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Feed Additives

JRC F.5/CvH/MGH/AS/Ares

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

Endo-1,4-beta-mannanase produced by Aspergillus niger (CBS 120604)

(FAD-2021-0053; CRL/200089)

European Union Reference Laboratory
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Dossier related to:	**FAD-2021-0053 - CRL/200089**
Name of Product:	***Endo-1,4-beta-mannanase produced by Aspergillus niger (CBS 120604)***
Active Agent (s):	**Endo-1,4-beta-mannanase**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
Report prepared by:	**María José González de la Huebra**
Report checked by: Date:	**Stefano Bellorini** **30/03/2022**
Report approved by: Date:	**Christoph von Holst** **31/03/2022**

EXECUTIVE SUMMARY

In the current application an authorisation is sought under Article 4 for *endo-1,4-beta-mannanase* under the category / functional group 4(a) "zootechnical additives / digestibility enhancers", according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, the authorisation is sought for chickens for fattening and other poultry for fattening.

According to the Applicant, the *feed additive* contains as *active substance endo-1,4-beta-mannanase* produced by the non-genetically modified microorganism *Aspergillus niger* (CBS 120604).

The *feed additive* is intended to be marketed under the trade name *Nutrixtend Optim* as a solid preparation with a minimum *endo-1,4-beta-mannanase* activity of 265 units (U) / g *feed additive*.

According to the Applicant, one mannanase unit (U) is defined as "the amount of enzyme required to release one micromole of mannose reducing sugar equivalents per minute at 40 ^{o}C and pH 4.5".

The *feed additive* is intended to be used directly into *feedingstuffs* or through *premixtures* at a recommended mimimum *endo-1,4-beta-mannanase* activity of 27 U / kg complete *feedingstuffs*.

For the quantification of the *endo-1,4-beta-mannanase* activity in the *feed additive*, *premixtures* and *feedingstuffs*, the Applicant submitted a single-laboratory validated and further verified colorimetric method based on the quantification of the water soluble dyed fragments produced by the action of *endo-1,4-beta-mannanase* on a commercially available (Beta-Mannazyme, Megazyme) azurine-crosslinked-carob galactomannan substrate.

Based on the overall available performance data, the EURL recommends for official control the colorimetric (Megazyme) method for the determination of the *endo-1,4-beta-mannanase* activity 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, as last amended by Regulation (EU) 2015/1761) is not considered necessary.

KEYWORDS

Endo-1,4-beta-mannanase, *Nutrixtend Optim*, zootechnical additives, digestibility enhancers, chickens for fattening, other poultry for fattening.

1. BACKGROUND

In the current application an authorisation is sought under Article 4(1) (new feed additive) for *endo-1,4-beta-mannanase* under the category / functional group 4(a) "zootechnical additives / digestibility enhancers", according to the classification system of Annex I of Regulation (EC) No 1831/2003 [1,2]. Specifically, the authorisation is sought for chickens for fattening, and other poultry for fattening [2].

According to the Applicant, the *feed additive* contains as an active substance *endo-1,4-beta-mannanase* produced by non-genetically modified microorganism *Aspergillus niger* (CBS 120604) [3].

The *feed additive* is intended to be marketed under the trade name *Nutrixtend Optim* as a solid preparation with a minimum activity of *endo-1,4-beta-mannanase* 265 units (U) / g *feed additive* [4].

According to the Applicant, one mannanase unit (U) is defined as "the amount of enzyme required to release one micromole of mannose reducing sugar equivalents per minute at 40 ^{o}C and pH 4.5" [5].

The *feed additive* is intended to be used directly into *feedingstuffs* or through *premixtures* at a recommended minimum *endo-1,4-beta-mannanase* activity of 27 U / kg complete *feedingstuffs* [5].

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761, 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 European Union Reference Laboratory concerning applications for authorisations of feed additives, the EURL is requested to submit a full evaluation report to the European Food Safety Authority for each application or group of applications. For this particular dossier, the methods of analysis submitted in connection with *endo-1,4-beta-mannanase* and their suitability to be used for official controls in the frame of the authorisation were evaluated.

