EUROPEAN COMMISSION
DIRECTORATE GENERAL
JOINT RESEARCH CENTRE
Directorate F – Health, Consumers and Reference Materials
European Union Reference Laboratory for Feed Additives

JRC F.5/CvH/ZE/mds/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

Polygalacturonase (EC. 3.2.1.15) produced by Aspergillus oryzae DSM 23104

(FAD-2016-0013; CRL/160000)

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-2016-0013 - CRL/160000**
Feed additive:	**Polygalacturonase (EC. 3.2.1.15) produced by Aspergillus oryzae DSM 23104**
Active Agent (s):	**Polygalacturonase**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA) JRC Geel, Belgium**
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Report approved by: Date:	**Christoph von Holst 16/01/2017**

EXECUTIVE SUMMARY

In the current application authorisation is sought under article 4(1) for *Polygalacturonase (EC 3.2.1.15) produced by Aspergillus oryzae DSM 23104*, 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, authorisation is sought for the use of the *feed additive* for chickens for fattening.

The Applicant is expressing the *polygalacturonase* enzymatic activity in units (U), where one *polygalacturonase* unit (U) is the amount of enzyme which liberates at pH 4.0 and 50 ^{o}C 1 μmol/min of reducing sugars (expressed as equivalent of glucose) from the poly-D-galacturonic acid methyl ester (citrus pectin) substrate.

The *feed additive* is to be marketed as beige to brown granules having a minimum *polygalacturonase* activity of 180 U/g product. The product also contains maize powder, sugar beet molasses, inactivated fungal biomass, organic acids and water. The *feed additive* is intended to be included through *premixtures* in *feedingstuffs* with a minimum *polygalacturonase* activity of 36 U/kg complete *feedingstuffs*.

For the quantification of the *polygalacturonase* activity in the *feed additive* the Applicant submitted a single-laboratory validated and further verified colorimetric method, based on the colorimetric reaction of 3,5-dinitrosalicylic acid (DNS) with reducing sugars released by the action of *polygalacturonase* on the poly-D-galacturonic acid methyl ester (citrus pectin) substrate. The following performance characteristics were reported for *feed additive* samples containing a *polygalacturonase* activity ranging from 233 to 322 U/g: a relative standard deviation for *repeatability* (RSD_r) ranging from 6.6 to 9.0%; a relative standard deviation for *intermediate precision* (RSD_{ip}) ranging from 7.0 to 9.7%; and a *recovery* rate (R_{Rec}) of 97%. Furthermore, the Applicant applied this colorimetric method for the quantification of *polygalacturonase* activity in *premixtures* and reported a RSD_r of 8.7% and a R_{Rec} of 100% for *premixtures* samples containing a *polygalacturonase* activity of 8.4 U/g. These performance characteristics are similar to those reported for the *feed additive*, thus confirming the suitability of this method to quantify the *polygalacturonase* activity in *premixtures*.

For the quantification of the *polygalacturonase* activity in *feedingstuffs* the Applicant submitted another single-laboratory validated and further verified viscosimetric method, based on viscosity reduction as a result of enzymatic hydrolysis by the action of *polygalacturonase* on the poly-D-galacturonic acid methyl ester (citrus pectin) substrate. The following performance characteristics were reported for *feedingstuffs* samples containing a *polygalacturonase* activity of 52 U/kg *feedingstuffs*: RSD_r and RSD_{ip} ranging from 6.6 to

7.9%; R_{Rec} of 94%; and a limit of quantification (LOQ) of 30 U/kg *feedingstuffs*, which is below the minimum activity specified by the Applicant for the conditions of use.

Based on the performance characteristics available, the EURL recommends for official control the two single-laboratory validated and further verified colorimetric and/or viscosimetric methods for the quantification of the *polygalacturonase* activity in the *feed additive*, *premixtures* and/or *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

Polygalacturonase (EC. 3.2.1.15), Fermentation product of Aspergillus oryzae DSM 23104 - Polygalacturonase (EC. 3.2.1.15), zootechnical additives, digestibility enchancers, chickens for fattening

1. BACKGROUND

In the current application authorisation is sought under article 4(1) (new feed additive) for *Polygalacturonase (EC 3.2.1.15) produced by Aspergillus oryzae DSM 23104*, 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]. Specifically, authorisation is sought for the use of the *feed additive* for chickens for fattening [1,2].

