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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-β-mannanase (E.C. 3.2.1.78) (Hemicell®)

(FAD-2021-0063; CRL/210014)

European Union Reference Laboratory
Feed Additives

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Dossier related to:	**FAD-2021-0063 - CRL/210014**
Name of Product:	**Endo-1,4-β-mannanase (E.C. 3.2.1.78) (Hemicell®)**
Active Agent (s):	**Endo-1,4-β-mannanase (E.C. 3.2.1.78)**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
Report prepared by:	**Zigmas Ezerskis**
Report checked by: Date:	**María José González de la Huebra** **22/08/2022**
Report approved by: Date:	**Christoph von Holst** **22/08/2022**

EXECUTIVE SUMMARY

In the current application authorisation is sought under article 4 for *endo-1,4-β-mannanase* (EC 3.2.1.78), 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 the use of the *feed additive* for chickens and turkeys for fattening, chickens reared for laying, turkeys reared for breeding, minor poultry species, piglets (weaned), pigs for fattening and minor porcine species.

According to the Applicant, the *feed additive* contains the active substance *endo-1,4-β-mannanase* (EC 3.2.1.78) produced by the genetically modified *Paenibacillus lentus* CMG3376 (DSM 33618) strain.

The *feed additive* is intended to be marketed as solid (*Hemicell®HT*) or liquid (*Hemicell®HT-L*) enzyme formulations with a minimum activity of *endo-1,4-β-mannanase* in the products of 1.6 x 10^8 U / kg and 5.9 x 10^8 U / L, respectively.

According to the Applicant, the activity of *endo-1,4-β-mannanase* is expressed in units (U) or in 10^6 units (MU), where 1 U is defined as the amount of *endo-1,4-β-mannanase* enzyme which generates 0.72 micrograms of reducing sugars per minute from a mannose containing substrate at pH 7.0 and 40 °C.

The *feed additive* is intended to be used in *premixtures* and *feedingstuffs* at a proposed minimum *endo-1,4-β-mannanase* activity of 32000 U / kg or 48000 U / kg complete *feedingstuffs*, depending on the animal species.

For the quantification of the *endo-1,4-β-mannanase* activity in the *feed additive* and *feedingstuffs*, the Applicant submitted several single-laboratory validated and further verified colorimetric methods based on the enzymatic hydrolysis of mannose containing substrate (locust bean gum, LBG) by the action of *endo-1,4-β-mannanase* followed by the reaction of the released reducing sugars (mannose equivalent) with 3,5-dinitrosalicylic acid (DNS).

For the quantification of *endo-1,4-β-mannanase* activity in *premixtures* the Applicant diluted premixture samples with blank feed and applied one of the above mentioned method for *feedingstuffs*.

Based on the performance characteristics obtained, the EURL recommends for official control the above mentioned single-laboratory validated and further verified colorimetric methods, based on the enzymatic hydrolysis and the reaction of the reducing sugars (mannose equivalent) with 3,5-dinitrosalicylic acid (DNS), for the quantification of *endo-1,4-β-mannanase* total 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-β-mannanase, *Hemicell*, zootechnical additives, digestibility enhancers, chickens and turkeys for fattening, chickens reared for laying, turkeys reared for breeding, minor poultry species, piglets (weaned), pigs for fattening and minor porcine species.

1. BACKGROUND

In the current application an authorisation is sought under article 4(1) (new feed additive) for *endo-1,4-β-mannanase* (EC 3.2.1.78), under the category / functional group 4(a) "zootechnical additives / digestibility enhancers" [1,2], according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, the authorisation is sought for the use of the *feed additive* for chickens and turkeys for fattening, chickens reared for laying, turkeys reared for breeding, minor poultry species, piglets (weaned), pigs for fattening and minor porcine species [2].

According to the Applicant, the *feed additive* contains the active substance *endo-1,4-β-mannanase* (EC 3.2.1.78) produced by the genetically modified *Paenibacillus lentus* CMG3376 (DSM 33618) strain [3,4].

The *feed additive* is intended to be marketed as solid (*Hemicell®HT*) or liquid (*Hemicell®HT-L*) enzyme formulations with a minimum activity of *endo-1,4-β-mannanase* in the products of 1.6×10^8 U / kg and 5.9×10^8 U / L, respectively [3,4].

According to the Applicant, the activity of *endo-1,4-β-mannanase* is expressed in units (U) or in 10^6 units (MU), where 1 U is defined as the amount of *endo-1,4-β-mannanase* enzyme, which generates 0.72 micrograms of reducing sugars per minute from a mannose containing substrate at pH 7.0 and 40 °C [3,4].

