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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-beta-D-mannanase

(FAD-2020-0107; CRL/200066)

European Union Reference Laboratory
Feed Additives

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

Dossier related to:	**FAD-2020-0107 - CRL/200066**
Name of Product:	***Endo-1,4-beta-D-mannanase***
Active Agent (s):	**Endo-1,4-beta-D-mannanase**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
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Report checked by: Date:	**María José González de la Huebra** **27/05/2021**
Report approved by: Date:	**Christoph von Holst** **27/05/2021**

EXECUTIVE SUMMARY

In the current application an authorisation is sought under Article 4(1) for *endo-1,4-beta-D-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, turkeys for fattening, other poultry for fattening, minor growing poultry species and ornamental birds.

According to the Applicant, the *feed additive* contains as an active substance *endo-1,4-beta-D-mannanase* produced by the genetically modified *Thermothelomyces thermophilus* strain LU19287 (DSM 33149).

The *feed additive* is intended to be marketed under the trade name *Natupulse*® as solid (*Natupulse*®*TS*) or liquid (*Natupulse*®*TS L*) formulations with a minimum activity of *endo-1,4-beta-D-mannanase* of 8000 thermostable mannanase units (TMU) / g *feed additive.*

According to the Applicant, one TMU is defined as the amount of enzyme that produces reducing carbohydrates, having a reducing power corresponding to one µmol of mannose, from locust bean gum (0.3 g/100 ml buffer solution) in one minute at 50 °C and pH 3.5.

The *feed additive* is intended to be used directly into *feedingstuffs* or through *premixtures* at a proposed minimum *endo-1,4-beta-D-mannanase* activity of 800 TMU/kg complete *feedingstuffs*.

For the quantification of the *endo-1,4-beta-D-mannanase* activity in the *feed additive*, *premixtures* and *feedingstuffs*, the Applicant submitted two single-laboratory validated and further verified methods based on the enzymatic hydrolysis of dyed carob galactomannan with the *endo-1,4-beta-D-mannanase*, where the amount of released low-molecular weight dyed fragments are measured by photometry. Based on this measurement, the enzyme activity of the sample is then quantified against a standard enzyme with known activity.

Based on the overall available performance data, the EURL recommends for official control the two above mentioned single-laboratory validated and further verified methods based on the enzymatic hydrolysis of dyed carob galactomannan with the *endo-1,4-beta-D-mannanase*, followed by photometric determination of dyed fragments, for official control for the determination of *endo-1,4-beta-D-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-D-mannanase, *Natupulse*®, zootechnical additives, digestibility enhancers, chickens for fattening, turkeys for fattening, other poultry for fattening, minor growing poultry species, ornamental birds.

1. BACKGROUND

In the current application an authorisation is sought under Article 4(1) (new feed additive) for *endo-1,4-beta-D-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, turkeys for fattening, other poultry for fattening, minor growing poultry species and ornamental birds [2].

According to the Applicant, the *feed additive* contains as an active substance *endo-1,4-beta-D-mannanase* produced by the genetically modified *Thermothelomyces thermophilus* strain LU19287 (DSM 33149) [3].

The *feed additive* is intended to be marketed under the trade name *Natupulse*® as solid (*Natupulse*®*TS*) or liquid (*Natupulse*®*TS L*) formulations [3] with a minimum activity of *endo-1,4-beta-D-mannanase* of 8000 thermostable mannanase units (TMU) / g *feed additive* [4].

According to the Applicant, one TMU is defined as the amount of enzyme that produces reducing carbohydrates, having a reducing power corresponding to one µmol of mannose, from locust bean gum (0.3 g/100 ml buffer solution) in one minute at 50 °C and pH 3.5 [4].

The *feed additive* is intended to be used directly into *feedingstuffs* or through *premixtures* at a proposed minimum *endo-1,4-beta-D-mannanase* activity of 800 TMU/kg complete *feedingstuffs* [2,5].