According to the Applicant, the active substance in the product is *polygalacturonase (EC 3.2.1.15)* produced by fermentation of non-genetically modified strain of *Aspergillus oryzae DSM 23104* [2,3].

The Applicant is expressing the *polygalacturonase* enzymatic activity in units (U), where one *polygalacturonase* unit (U) is the amount of enzyme which liberates at pH 4.0 and 50 ^{o}C 1 μmol/min of reducing sugars (expressed as equivalent of glucose) from the poly-D-galacturonic acid methyl ester (citrus pectin) substrate.

The *feed additive* is to be marketed as beige to brown granules having a minimum *polygalacturonase* activity of 180 U/g product. The product also contains maize powder, sugar beet molasses, inactivated fungal biomass, organic acids and water [3].

The *feed additive* is intended to be included through *premixtures* into *feedingstuffs* with a minimum *polygalacturonase* activity of 36 U/kg complete *feedingstuffs* [2].

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 *Polygalacturonase (EC 3.2.1.15) produced by Aspergillus oryzae DSM 23104* and their suitability to be used for official control 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 European Union Reference Laboratories [4].

Description of the analytical methods for the determination of the active substance in feed additive, premixtures and feedingstuffs

For the quantification of *polygalacturonase* activity in the *feed additive* and *premixtures* the Applicant submitted a single-laboratory validated and further verified colorimetric method, based on the colour formation of released reducing sugars, expressed as glucose equivalents, with 3,5-dinitrosalicylic acid (DNS). The assay is based on the enzymatic hydrolysis of *polygalacturonase* on the poly-D-galacturonic acid methyl ester (citrus pectin) substrate at pH 4.0 and 50 °C [5].

The *feed additive* sample (1 g) is mixed with 0.1 M citrate buffer (pH 4.0), the solution is stirred for 10 min and further centrifuged. After a 20 fold dilution, the supernatant (0.1 ml) is placed into a test tube together with 0.2 ml of the citrus pectin substrate (poly-D-galacturonic acid methyl ester at 0.01 g/ml citrate buffer) and incubated at 50 °C for 10 min. The *premixtures* sample (2 g) is mixed with the citrate buffer, the solution is stirred for 35 min and centrifuged. After a 2 fold dilution, the supernatant (0.1 ml) is placed into a test tube together with 0.2 ml of the citrus pectin substrate and incubated at 50 °C for 10 min. After incubation, 0.8 ml of a 10 % DNS solution is added to the test samples (*feed additive* and *premixtures*) and boiled at 100 °C for 10 min. After that, the samples are cooled down and centrifuged. In addition a "reagent blank" and a "sample blank" (*feed additive* and *premixtures* containing

inactivated enzyme) are prepared using the same conditions as for the test samples. Analysis of the solutions is performed by colorimetry at 550 nm. The absorption of the *feed additive* and *premixtures* samples are corrected by subtracting the absorptions of the "reagent blank" and of the "sample blank". The content of *polygalacturonase* is quantified using a standard glucose (external) calibration curve, prepared from the glucose calibration solutions, which undergo the same sample treatment conditions as described above – only without the presence of the *feed additive* or *premixtures* [5].

For the quantification of the *polygalacturonase* activity in *feedingstuffs* the Applicant submitted another single-laboratory validated and further verified viscosimetric method, based on viscosity reduction as a result of enzymatic hydrolysis by the action of *polygalacturonase* on the poly-D-galacturonic acid methyl ester (citrus pectin) substrate [6].

The *feedingstuffs* sample (40 g) is mixed with a 0.1 M citrate buffer solution (pH 4.0) for 35 min. The extract is then centrifuged and filtered. An aliquot of the extract from the test sample is incubated with the pectin substrate at 50 ^{o}C for 4 h. After cooling the reaction mixture, the viscosity is measured at 25 ^{o}C with a shear rate of 50 s^{-1}, using a commercially available rheometer. The "sample blank" prepared by autoclaving an aliquot of a *feedingstuffs* sample undergoes the same sample preparation and measurement conditions as the corresponding test sample.

