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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-2010-0001
CRL/ 090042

Name of Product: Hostazym X

Active Agent (s): Endo-1,4-beta-xylanase (E.C. 3.2.1.8)

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EXECUTIVE SUMMARY

In the current application authorisation is sought for Hostazym X under article 4(1) and article 10(2) for *Hostazym X* under the category "zootechnical additives", functional groups 4(a) "digestibility enhancers", according to Annex I of Regulation (EC) No 1831/2003. The authorisation for chickens for fattening, turkeys for fattening, laying hens, weaned piglets, pigs for fattening and other birds for fattening or laying is requested. *Hostazym X* contains *endo-1,4-β-xylanase* (EC 3.2.1.8) as active agent, produced by the strains of *Trichoderma longibrachiatum* (IMI 356040). This feed additive is already authorised with the number E 1617 by the Commission Regulations (EC) 2148/2004, (EC) 828/2007 and (EC) 322/2009.

The enzymatic activity of *endo-1,4-β-xylanase* is expressed in *endo-pentosanase* units (EPU). According to the applicant, one EPU is the amount of enzyme which releases 0.0083 μmol of reducing sugars (xylose equivalent) per minute from oat spelt xylan at pH 4.7 and 50 °C.

The product is intended to be marketed as solid (*Hostazym X Microgranulate*) and liquid (*Hostazym X Liquid*) formulations used in compound feed rich in non-starch polysaccharides (mainly arabinoxylans). Two solid formulations have a guaranteed minimum activity of *endo-1,4-β-xylanase* of 6000 and 30000 EPU/g. Two liquid formulations have a guaranteed minimum activity of *endo-1,4-β-xylanase* of 6000 and 15000 EPU/g. *Hostazym X* is intended to be mixed into *premixtures* and/or complete *feedingstuffs*: to obtain minimum activities of *endo-1,4-β-xylanase* in *feedingstuffs* of:

- 1050 EPU/kg for turkeys for fattening, laying hens and other birds for fattening or laying, and
- 1500 EPU/kg for chickens for fattening, weaned piglets and pigs for fattening.

For the determination of the activity of *endo-1,4-β-xylanase* in the *feed additive*, *premixtures* and *feedingstuffs*, the applicant proposes a single laboratory validated and further verified 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, available from the applicant upon request. 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 1.9 to 3.3 %; - a relative standard deviation for *intermediate precision* (RSD_{int}) ranging from 1.9 to 3.3 %; and - a *recovery rate* (R_{Rec}) ranging from 101 to 104 %;

for *premixtures*: - RSD_r ranging from 3.2 to 8.2 %; - RSD_{int} = 3.2 %, and - R_{Rec} ranging from 96 to 103 %;

for *feedingstuffs*: - RSD_r ranging from 7.6 to 16 %; - RSD_{int} ranging from 8.9 to 16 %; - R_{Rec} ranging from 93 to 112 %; - a limit of detection (LOD) and quantification (LOQ) of 107 and 358 EPU/kg, respectively.

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

Hostazym X, *endo-1,4- β -xylanase*, *Trichoderma longibrachiatum*, digestibility enhancers, chickens for fattening, turkeys for fattening, laying hens, weaned piglets, pigs for fattening and other birds for fattening or laying

1. BACKGROUND

In the current application authorisation is sought under article 4(1) and 10(2) for *Hostazym X* under the category "zootechnical additives", functional groups 4(a) "digestibility enhancers", according to Annex I of Regulation (EC) No 1831/2003 [1]. Specifically, authorisation is sought for the use of *Hostazym X* for chickens for fattening, turkeys for fattening, laying hens, weaned piglets, pigs for fattening and other birds for fattening or laying. *Hostazym X* contains *endo-1,4- β -xylanase* (EC 3.2.1.8) as the active agent [2] produced by the strain *Trichoderma longibrachiatum* IMI 356 040 (SD 135). The production strain have been deposited at the International Mycological Institute (IMI) in Surrey, UK [3]. This additive has been already authorized as feed additive by Commission Regulation (EC) 2148/2004, (EC) 828/2007 and (EC) 322/2009.

The activity of *endo-1,4- β -xylanase* is expressed as *endo-pentosanase* units (EPU). According to the applicant, one EPU is the amount of enzyme which releases 0.0083 μ mol of reducing sugars (xylose equivalent) per minute from oat spelt xylan at pH 4.7 and 50 °C [5].

