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CRL Evaluation Report on the Analytical Methods submitted in connection with Section II, 2.5 (Control Methods) of the Application for Authorisation as a Feed Additive according to Regulation (EC) No 1831/2003

Dossier related to:	EFSA-Q-2005-115
Name of Additive:	Belfeed B1100 MP/ML
Active Subtance(s):	Endo-1,4-beta-xylanase
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Date:	19/01/2006

EXECUTIVE SUMMARY

In the current application authorisation is sought for Belfeed B1100M® 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 to use Belfeed B1100M® as a digestibility enhancer for ducks for fattening. The active substance of Belfeed B1100M® is endo-1,4-β-xylanase (produced by the genetically modified microorganism *Bacillus subtilis* LMG S-15136), which degrades the polysaccharide β-1,4-xylan. The additive is intended to be marketed in two forms, as a granulated powder (Belfeed B1100MP®) and as liquid formulation (Belfeed B1100ML®).

The activity of endo-1,4-β-xylanase is expressed as international units (IU). According to the applicant, one IU is the amount of enzyme which liberates 1 μmole of reducing sugars (xylose equivalents) from birchwood xylan per minute at pH 4,5 and 30°C. Belfeed B1100MP® and Belfeed B1100ML® have a minimum specific activity of 100 IU endo-1,4-β-xylanase /g of product. The product also has other limited enzymatic activities (β-glucanase, α-amylase and pectinase). Belfeed B1100M® is intended to be mixed into feedingstuffs obtaining a minimum enzyme activity of 10 IU/kg.

For the determination of the xylanase activity in the *feed additive* the applicant proposes an in-house developed test which is based on the principle that xylanase releases xylose from the substrate xylane, which in the presence of copper neocuproin forms a yellow coloured complex. The yellow complex is measured with a spectrophotometer using an enzyme standard curve. Based on the obtained method performance characteristics, that include a limit of detection of 5 IU/g and a relative standard deviation for within-laboratory reproducibility of 3.2 %, the method is considered suitable for the intended purpose.

For the determination of the xylanase activity in *premixtures* the applicant proposes the same method obtaining relative standard deviations for within-laboratory reproducibility ranging from 0.8 to 2.3 % for the solid form and from 1.7-6.5% for the liquid form of the feed additive. The method is considered suitable for the intended purpose.

For the determination of the xylanase activity in *feedingstuffs* a modified method of a commercially available test kit is proposed. The method is based on the principle that xylanase releases water soluble dyed fragments from a substrate, which are directly related to the enzyme activity. The formed dyed fragments are then measured with a spectrophotometer and the enzyme activity is quantified against matrix matched standards. The linear range of the test, in which the activity of the enzyme can be quantified, is from 5 to 75 IU/kg. The relative standard deviations for within-laboratory reproducibility range from 2.8-5.9% for the solid form of the feed additive and 3.1-7.4% for the liquid form of the product. Taking into account the target level of application which is equal to 10 IU /kg of feedingstuffs and the

acceptable values of method performance characteristics, in the opinion of the CRL the proposed method is fit for official control purposes to determine the activity of the xylanase in target feedingstuffs at the target activity level.

Further testing or validation is not considered necessary.

KEYWORDS

Belfeed B1100M®, endo-1,4-beta-xylanase, *Bacillus subtilis*, digestibility enhancer, fattening ducks

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1. BACKGROUND

Belfeed B1100M® is a feed additive belonging to the 'zootechnical additives' group (4a). It contains endo-1,4-β-xylanase (a minimum specific activity of 100 IU/g product) as the active ingredient. Endo-1,4-β-xylanase (produced by *Bacillus subtilis* (LMG S-15136)) is already permanently authorised for chickens for fattening (Commission Regulation (EC) N° 1259/2004) and for piglets (Commission Regulation (EC) N° 1206/2005) and provisionally authorized for turkeys for fattening (Commission Regulation (EC) N° 2188/2002), pigs for fattening (Commission Regulation (EC) N° 2611/2003) and laying hens (Commission Regulation (EC) N° 358/2005).