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-D-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-D-mannanase* activity in the *feed additive*, *premixtures* and *feedingstuffs*, the Applicant submitted two single-laboratory validated and further verified methods [6,7] based on the enzymatic hydrolysis of dyed carob galactomannan with the *endo-1,4-beta-D-mannanase*, where the amount of released low-molecular weight dyed fragments are measured by photometry. Based on this measurement, the enzyme activity of the sample is then quantified against a standard enzyme with known activity.

For the quantification of the *endo-1,4-beta-D-mannanase* activity in the *feed additive*, the samples (0.5 to 2 g), for which the carrier is soluble in water, are diluted with citrate buffer containing Tween® 20 (pH 3.5). The samples, for which the carrier of the *feed additive* preparation is not soluble in water, are suspended by mixing them in the above mentioned buffer solution for 30 min followed by filtration. Aliquots of the prepared samples, after their pre-incubation at 40 °C for 5 min, are mixed with the Azo-Carob-Galactomannan substrate solution in the citrate buffer (2 g/ml) and additionally incubated at 40 °C for 20 min. The enzymatic reaction is stopped by adding the precipitation solution composed of industrial methylated spirits (95 %, v/v) or pure ethanol (95 %, v/v) into the reaction mixture followed by mixing. The resulting solutions are centrifuged after equilibration at room temperature for 10 min at 3000 *g* for 15 min. The supernatants, containing low-molecular weight dyed fragments formed from the reaction of the *endo-1,4-beta-D-mannanase* on the substrate, are analysed by photometry at 590 nm [6].

The blank samples are prepared from the above mentioned citrate buffer, the substrate and the precipitation solution. The absorbance of the blank samples is subtracted from the absorbance values of the sample and the standard solutions, respectively. In addition, for quality control purposes, control samples containing a known activity of the enzyme are prepared. The quantification of the *endo-1,4-beta-D-mannanase* activity is performed using an external calibration curve prepared from the standard samples of the *endo-1,4-beta-D-mannanase* [6] with known activity, which were subjected to identical experimental conditions as the blank, the control and target samples.

For the quantification of the *endo-1,4-beta-D-mannanase* activity in *premixtures* and *feedingstuffs*, the ground samples of the *premixtures* (0.5 g) or *feedingstuffs* (50 g) are stirred with added citrate buffer containing Tween® 20 (pH 3.5) for 30 min. Aliquots of the extracts are centrifuged at 3000 *g* for 15 min and the supernatants are filtered. The prepared samples

are mixed, after their pre-incubation at 50 °C for 5 min, with a Beta-Mannazyme tablet (T-MNZ, Megazyme) and left for an enzymatic reaction at 50 °C for 30 min without stirring. The reaction is stopped by adding the above mentioned precipitation solution followed by vigorous mixing. Afterwards, the mixtures are filtered for further analysis by photometry at 590 nm. The blank and control samples are prepared similarly as in case of the *feed additive*. For the quantification of the *endo-1,4-beta-D-mannanase* activity, the absorbance of the blank samples is subtracted from the absorbance values of the target and standard samples, respectively. The quantification is performed using an external calibration curve prepared from the standard samples of the *endo-1,4-beta-D-mannanase* with known activity, which were subjected to identical experimental conditions as the blank, the control and target samples [7].

The performance characteristics derived from the validation and verification studies [8-15] for the quantification of the *endo-1,4-beta-D-mannanase* activity in the *feed additive*, *premixtures* and *feedingstuffs* are presented in Table 1. In addition, the Applicant reported a limit of quantification (LOQ) of 33 TMU/kg *feedingstuffs* [12], which is below the minimum activity recommended by the Applicant in the conditions of use [2,5].

In addition, the Applicant presented stability data for the different batches of the *feed additive* at different storage conditions [16,17]. An RSD_r ranging from 0.1 to 2.2 % and 0.4 to 1.3 %, respectively, was derived for the *feed additive* samples with the enzymatic activity ranging from 8690 to 9631 TMU/g and 167371 to 247971 TMU/g [16,17]. Furthermore, the Applicant presented data from homogeneity studies for *premixtures* [18], mash and pelleted *feedingstuffs* [19].