The product is intended to be marketed as solid (*Hostazym X Microgranulate*) and liquid (*Hostazym X Liquid*) formulations used in compound feed rich in non-starch polysaccharides (mainly arabinoxylans). The main carriers of the solid and liquid formulations are wheat meal and water + sorbitol, respectively. Two solid formulations have a guaranteed minimum

activity of *endo-1,4- β -xylanase* of 6000 and 30000 EPU/g. Two liquid formulations have a guaranteed minimum activity of *endo-1,4- β -xylanase* of 6000 and 15000 EPU/g [2].

The applicant recommended *endo-1,4- β -xylanase* activities in complete *feedingstuffs* [2,3]:

- ranging from 1050 to 3000 EPU/kg, for turkeys for fattening, laying hens and other birds for fattening or laying and
- ranging from 1500 to 3000 EPU/kg, for chickens for fattening, weaned piglets and pigs for fattening.

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 *Hostazym X*, 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

For the determination of the activity of *endo-1,4- β -xylanase* in the *feed additive, premixtures* and *feedingstuffs*, the applicant proposes spectrophotometric methods [3] based on the quantification of water soluble dyed fragments produced at pH 4.7 and 50 °C, by the action of *endo-1,4 β -xylanase* on commercially available azurine cross-linked wheat arabinoxylan substrates from Megazyme.

For the determination of the activity of *endo-1,4-β-xylanase* in the *feed additive* and in *premixtures*, the samples are prepared by extracting of 1.0 g in 100 ml of acetate buffer. After centrifugation the sample solution is diluted with buffer to an enzyme concentration within 400 – 800 EPU/ml. After 30 minutes incubation at pH 4.7 and 50 °C, the reaction is stopped with TRIS solution. The rate of dye release is measured on a spectrophotometer at 590 nm and quantified against a reference enzyme standard with certified activity, available from the applicant upon request.

For the determination of the activity of *endo-1,4-β-xylanase* in *feedingstuffs*, the applicant proposes a single laboratory validated and further verified enzyme standard addition method. The feed sample (10 g) is extracted 15 minutes with 200 ml acetate buffer containing the spiking solution. After centrifugation, the solution are incubated at 50 °C and pH 4.7 for 150 minutes. The reaction is stopped by addition of TRIS solution and the absorbance is measured at 590 nm. The quantification is carried out with the usual standard addition technique [3], where subsamples are taken and (1) a part of these subsamples are analysed as such and (2) the other subsamples are analysed after being fortified with known amounts of the reference standard. The method performance characteristics derived from the validation [5,6] and verification studies [7,8], were recalculated by the Rapporteur [9] and are presented in Table 1. Furthermore, a limit of detection (LOD) and quantification (LOQ) of 107 and 358 EPU/kg of *feedingstuffs*, were recalculated by the Rapporteur [9], based on the validation data provided by the Applicant [5].

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

	RSD _r (%)		RSD _{int} (%)		R _{Rec} (%)	
	Validation	Verification	Validation	Verification	Validation	Verification
FA	3.3 [5]	1.9 [7]	3.3 [5]	1.9 [7]	101-104 [5]	101-102 [7]
PM	3.2 [5]	8.2 [9]	3.2 [5]	np	99-103 [5]	96 [7]
FS	7.6 [9]	16 [9]	8.9 [9]	16 [9]	98-109 [6]	93-112 [7]

RSD_r, RSD_{int} - relative standard deviation for *repeatability* and *intermediate precision*,

R_{Rec} – recovery rate (calculated as activity measured/activity expected)

np – not provided

Based on the satisfactory performance characteristics presented in Table 1, the CRL recommends for official control the single laboratory validated and further verified colorimetric methods submitted by the applicant for the determination of *endo-1,4-beta-xylanase* 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.

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-β-xylanase* in the *feed additive, premixtures* and *feedingstuffs*.

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.

5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Hostazym X* 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. 1831/001-2010
 - [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, Reference_II.29
 - [6] *Technical Dossier, Section II, Reference_II.30
 - [7] *Technical Dossier, Section II, Reference_II.36
 - [8] *Supplementary information provided by the applicant upon request CRL
 - [9] *Additional Information – Precision data as recalculated by the CRL_AGES.xls
- * Refers to Dossier No. FAD-2010-0001

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation was the Agency for Food and Health Safety (AGES), Vienna, Austria. 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)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (SK)