The intended use (*cf.* EFSA-Q-2005-115) of the current application is to enhance the digestibility of ducks for fattening, by mixing the feed additive into complete feedingstuffs in a concentration of 10 IU/kg.

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005 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 for each application. For this particular dossier, the suitability of the control methods and validation studies submitted in connection with Belfeed B1100M®, *cf.* EFSA-Q-2005-115, was evaluated.

3. EVALUATION

The numbering system under this point refers to that of Section II of the "Guidelines for the assessment of additives in feedingstuffs, PART II: Enzymes and Micro-organisms (2.5 Control methods)", adopted by the Scientific Committee on Animal Nutrition on 22 October 1999, in the following referred as "the Guidelines".

Description of the analytical methods listed under 2.5.1 of the Guidelines

Qualitative and quantitative composition

In addition to the main enzyme xylanase the feed additive also contains other enzymes such as β-glucanase and α-amylase. For the determination of the activity of β-glucanase in the feed additive a commercial test kit is available which is considered suitable for the intended purpose. For the determination of the activity of α-amylase a commercial Phadebas® Amylase Test (Magle AB, Sweden; formerly available from Pharmacia AB, Sweden) is proposed. However, since this test is convenient for the determination of the activity of α-amylase in body fluids, it is not possible to evaluate a suitability of the test for feed additive. For that purpose, CRL recommends a commercial Ceralpha (Alpha-Amylase) kit available from Megazyme (AOAC Method 2002.01).

Determination of heavy metals

For the determination of various heavy metals (arsenic, lead, cadmium and mercury) an atomic absorption spectroscopy method from the Joint FAO/WHO Committee on Food Additives [1] is proposed, which is considered suitable for this purpose.

Determination of micro-organisms

Colony count technique on plate count agar is used for mesophylic aerobic bacteria, yeasts and moulds [ISO 4833]. Specific isolation and confirmation of *Salmonella* is performed according FDA Bacteriological Analytical Manual, 6th ed. [2]. Coliforms and *E. coli* are isolated and identified according ISO 4832 and FDA Bacteriological Analytical Manual, 6th ed. [3]. *Staphylococcus aureus* is isolated and identified according ISO 6887. The proposed methods are commonly used and are considered suitable for the intended purpose.

Determination of mycotoxins

The applicant proposed an analytical method based on thin layer chromatography [4] which is considered suitable for the intended purposes.

Quantitative analysis of the activity of endo-1,4-beta-xylanase in the feed additive

For the determination of the activity of xylanase in the feed additive the applicant proposed the in-house developed modified method "Skalar test" [5] which has been modified to make it suitable for the determination of the activity of *bacterial* xylanases and not only of *fungal* xylanases. The method works on both solid and liquid forms of the additive.

The sample is extracted with an acetate buffer and after addition of the substrate xylan incubated at 50°C for 30 min. The incubation is stopped by the addition of carbonate solution and copper neocuproin solution is added for the reduction of the formed sugars to a yellow coloured complex. After addition of potassium sodium tartrate solution the absorbance of the complex is measured at 460 nm. The results are presented as absorbance values converted into enzyme activity units (IU/g).

The working range of the method was 10-1000 IU xylanase/g product. The obtained method performance characteristics include a limit of detection (LOD) of 5 IU /g product, a recovery rate of 99 % and a relative standard deviation for within-laboratory reproducibility of 3.2 %. Taking into account the target level of application which is equal to 100 IU enzyme/g of additive and the acceptable value of performance parameters, in the opinion of the CRL the proposed method is fit for purpose of official control to determine the activity of xylanase in in pure Belfeed B1100M® samples.

Description of the qualitative and quantitative analytical methods for routine control of the active substance in premixtures and feedingstuffs (cf. pt. 2.5.2 of the Guideline)

For the determination of xylanase activity in *premixtures* the applicant uses the modified Skalar method, which is identical to the method applied for the determination of the xylanase activity in the feed additive. The method is considered suitable for this purpose, since the relative standard deviations for within-laboratory reproducibility ranged from 0.8 to 2.3% for Belfeed B1100MP® and from 1.7 to 6.5% for Belfeed B1100ML®.

