enzymatic activities of *α-galactosidase* and *endo-1,4-β-glucanase* are expressed in enzyme units (U). According to the applicant (i) one *α-galactosidase* unit (U) is the quantity of enzyme which liberates 1 μmol of p-nitrophenol per minute from p-nitrophenyl-α-galactopyranoside (pNPG) at pH = 5.0 and 37^{o}C, whereas (ii) one *endo-1,4-β-glucanase* unit (U) is the quantity of enzyme which liberates 1 mg of reducing sugar (glucose equivalent) per minute from β-glucan at pH = 5.0 and 50^{o}C. *Biogalactosidase BL* has an *α-galactosidase* and *endo-1,4-β-glucanase* target activity of 1000 U/g and 5700 U/g. The product is intended to be mixed into *premixtures* and/or *feedingstuffs* to obtain activity levels of 50 to 100 U *α-galactosidase* /kg and 285 to 570 U *endo-1,4-β-glucanase* /kg in complete *feedingstuffs*.

For the determination of *α-galactosidase* the applicant proposes two single laboratory validated and further verified colorimetric methods for the analysis: - in *feed additives*; and in *premixtures* and *feedingstuffs*. These methods are based on the production of p-nitrophenol (pNP) and D-galactose from p-nitrophenyl-α-galactopyranoside (pNPG) at pH = 5.0 at 37^{o}C. The following performance characteristics were reported: (a) relative standard deviations for *repeatability* (RSD_r) ranging from 2.7 to 10 %; (b) relative standard deviations for *reproducibility* (RSD_R) ranging from 3 to 6 %; (c) recovery rates (R_{Rec}) ranging from 81 to 103 %; and a limit of quantification (LOQ) of 10 U/kg *feedingstuffs*.

For the determination of the activity of *endo-1,4-β-glucanase* in the *feed additive* the applicant proposes a single laboratory validated and further verified colorimetric method, based on the hydrolyses of a barley beta-D-glucan at 50^{o}C and pH = 5.0 to release reducing sugars reacting with 3.5-dinitro salicylic acid (DNS). As for the determination of the enzyme activity of *endo-1,4-β-glucanase* in the *premixtures* and *feedingstuffs* the applicant proposes another single laboratory validated colorimetric method using azurine cross-linked barley glucan substrate.

The following performance characteristics were reported: (a) RSD_r ranging from 3.3 to 10 %; (b) RSD_R ranging from 1.3 to 15 %; (c) R_{Rec} around 100 %; and a limit of detection (LOD) and a limit of quantification (LOQ) of 66 and 132 U/kg *feedingstuffs,* respectively.

Based on the above mentioned performance characteristics, the CRL recommends for official control the single laboratory validated and further verified colorimetric methods submitted by the applicant for the determination of *α-galactosidase* and *endo-1,4-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

Biogalactosidase BL, *α-galactosidase*, *Sacharomyces cerevisiae, endo-1,4-β-glucanase, Aspergillus niger,* digestibility enhancer, chickens for fattening

1. BACKGROUND

Biogalactosidase BL is a product for which authorisation is sought under the category 'zootechnical additives', functional group 4(a) 'digestibility enhancer', according to Annex I of Regulation (EC) No 1831/2003 [1]. Authorisation is sought to use this product as a digestibility enhancer for chickens for fattening. *Biogalactosidase BL* contains two active agents: *α-galactosidase* (EC 3.2.1.22) and *endo-1,4-β-glucanase* (EC 3.2.1.4) produced by a genetically modified *Sacharomyces cerevisiae* strain (CBS 615.94) and an *Aspergillus niger* strain (CBS 120604), respectively [2]. Both strains have been deposited at the Centraal Bureau voor Schimmelcultures (CBS) in Baarn, The Netherlands [3]. The enzymatic activities of *α-galactosidase* and *endo-1,4-β-glucanase* are expressed in enzyme units (U). According to the applicant, (i) one *α-galactosidase* unit (U) is the amount of enzyme which releases 1 μmol of p-nitrophenol per minute from p-nitrophenyl-α-galactopyranoside (pNPG) at pH = 5.0 and 37^{o}C, whereas (ii) one *endo-1,4-β-glucanase* unit (U) is the amount of enzyme which releases 1 mg of reducing sugar (glucose equivalent) per minute from β-glucan at pH = 5.0 and 50^{o}C [3]. *Biogalactosidase BL* is intended to be marketed as solid formulation containing *α-galactosidase* and *endo-1,4-β-glucanase* activities of 1000 U/g and 5700 U/g, respectively. The product is intended to be mixed into *premixtures* and/or *feedingstuffs* to obtain activities in complete *feedingstuffs* ranging from 50 to 100 U/kg for *α-galactosidase* and from 285 to 570 U/kg for *endo-1,4-β-glucanase* [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 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. For this particular dossier, the methods of analysis submitted in connection with *Biogalactosidase BL* 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 such as heavy metals (lead), dioxins, microbiological agents and mycotoxins are available from the respective Community Reference Laboratories [4].