The matrix-matched calibration standards are prepared from sub-samples of a "blank sample" (*feedingstuffs* with inactivated enzyme) fortified with different amounts of the *feed additive* of known *polygalacturonase* activity and submitted to the same sample preparation and measurement conditions as the test *feedingstuffs* samples. A calibration curve is established from these measurements and used to quantify the *polygalacturonase* activity of the *feedingstuffs* sample [6].

Table 1 presents the performance characteristics of the above described colorimetric and viscosimetric methods obtained in the frame of the validation and verification studies [7, 8]. In addition, the Applicant reported the limit of quantification (LOQ) of 30 U/kg *feedingstuffs* (see Fig.5 in [8]), which is below the minimum activity specified by the Applicant for the conditions of use [2]. In addition, the Applicant performed the tests for selectivity, matrix interferences of the method using protease or amylase enzymes. The results of viscosimetry measurements of the test samples, containing *polygalacturonase* showed no significant differences in the presence or absence of the mentioned non-target enzymes [8]. Furthermore, according to the Applicant, the presence of the other (than protease and amylase) non-target enzymes in the test samples, does not influence significantly the determination of *polygalacturonase* content in feed.

Table 1: Performance characteristics for the quantification of *polygalacturonase* activity in the *feed additive*, *premixtures* and *feedingstuffs*

	Feed additive		*Premixtures*	*Feedingstuffs*	
	Validation	Verification	Validation	Validation	Verification
Activity, U/kg	233000 – 322000		8400	52	
RSD_r, %	6.6 – 8.2	6.6 – 9.0	8.7	7.3	6.6
RSD_{ip}, %	7.3 – 9.5	7.0 – 9.7	–	7.9	7.4
R_{Rec}, %	97	–	100	95	94
Reference	[7]		[9]	[8]	

RSD_r and RSD_{ip}: relative standard deviation for *repeatability* and *intermediate precision*, respectively; R_{Rec}: a recovery rate.

Based on the performance characteristics available, the EURL recommends for official control the two single-laboratory validated and further verified colorimetric and/or viscosimetric methods described above for the quantification of *polygalacturonase* activity in the *feed additive*, *premixtures* and/or *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 EURL recommends for official control two single-laboratory validated and further verified methods:

- colorimetric method, based on the enzymatic hydrolysis by the action of *polygalacturonase* on the poly-D-galacturonic acid methyl ester (citrus pectin) substrate and the reaction of reducing sugars with 3,5-dinitrosalicylic acid (DNS), for the quantification of *polygalacturonase* activity *feed additive* and *premixtures*; and
- viscosimetric method, based on viscosity reduction as a result of enzymatic hydrolysis by the action of *polygalacturonase* on the poly-D-galacturonic acid methyl ester (citrus pectin) substrate, for the quantification of *polygalacturonase* activity in the *feedingstuffs*.

Recommended text for the register entry (analytical method)

For the quantification of *polygalacturonase* activity in the *feed additive* and *premixtures*:

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

For the quantification of *polygalacturonase* activity in *feedingstuffs*:

- viscosimetric method, based on viscosity reduction as a result of enzymatic hydrolysis

One polygalacturonase unit (U) is the amount of enzyme which liberates at pH 4.0 and 50 ^{o}C 1 μmol/min of reducing sugars (expressed as equivalent of glucose) from the poly-D-galacturonic acid methyl ester (citrus pectin) substrate.

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Polygalacturonase (EC 3.2.1.15) produced by Aspergillus oryzae DSM 23104* 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. 1831/0012-2016
[2] *Application, Proposal for Register Entry – Annex A
[3] *Technical dossier, Section II
[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 – Annex II.6-1
[6] *Supplementay information – 2016-12-21 - FAD-2016-0013_Polygalacturonase - Annex II.6-4
[7] *Technical dossier, Section II – Annex II.6-2
[8] *Technical dossier, Section II – Annex II.6-5
[9] *Technical dossier, Section II – Annex II.6-3

*Refers to Dossier no: FAD-2016-0013

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)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Instytut Zootechniki – Państwowy Instytut Badawczy, Krajowe Laboratorium Pasz, Lublin (PL)
- Thüringer Landesanstalt für Landwirtschaft (TLL). Abteilung Untersuchungswesen. Jena (DE)
- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia, PESCA, Alimentació i Medi Natural. Generalitat de Catalunya, Cabrils (ES)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Państwowy Instytut Weterynaryjny, Pulawy (PL)