The *feed additive* is intended to be used in *premixtures* and *feedingstuffs* at a proposed minimum *endo-1,4-β-mannanase* activity of 32000 U / kg or 48000 U / kg complete *feedingstuffs*, depending on the animal species [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-β-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-β-mannanase* activity in the *feed additive* and *feedingstuffs*, the Applicant submitted several single-laboratory validated and further verified colorimetric methods based on the enzymatic hydrolysis of mannose containing substrate (locust bean gum, LBG) by the action of *endo-1,4-β-mannanase* followed by the reaction of the released reducing sugars (mannose equivalent) with 3,5-dinitrosalicylic acid (DNS) [6-9].

For the quantification of *endo-1,4-β-mannanase* activity in *Hemicell®HT-L*, the sample (1 g) is diluted with Glycine buffer (pH 8.5) containing Bovine Serum Albumin (BSA) and pre-incubated at 40 °C for 15 min [6]. For *Hemicell®HT*, the sample (10 g) is mixed with distilled water and shaken at 250 rpm for 40 min at room temperature. An aliquot of the latter extract is diluted with the above mentioned Glycine buffer containing BSA and pre-incubated under the same conditions as the sample of *Hemicell®HT-L*. All samples are then incubated with LBG substrate at 40 °C for 10 min. DNS is added to the incubated and non-incubated (blank) samples to stop the enzymatic reaction. Samples are further reacted for 5 to 15 min in boiling bath and centrifuged, then cooled to room temperature. The absorbance of the yellow-orange coloured solutions is measured spectrophotometrically at 550 nm. The *endo-1,4-β-mannanase* activity is derived from the difference in absorbance of the incubated and non-incubated (blank) with LBG aliquots. The quantification is performed using *endo-1,4-β-mannanase* enzyme external reference standard calibration curve prepared from the reference standards, which undergo the standard operating procedure described above [6].

According to another protocol of the method for the quantification of *endo-1,4-β-mannanase* activity in the *feed additive*, which is a similar to the one described above, an automatised liquid handling system is used [7].

For the quantification of *endo-1,4-β-mannanase* activity in *feedingstuffs*, the sample (10 g) is at first ground and mixed with distilled water, shaken for 40 min and centrifuged [8]. An aliquot of the supernatant is incubated with LBG mannose substrate in Tris buffer (pH 7.0) at 40 °C for 60 min. Aliquots of the incubated samples (for 5 to 15 min or 50 to 60 min) are treated with DNS at 100 °C for 5 to 15 min, centrifuged, then cooled down to room temperature. The absorbance of the yellow-orange coloured solutions is measured spectrophotometrically at 550 nm. The difference in absorbance of the two sample aliquots (ΔOD) with different incubation times is proportional to the total *endo-1,4-β-mannanase* activity. The quantification is performed using an external standard calibration curve, prepared from mannose standards treated with DNS in the same manner as in case of samples [8].

In addition, in the frame of supplementary information [9] the Applicant has provided another similar method for the quantification of *endo-1,4-β-mannanase* activity in *feedingstuffs* [10].

Following the protocol of this method, the ground sample (19.5 to 20.5 g) is mixed with 0.1 % aqueous solution of Bovine Serum Albumin, shaken for 60 ± 15 min and centrifuged. An aliquot of the supernatant is purified by the PD-10 desalting column using Tris buffer (pH 7.0) for the elution. The aliquot of the eluent is incubated with LBG mannose substrate in Tris buffer (pH 7.0) at 40 °C for 180 min. The aliquots of the incubated samples (for 30 min or 180 min) are reacted with DNS at 95 ± 5 °C for 30 min, then cooled down to room temperature and centrifuged. The absorbance of the yellow-orange coloured solutions is measured spectrophotometrically at 550 nm. The difference in absorbance of the two sample aliquots (ΔOD) with different incubation times is proportional to the total *endo-1,4-β-mannanase* activity. The quantification is performed using an external standard calibration curve prepared from the reference standards of *endo-1,4-β-mannanase* enzyme, which undergo the standard operating procedure as the samples [10].

The performance characteristics of the above described methods [6-8,10] reported by the Applicant and recalculated by the EURL [11] in the frame of the validation [12-14] and the verification studies [15-18] for the quantification of *endo-1,4-β-mannanase* total activity in the *feed additive*: *Hemicell®HT* (dry) & *Hemicell®HT-L* (liquid) and *feedingstuffs* are presented in Table 1.

In addition, the Applicant has set the limits of quantification (LOQ) of 300 and 15000 U / kg *feedingstuffs* [13,18] for the two different methods [8,10] for the quantification of *endo-1,4-β-mannanase* activity in *feedingstuffs*. These LOQ values are below the minimum activity recommended by the Applicant in the conditions of use [5].