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

α-galactosidase

For the determination of *α-galactosidase* in the *feed additive, premixtures* and *feedingstuffs* the applicant proposes single laboratory validated colorimetric methods [5, 6]. These methods are based on the production of p-nitrophenol (pNP) and D-galactose from p-nitrophenyl-α-galactopyranoside (pNPG) at pH = 5.0 and 37^{o}C. The *feed additive* sample (1.0 g) is extracted and suitably diluted with deionised water according to its expected enzyme activity [5]. Then 200 µl of the enzyme dilution is incubated at 37^{o}C for 5 min with 1.8 ml of the pNGP substrate prepared in 0.1 M acetate buffer, adjusted at pH = 5.0. The incubation is stopped by the addition of 4 ml of a sodium carbonate solution and the optical density of the reaction solution is measured at 405 nm. The enzyme activity of the sample is calculated as the difference between the absorbance of the sample and the absorbance of the blank reagent.

For the analysis of *premixtures* and *feedingstuffs* the protocol [6] foresees the extraction of 5.0 g of sample with 0.1 M acetate buffer adjusted at pH 5.0. An aliquot of 0.5 ml of centrifuged extract is incubated at 37°C for 5 minutes with 0.45 ml of the pNPG substrate prepared in a solution of 0.1 M sodium acetate. The incubation is stopped by the addition of 1 ml of a 10% sodium carbonate solution and the optical density of the reaction solution is measured at 405 nm. The enzyme activity of the sample is calculated as the difference between the absorbance of the sample and the absorbance of the blank reagent.

The two single laboratory methods were further validated by an independent laboratory. The performances characteristics of the method for *α-galactosidase* in *feed additives*, *premixtures* and *feedingstuffs* are presented in Table 1. Furthermore, the applicant reported a limit of quantification (LOQ) of 10 U/kg *feedingstuffs* [7].

The verification studies [8,11,14] performed by an independent laboratory confirm most of the performance characteristics obtained by the applicant during the method validation study. However, significantly different measurement results were reported for the reproducibility experiment of the verification study [14]: (91.5 ± 4.9) U/kg for Day 1 and (56.0 ± 9.6) U/kg for Day 2, for an expected value of 59 U/kg. The results obtained on Day 2 confirm the *repeatability* and *recovery* rates obtained by the applicant, while those obtained on the first day seem to be overestimated. Consequently, the reported RSD_R [14] - calculated pooling both set of data - is considered unreliable.

Based on the performance characteristics presented in Table 1, the CRL recommends for official control the single laboratory validated and further verified methods submitted by the applicant for the determination of *α-galactosidase* activity in the *feed additive, premixtures* and *feedingstuffs*.

Table 1: Method performance characteristics for the determination of *α-galactosidase* in the *feed additive* (FA), *premixtures* (PM) and *feedingstuffs* (FS)

	RSD_r (%)		RSD_R (%)		R_{Rec} (%)	
	Validation	Verification	Valid.	Verif.	Valid.	Verif.
FA	3.8 [7]	2.7 [8]	4.9 [9]	2.7 [8]	90-100 [8]	84 [8]
PM	3-4.9 [10]	5.9 [11]	np	5.9 [11]	*94 [12]	82 [11]
FS	4.3-5.3 [13]	10 [14]	np	#35 [14]	*81-103 [13]	Day1: *155 # Day 2: *95 [14]

RSD_r and RSD_R: relative standard deviation for *repeatability* and *reproducibility*
R_{Rec}: recovery rate ; np: not provided; (#) Questionable value
(*) calculated by the CRL as: $R_{Rec} = Activity_{measured} / Activity_{expected}$

endo-1,4-β-glucanase

For the determination of the activity of *endo-1,4-β-glucanase* in the *feed additive* the applicant proposes a single laboratory validated colorimetric method, based on the hydrolyses of a barley beta-D-glucan at 50°C and pH = 5.0 to release reducing sugars reacting with 3.5-dinitro salicylic acid (DNS). When analysing the *feed additive*, three replicates of 1.0 g of *feed additive* are extracted in 100 ml deionised water, diluted and incubated with buffered barley beta-D-glucan at 50°C and pH = 5.0 for 10 minutes. Then the reaction is stopped by adding DNS reagent. The colour change produced is proportional to the amount of reduced sugar released and to the activity of *endo-1,4-β-glucanase* present in the sample. The optical density (OD) is measured with a spectrophotometer at 540 nm and quantified against a gravimetrically prepared reference enzyme standard [15].