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

	Feed additive		***Premixtures***		***Feedingstuffs***	
	Validation	**Verification**	**Validation**	**Verification**	**Validation**	**Verification**
Activity, TMU/g (or kg)	8144 – 241534(*)	224750(*)	22000(**)	23487(**)	461 – 1950000(**)	1950000(**)
RSD_r, %	1.7 – 4.8	3.4	11.3	8.8	3.3 – 6.1	1.8
RSD_{ip}, %	2.3 – 5.0	8.8	17.1	11.6	3.8 – 6.5	2.8
R_{Rec}, %	94 – 111	95	107	100	100 – 102	107
Reference	[8-11,13]	[13]	[15]	[15]	[12,14]	[14]

RSD_r and RSD_{ip}: relative standard deviations for *repeatability* and *intermediate precision*, respectively; R_{Rec}: a recovery rate; (*) TMU/g; (**) TMU/kg.

The following performance characteristics were obtained in the frame of the homogeneity studies: (i) an RSD_r ranging from 9.6 to 13.7 % and an R_{Rec} ranging from 94 to 104 % for *premixtures* with the enzymatic activity ranging from 78386 to 84489 TMU/kg [18]; and (ii) an RSD_r ranging from 3.6 to 10.4 % and an R_{Rec} ranging from 81 to 111 % for *feedingstuffs* with the enzymatic activity ranging from 728 to 1006 TMU/kg [19].

The obtained performance characteristics from the stability and homogeneity studies were additionally used by the EURL when evaluating the suitability of the proposed methods for official control.

Based on the overall available performance data, the EURL recommends for official control the two above mentioned single-laboratory validated and further verified methods for official control for the determination of the *endo-1,4-beta-D-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)

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

A 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 two single-laboratory validated and further verified methods based on the enzymatic hydrolysis of dyed carob galactomannan with the *endo-1,4-beta-D-mannanase*, followed by a photometric determination of dyed fragments, for official control for the determination of the *endo-1,4-beta-D-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-β-mannanase* activity in the *feed additive, premixtures* and *feedingstuffs:*

- Enzymatic hydrolysis of dyed carob galactomannan with the *endo-1,4-beta-D-mannanase* followed by photometry

One TMU is defined as the amount of enzyme that produces reducing carbohydrates, having a reducing power corresponding to one μmol of mannose, from locust bean gum (0.3 g/100 ml buffer solution) in one minute at 50 °C and pH 3.5.

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *endo-1,4-β-mannanase* 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. 1831-0002-2021

[2] *Application, Annex 1 – submission number 1607680358379-2752

[3] *Technical dossier, Section II: 2.1.1. Name of the additive

[4] *Technical dossier, Section II: 2.1.3. Qualitative and Quantitative composition of the additive

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

[6] *Technical dossier, Section II – Annex_II_19

[7] *Technical dossier, Section II – Annex_II_165

[8] *Technical dossier, Section II – Annex_II_167

[9] *Technical dossier, Section II – Annex_II_168

[10] *Technical dossier, Section II – Annex_II_169

[11] *Technical dossier, Section II – Annex_II_170

[12] *Technical dossier, Section II – Annex_II_172

[13] *Technical dossier, Section II – Annex_II_173

[14] *Technical dossier, Section II – Annex_II_174

[15] Supplementary information – Annex I_Verification Report_Mannanase-Natupulse Premix

[16] *Technical dossier, Section II – Annex_II_153

[17] *Technical dossier, Section II – Annex_II_154

[18] *Technical dossier, Section II – Annex_II_159

[19] *Technical dossier, Section II – Annex_II_160

*Refers to Dossier no: FAD-2020-0107

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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- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Instytut Zootechniki — Państwowy Instytut Badawczy, Krajowe Laboratorium Pasz, Lublin (PL)
- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia, PESCA, Alimentació i Medi Natural. Generalitat de Catalunya, Cabrils (ES)