For the routine measurements of the activity of xylanase in *feedingstuffs* the applicant proposed a slightly modified version of a commercially available test kit (Xylazyme AX Tablets from Megazyme International Ireland), which has been validated by an external and accredited laboratory for the specific purpose of the analysis in the frame of this authorisation.

The protocol foresees the extraction of 5.0 g of the feed sample with acetate buffer. However, the CRL recommends increasing the sample amount to 20 g, considering potential heterogeneity of the test material as proposed in another paper [6]. A specific substrate based on xylane (Azurine crosslinked wheat arabinoxylan) and which is part of the test kit is added and the solution is incubated at 50°C for 30 min. Due to the reaction between this substrate

and xylanase a water soluble dye fragments is released. The reaction is stopped by the addition of a specific basic buffer and the formed colour is measured at 590 nm. The enzyme activity is measured against the target blank feedingstuffs samples fortified with different concentrations of the enzyme. To further improve the robustness of the method the CRL, however, recommends applying the standard addition method performed on the target matrix.

Validation experiments were carried out on four different feedingstuff matrices. The linear range of the test, in which the activity of the enzyme can be quantified, is from 5 to 75 IU/kg. The relative recovery rate 99% and the relative standard deviation for reproducibility ranged from 3.1-7.4% for the liquid form and 2.8-5.9% for the solid form of the enzyme. Taking into account the target level of application, which is equal to 10 IU enzyme/kg of feedingstuffs, and the acceptable value of performance parameters, the proposed method is considered suitable for official control to determine the activity of the xylanase in target feedingstuffs at the target activity level.

CHECKLIST

		Y	N	N/A	Comments
1.1	Is/Are the method(s) mentioned on Premixtures accompanied by information on:				
	- Sampling Method used		**X**		
	- Percentage Recovery	**X**			
	- Specificity		**X**		
	- Accuracy	**X**			
	- Precision	**X**			
	- Limits of detection				Linear range for quantification provided
	- Limits of quantification				
	- Validation procedure used	**X**			
1.2	Is/Are the method(s) mentioned on Feedingstuffs accompanied by information on:				
	- Sampling Method used		**X**		
	- Percentage Recovery	**X**			
	- Specificity		**X**		
	- Accuracy	**X**			
	- Precision	**X**			
	- Limits of detection				Linear range for quantification provided
	- Limits of quantification				
	- Validation procedure used	**X**			

4. CONCLUSIONS AND RECOMMENDATIONS

For the determination of the xylanase activity in the feed additive, premixtures and feedingstuffs, the applicant proposed two different spectrometric methods, which, however, are based on the same principle: the enzyme is dissolved in a buffer solution and reacts on an added substrate. In the case of the method for the determination of the enzyme activity in the feed additive the produced sugar forms with another agent a coloured complex which is measured with a spectrophotometer. For the determination of the enzyme activity in feedingstuffs a commercially available test kit is used. This method utilises a substrate which releases a coloured compound due to the reaction with the enzyme. The coloured product of this reaction can be directly measured with a spectrophotometer. For both methods acceptable method performance characteristics were obtained, showing that the methods are suitable for official control in target feedingstuffs at the target activity level. However, for the measurements of the activity of xylanase in feedingstuffs the applicant proposed extraction of 5.0 g of the feed sample. The CRL recommends increasing the sample amount to 20 g, considering potential heterogeneity of the test material as proposed in another paper. To further improve the robustness of the method the CRL also recommends applying the standard addition method performed on the target matrix.

For the determination of the activity of α-amylase the CRL recommends a commercial Ceralpha (Alpha-Amylase) kit available from Megazyme (AOAC Method 2002.01).

5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

The dossier, provided by the applicant, is divided into various documents structured according to the "Guidelines for the assessment of additives in feedingstuffs, PART II: Enzymes and Micro-organisms", adopted by the Scientific Committee on Animal Nutrition on 22 October 1999 and has been made available to the CRL by EFSA.