For the determination of the enzyme activity of *endo-1,4-β-glucanase* in the *premixtures* and *feedingstuffs* the applicant proposes another single laboratory validated colorimetric method with azurine cross-linked barley glucan substrate [16], derived from the Megazyme protocol [22]. The protocol foresees the extraction of 5.0 g of sample with 0.1 M acetate buffer adjusted at pH 4.5 for 30 minutes at room temperature. After centrifugation, supernatant is diluted and used for determination of activity. Diluted extract is treated by a commercially available Beta-Glucozyme tablet and incubated at 40°C for 30 minutes. The incubation is stopped by the addition of 10 ml of a 2% trisodium phosphate (Trizma) solution and after filtration the optical density of clear colour extract is measured with a spectrophotometer at 590 nm and quantified against a *Biogalactosidase BL* sample (characterised with the *feed additive* protocol [15] described above).

The two single laboratory methods were further validated by an independent laboratory. The performances characteristics of the method for *endo-1,4-β-glucanase* in *feed additives*, *premixtures* and *feedingstuffs* are presented in Table 2. The recovery rates of the order of 120 to 130 % reported in the frame of the verification studies [19, 21] are considered acceptable for an enzymatic analysis. Furthermore, the applicant reported the following limits of detection (LOD) and quantification (LOQ) [20]:

LOD and LOQ = 66 and 132 U/kg *feedingstuffs*, respectively.

Based on the performance characteristics presented in Table 2, the CRL recommends for official control the analytical methods submitted by the applicant for the determination of *endo-1,4-β-glucanase* activity in the *feed additive, premixtures* and *feedingstuffs*.

Table 2: Method performance characteristics for the determination of *endo-1,4-β-glucanase* in the *feed additive* (FA), *premixtures* (PM) and *feedingstuffs* (FS)

	RSD_r (%)		RSD_R (%)		R_{Rec} (%)	
	Validation	Verification	Valid.	Verif.	Valid.	Verif.
FA	np	4.1 [18]	3-4 [17]	16 [18]	*101 [17]	97 [18]
PM	np	3.3 [19]	1.3 [12,20]	6.9 [19]	*100 [20]	*130 [19]*
FS	np	10 [21]	2.3-5.4 [13,20]	15 [21]	*100 [13,20]	*121 [21]*

RSD_r and RSD_R: relative standard deviation for *repeatability* and *reproducibility*
R_{Rec}: recovery rate
np: not provided
(*) calculated by the CRL as: $R_{Rec} = Activity_{measured} / Activity_{expected}$

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of *Biogalactosidase BL* authorisation, the CRL recommends for official control the single laboratory validated and further verfied colorimetric methods for determination of α-*galactosidase* and *endo-1,4-β-glucanase* in *feed additives, 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.

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

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

- Colorimetric method measuring p-nitrophenol released by action of *α-galactosidase* from p-nitrophenyl-α-galactopyranoside substrate;
- Colorimetric method measuring water soluble dye released by action of *endo-1,4-β-glucanase* from azurine-crosslinked barley glucan substrate.

5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Biogalactosidase BL* 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. 1831/012-2009
[2] *Application, FAD-2009-0014_RegEntry.pdf
[3] *Technical Dossier, Section II, Sec_II_Identity.pdf
[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 Community Reference Laboratories
[5] *Technical Dossier, Section II - Annex II.1.3.B
[6] *Technical Dossier, Section II - Annex II.6.1.A
[7] *Supplementary information, SupInfo-FAD-2005-0020-Section_II
[8] *Technical Dossier, Section II - Annex II.6.1.F
[9] *Supplementary information, Validation_alpha-Gal_FA
[10] *Supplementary information, SupInfo-FAD-2005-0020-PM-valid
[11] *Supplementary information, Verification_alpha-Gal_PM
[12] *Technical Dossier, Section II - Annex II.4.2.A
[13] *Technical Dossier, Section II - Annex II.4.2.B
[14] *Supplementary information, Verification_alpha-Gal_FS
[15] *Technical Dossier, Section II - Annex II.1.3.C
[16] *Technical Dossier, Section II - Annex II.6.1.B
[17] *Technical Dossier, Section II - Annex II.6.1.C
[18] *Technical Dossier, Section II - Annex II.6.1.G
[19] *Supplementary information, Verification_beta-Gluc_PM
[20] *Technical Dossier, Section II - Annex II.6.1.D
[21] *Supplementary information, Verification_beta-Gluc_FS
[22] *Supplementary information, Megazyme protocol

*Refers to Dossier number: FAD-2009-0014

6. RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was National Research Institute of Animal Production, National Laboratory for Feedingstuffs, Lublin, Poland. 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.

7. ACKNOWLEDGEMENTS

The following National Reference Laboratories contributed to this report:

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