Table 1: Performance characteristics of the methods [6-8,10] reported by the Applicant and recalculated by the EURL [11] in the frame of the validation [12-14] and the verification studies [15-18] for the quantification of *endo-1,4-β-mannanase* total activity in the *feed additive*: *Hemicell®HT* (dry) & *Hemicell®HT-L* (liquid) and *feedingstuffs*

	Feed additive (Hemicell® HT)		***Feed additive (Hemicell® HT-L)***		***Feedingstuffs***	
	Validation	**Verification**	**Validation**	**Verification**	**Validation**	**Verification**
Activity, MU/kg	264		704		0.015 – 10	
RSD_r, %	1.6	2.5 – 5.9	2.8	2.5 – 6.1	1.9 – 12.3	3.5 – 10.4
RSD_{ip}, %	6.8	5.9 – 7.0	4.6	6.3 – 7.6	3.0 – 22.6	5.5 – 11.3
R_{Rec}, %	93	91 – 94	96	95 – 98	86 – 119	77 – 126
Reference	[11,12]	[11,15,16]	[11,12]	[11,15,16]	[11,13,14]	[11,17,18]

MU: mega ($x10^6$) units; RSD_r and RSD_{ip}: relative standard deviation for *repeatability* and *intermediate precision*, respectively; R_{Rec}: a recovery rate.

For the quantification of *endo-1,4-β-mannanase* activity in *premixtures* the Applicant diluted premixture samples with blank feed [19] and applied the above mentioned method for *feedingstuffs* [8]. Two vitamin/mineral *premixtures* for poultry and swine were analysed in the frame of homogeneity studies and a relative standard deviation for *repeatability* (RSD_r) ranging from 15.8 to 17.2 % was reported for an average *endo-1,4-β-mannanase* activity ranging from 50 to 59 MU/kg *premixtures* [19].

Based on the performance characteristics presented, the EURL recommends for official control the above mentioned single-laboratory validated and further verified colorimetric methods, based on the enzymatic hydrolysis and the reaction of the reducing sugars (mannose equivalent) with 3,5-dinitrosalicylic acid (DNS), for the quantification of *endo-1,4-β-mannanase* total 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)

An 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 methods, based on the enzymatic hydrolysis and the reaction of the reducing sugars (mannose equivalent) with

3,5-dinitrosalicylic acid (DNS), to quantify the total activity of *endo-1,4-β-mannanase* in the *feed additive*, *premixtures* and *feedingstuffs*.

Recommended text for the register entry (analytical method)

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

- colorimetric methods based on enzymatic hydrolysis and the reaction of reducing sugars (mannose equivalent) with 3,5-dinitrosalicylic acid (DNS)

One unit of *endo-1,4-β-mannanase* activity (U) is the amount of the enzyme which generates 0.72 micrograms of reducing sugars per minute from a mannose containing substrate at pH 7.0 and 40 °C.

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Hemicell®HT* and *Hemicell®HT-L* 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_FWD. APPL. 1831-0056-2021

[2] *Application, Annex 1 – Submission No. 1615563827555-2894

[3] *Technical dossier, Section II: 2. Introduction

[4] *Technical dossier, Section II: 2.1.3. Qualitative and quantitative composition (active substance/agent, other components, impurities, batch to batch variation)

[5] *Technical dossier, Section II: 2.5.1. Proposed mode of use in animal nutrition

[6] *Technical dossier, Section II – Annex II_6_1_1

[7] *Technical dossier, Section II – Annex II_6_1_7

[8] *Technical dossier, Section II – Annex II_6_1_4

[9] *Supplementary information – 2_Spontaneus submission Hemicell HT

[10] *Supplementary information – Annex II_6_1_9

[11] *Supplementary information – Performance characteristics calculation by EURL

[12] *Technical dossier, Section II – Annex II_6_1_2

[13] *Technical dossier, Section II – Annex II_6_1_5

[14] *Supplementary information – Annex II_6_1_10

[15] *Technical dossier, Section II – Annex II_6_1_3

[16] *Technical dossier, Section II – Annex II_6_1_8

[17] *Technical dossier, Section II – Annex II_6_1_6

[18] *Supplementary information – Annex II_6_1_11

[19] *Technical dossier, Section II – Annex II_4_1_5

*Refers to Dossier no: FAD-2021-0063

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

The following National Reference Laboratories contributed to this report:

- Centro di referenza nazionale per la sorveglienza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia, PESCA, Alimentació i Medi Natural. Generalitat de Catalunya, Cabrils (ES)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)