Further information provided by applicant:

- Supplement to the Section II 2.3 (Physico-chemical, technological and biological properties of the additive); 2.5 (Control methods); Section V. Form of monograph; Summary for publication – dated 15 September 2005.
- Other supplementary data submitted by mail dated 9/11/2005, e-mail dated 21/11/2005 and fax dated 19/12/2005:

Copies of the following publications:

- Bailey M.J., Biely P. and Poutanen K. 1992. Interlaboratory testing of methods for assay of xylanase activity. *J. of Biotechnology*, 23:257-270.

- Bailey M.J. and Poutanen K. 1992. Collaborative testing of a xylanase assay method. In *Xylans and xylanases* (Visser, J., Beldman, G., Kusters-van-Someren, M.A. and Voragen, A.G.J. (eds) Elsevier Science Publishers, Amsterdam, pp. 155-160.
- Cosson T., Perez Vendrell A.M., Gonzalez Teresa B., Rene D., Taillade P. and Brufau J. 1999. Enzymatic assays for xylanase and β-glucanase feed enzymes. *Anim. Feed Sci. Techn.* 77:345-353.
- Cole S.C.J. and Sheenan N. 2000. The analysis of xylanase and beta-glucanase in feed enzymes. In www.feedinfo.com or in www.enzymes.co.uk.
- König J., Grasser R., Pikor H. and Vogel K. 2002. Determination of xylanase, β-glucanase and cellulase activity. *Anal. Bioanal. Chem.*, 374:80-87.
- McCleary B.V. 1992. Measurement of endo-1,4-β-D-xylanase. In *Xylans and xylanases* (Visser, J., Beldman, G., Kusters-van-Someren, M.A. and Voragen, A.G.J. (eds) Elsevier Science Publishers, Amsterdam, pp. 161-170.
- McCleary B.V. 2001. Analysis of feed enzymes. In *Enzymes in farm animal nutrition* (Bedford and Partridge (eds). Cabi Publishing, p. 85-107.
- McCleary B.V. et al. 2003. Endo-1,4-beta-xylanase activity measurement: a state of the art. In *Recent advances in enzymes in grain processing*. Courtin et el. (eds). Proceedings of the 3rd European Symposium on Enzymes in Grain Processing, KUL, Leuven, 2002, p. 47-51.
- Recent documents from the website of Megazyme (www.megazyme.com) concerning the endo-1,4-beta-xylanase assay procedures.
- An updated version of the document "Determination of endo-1,4-beta-xylanase activity in complete feed – modified Megazyme".
- Validation data on Skalar and Megazyme methods.
- In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of Belfeed B1100M® have been sent to the Community Reference Laboratory for feed additives authorisation in June 06, 2005.

6. REFERENCES

[1] Joint FAO/WHO Committee on Food Additives (JEFCA). Guide to JEFCA specifications (FAO Food and Nutrition Papers 5 Rev. 2). 1991.

[2] Chapter 7: Isolation and identification of *Salmonella*. In: U.S. Food and Drug Administration (FDA) Bacteriological Analytical Manual. 6th edition, 1984

[3] Chapter 6: Enteropathogenic *Escherichia coli*. In: U.S. Food and Drug Administration (FDA) Bacteriological Analytical Manual. 6th edition, 1984..

[4] Patterson D.S. and Roberts B.A. 1979. Mycotoxins in animal feedstuffs: sensitive thin layer chromatographic detection of aflatoxin, ochratoxin A, sterigmatocystin, zearalenone, and T-2 toxin. *J. Assoc. Off. Anal. Chem*. 62(6):1265-7.

[5] Bailey M.J., Biely P. and Poutanen K. 1992. Interlaboratory testing of methods for assay of xylanase activity. *J. of Biotechnology*, 23:257-270.

[6] Cosson T., Perez Vendrell A.M., Gonzalez Teresa B., Rene D., Taillade P. and Brufau J. 1999. Enzymatic assays for xylanase and β-glucanase feed enzymes. *Anim. Feed Sci. Techn*. 77:345-353.

7. RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was Community Reference Laboratory for Feed Additives Authorization, IRMM, Geel, Belgium.