3. EVALUATION

Description of the analytical methods for the determination of the active substance in the feed additive, premixtures, feedingstuffs and when appropriate water (section 2.6.1 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

For the quantification of the *endo-1,4-beta-mannanase* activity in the *feed additive*, *premixtures* and *feedingstuffs*, the Applicant submitted a single-laboratory validated [6, 7] and further verified colorimetric method [8,9] based on the quantification of the water soluble dyed fragments produced by the action of *endo-1,4-beta-mannanase* on a commercially available (Beta-Mannazyme, Megazyme) azurine-crosslinked-carob galactomannan (AZCL-galactomannan substrate) [10,11].

For the quantification of the *endo-1,4-beta-mannanase* activity in the *feed additive* and *premixtures* (1 g), the samples are extracted at room temperature with 50 mM sodium acetate buffer at pH 4.5, filtered and properly diluted.

Aliquots (0.5 ml) of the diluted samples, are pre-incubated at 40 °C for 5 min, mixed with the AZCL-galactomannan substrate and additionally incubated at 40 °C for 10 min [10, 11]. After the incubation time the stop solution (2 % Trizma base) is added followed by vortex mixing in order to stop the enzymatic reaction. A reagent blank is then prepared by adding to 0.5 ml of sodium acetate buffer, the stop solution and finally the beta-mannazyne tablet. The resulting solutions (blank and samples) are equilibrated at room temperature for 5 min, vortex mixed and filtered through paper filter. The absorbance of the samples are finally measured at 590 nm against the reagent blank. [10, 11].

Table 1. Performance characteristics of the methods for the quantification of *endo-1,4-beta-mannanase* activity in the *feed additive*, *premixtures* and *feedingstuffs*

	Feed additive		***Premixtures***		***Feedingstuffs***	
	Validation	**Verification**	**Validation**	**Verification**	**Validation**	**Verification**
Activity, U/g (or U/kg)	31908*	28580*	441**	414**	52-95**	53-102**
[(a)]RSD_r, %	3.9	1.7	5.5	12.8	11.0-13.8	13.9-15.0
[(a)]RSD_{ip}, %	5.1	1.8	5.9	12.8	13.5-13.8	13.9-15.0
[(a)]R_{Rec}, %	93-109	92	93	103	97-102	89-102
Reference	[7]	[9]	[8]	[10]	[8]	[10]

RSD_r and RSD_{ip}: relative standard deviations for *repeatability* and *intermediate precision*, respectively; R_{Rec}: a recovery rate; [(*)]U/g; [(**)]U/kg. [(a)]Recalculated by EURL [12].

For the quantification of the *endo-1,4-beta-mannanase* activity in *feedingstuffs*, the ground *feedingstuffs* samples (10 g) are stirred with sodium acetate buffer (pH 4.5) for 30 min at room temperature. The mixture is ultraturrax homogenised, stirred and centrifuged before filtrate them through a 0.45 μm filter. The obtained filtrates are then appropriately diluted with the acetate buffer [11].

An aliquot (1.0 ml) of the obtained diluted *feedingstuffs* filtrates, are pre-incubated at 40 °C for 10 min. Then the AZCL-galactomannan substrate is added to the samples and additionally incubated at 40 °C for 30 min. After the incubation time the stop solution (2 % Trizma base) is added followed by vortex mixing for stopping the enzymatic reaction. A substrate/enzyme blank is then prepared by adding the stop solution to 1.0 ml of buffer before the addition of the Beta-Mannazyne tablet. The blanks are kept at room temperature during the reaction. All solutions are further equilibrated at room temperature for another 5 min after stopping the reaction following by filtration through paper filter. The *endo-1,4-beta-mannanase* activity is finally determined by colorimetry at 590 nm using a calibration curve prepared with blank feed sample supplemented with a reference *endo-1,4-beta-mannanase* enzyme of known activity, available from the Applicant upon request. The calibrant solutions undergo a similar procedure than the *feedingstuffs* samples [11].

However, this approach requires the use of blank feed sample for the quantification of the *endo-1,4-beta-mannanase* activity in *feedingstuffs*. Therefore, whenever a blank feed sample is not available, the Applicant upon request of the EURL, proposed to prepare the calibration curve replacing the blank feed extract by a *feedingstuff* sample extract submitted beforehand to a heating treatment (at 100 ºC for 8 min.) to ensure that any enzyme present is deemed inactive [13]. The EURL considers this a suitable alternative to determine the *endo-1,4-beta-mannanase* enzyme activity in *feedingstuffs*.

The performance characteristics derived from the validation and verification studies [6-9] for the quantification of the *endo-1,4-beta-mannanase* activity in the *feed additive, premixtures* and *feedingstuffs* are presented in Table 1. In addition, the Applicant reported a limit of detection (LOD) and a limit of quantification (LOQ) of 3.3 and 4.8 U / kg *feedingstuffs*, respectively [7], which are far below the minimum *endo-1,4-beta-mannanase* activity recommended by the Applicant [4].

Based on the overall available performance data, the EURL recommends for official control the single-laboratory validated and further verified colorimetric method described above for the quantification of the *endo-1,4-beta-mannanase* activity in the *feed additive, premixtures* and *feedingstuffs*.

Methods of analysis for the determination of the residues of the additive in food (section 2.6.2 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

The evaluation of corresponding methods of analysis is not relevant for the present application.

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. as last amended by Regulation (EU) 2015/1761) is not considered necessary.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation the EURL recommends for official control the single-laboratory validated and further verified colorimetric method based on the quantification of water soluble dyed fragments produced by the action of *endo-1,4-beta-mannanase* on a commercially available (Beta-Mannazyme, Megazyme) azurine-crosslinked-carob galactomannan (AZCL-galactomannan substrate) for the determination of the *endo-1,4-beta-mannanase* activity in the *feed additive*, *premixtures* and *feedingstuffs*.

Recommended text for the register entry (analytical method)

For the determination of the *endo-1,4-beta-mannanase* activity in the *feed additive, premixtures* and *feedingstuffs:*

- colorimetric method based the enzymatic reaction of *endo-1,4-beta-mannanase* on the azurine cross-linked-carob galactomannan substrate

One mannanase unit (U) is defined as the amount of enzyme required to release one micromole of mannose reducing sugar equivalents per minute at 40 ^{o}C and pH 4.5.

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *endo-1,4-beta-mannanase* produced by *Aspergillus niger* (CBS 120604) have been sent to the European Union Reference Laboratory for Feed Additives. The dossier has been made available to the EURL by EFSA.

6. REFERENCES

[1] *Application, Reference SANTE/E5: FORW. APPL. 1831-0020-2021

[2] *Application, Annex 1 – submission number 1615453419773-2864

[3] *Technical dossier, Section II: 2.1 Identity of the additive

[4] *Technical dossier, Section II: 2.5 Conditions of use of the additive

[5] *Technical dossier, Section II: 2.6 Methods of analysis and reference samples

[6] *Technical dossier, Section II – Annex_II_62

[7] *Technical dossier, Section II – Annex_II_63

[8] *Technical dossier, Section II – Annex_II_66 & Annex II_64

[9] *Technical dossier, Section II – Annex_II_67 & Annex II_65

[10] *Technical dossier, Section II – Annex_II_6

[11] *Technical dossier, Section II – Annex_II_61

[12] Supplementary information : «eurl-anova.pdf»

[13] Supplementary information «Feed Blank Enzyme Inactivation-test.pdf»

*Refers to Dossier no: FAD-2021-0053

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation is the European Union Reference Laboratory for Feed Additives, JRC, 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 (EU) 2015/1761.

8. ACKNOWLEDGEMENTS

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- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Centro di referenza nazionale per la sorveglienza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia, Pesca, Alimentació i Medi Natural. Generalitat de Catalunya, Cabrils (ES)
- Instytut Zootechniki - Państwowy Instytut Badawczy, Krajowe Laboratorium Pasz, Lublin (PL)