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**Food and Feed Compliance**

European Union Reference Laboratory
Feed Additives

JRC F.5/CvH/SB/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

***Fumonisin B1 esterase (3.1.1.87)***
***produced by Komagataella phaffii NCAIM (P) Y001485***
***(FAD-2021-0055; CRL/210039)***

European Union Reference Laboratory
Feed Additives

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| Dossier related to: | **FAD-2021-0055 - CRL/210039** |
| Name of Product: | ***Fumonisin B1 esterase (3.1.1.87) produced by Komagataella phaffii NCAIM (P) Y001485*** |
| Active Agent (s): | **Fumonisin esterase (EC 3.1.1.87)** |
| Rapporteur Laboratory: | **European Union Reference Laboratory for Feed Additives (EURL-FA)**<br>**JRC Geel, Belgium** |
| Report prepared by: | **Stefano Bellorini** |
| Report checked by:<br>Date: | **Zigmas Ezerskis**<br>**22/02/2022** |
| Report approved by:<br>Date: | **Christoph von Holst**<br>**22/02/2022** |

## EXECUTIVE SUMMARY

In the current application an authorisation of *fumonisin B1 esterase (3.1.1.87) produced by Komagataella phaffii NCAIM (P) Y001485* is sought under Article 4 and under the category/functional group 1(m) "technological additives"/"substances for reduction of the contamination of feed by mycotoxins: substances that can suppress or reduce the absorption, promote the excretion of mycotoxins or modify their mode of action" according to Annex I of Regulation (EC) No 1831/2003. The authorisation is sought for the use of the *feed additive* for all pigs (piglets, pigs for fattening, sows and minor growing and reproductive porcine species). According to the Applicant, the *feed additive* contains as active substance *fumonisin esterase (EC 3.1.1.87)* which is produced by *Komagataella phaffii NCAIM Y001485*.

The activity of *fumonisin B1 esterase* is expressed in fumonisin esterase units (U), where "one U unit is the amount of enzyme that releases one μmol of hydrolyzed fumonisin B1 (HFB1) per minute from 100 μM of fumonisin B1 in 50 mM phosphate buffer pH 6.0 at 37 °C". The *feed additive* is intended to be marketed as solid preparation having a guaranteed minimum activity of *fumonisin B1 esterase* corresponding to 1200 U/g. The marketed product (*Free Yeast® F*) is intended to be incorporated through *premixtures* or directly into *feedingstuffs*. The Applicant recommended to include the *feed additive* in *feedingstuffs* in order to obtain its content ranging from 50 to 300 g/ton *feedingstuffs*.

For the quantification of the *fumonisin B1 esterase* activity in the *feed additive*, *premixtures* and *feedingstuffs* the Applicant submitted a single-laboratory validated and further verified method based on the selective quantification of the reaction product HFB1 by High Performance Liquid Chromatography coupled with fluorescence detection (HPLC-FLD).

The following method performance characteristics were derived from the validation and verification studies:

- for the *feed additive* in a range of enzyme activity between 167 and 1667 U/g: a relative standard deviation for *repeatability* ($RSD_r$) ranging from 1.8 to 5.1 %, a relative standard deviation for *intermediate precision* ($RSD_{ip}$) ranging from 4.1 to 9.4 % and a *recovery rate* ($R_{Rec}$) ranging from 88 to 102 %;
- for *premixtures* in a range of enzyme activity between 417 and 4167 U/kg: a $RSD_r$ ranging from 1.6 to 7.6 %, a $RSD_{ip}$ ranging from 4.7 to 9.8 % and a $R_{Rec}$ ranging from 89 to 109 %; and
- for *feedingstuffs* in a range of enzyme activity between 21 and 208 U/kg: a $RSD_r$ ranging from 3.1 to 4.1 %, a $RSD_{ip}$ ranging from 3.4 to 4.4 % and a $R_{Rec}$ ranging from 90 to 101 %.

Furthermore, a limit of detection (LOD) and a limit of quantification (LOQ) of 0.21 and 0.67 U of *fumonisin B1 esterase* /kg of *feedingstuffs*, respectively, were reported by the Applicant.

Based on the performance characteristics available, the EURL recommends for official control the single-laboratory validated and further verified HPLC-FLD method, based on the selective quantification of the reaction product HFB1 produced at pH 6.0 and 37 °C by the action of *fumonisin B1 esterase (3.1.1.87)* to fumonisin B1, for the quantification of the *fumonisin B1 esterase* 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**

*Fumonisin B1 esterase (3.1.1.87) produced by Komagataella phaffii NCAIM (P) Y001485*, technological additives, substances for reduction of the contamination of feed by mycotoxins: substances that can suppress or reduce the absorption, promote the excretion of mycotoxins or modify their mode of action; all pigs (piglets, pigs for fattening, sows and minor growing and reproductive porcine species).

## 1. BACKGROUND

In the current application an authorisation of *fumonisin B1 esterase (3.1.1.87) produced by Komagataella phaffii NCAIM (P) Y001485* is sought under Article 4(1) under the category/functional group 1(m) "technological additives"/"substances for reduction of the contamination of feed by mycotoxins: substances that can suppress or reduce the absorption, promote the excretion of mycotoxins or modify their mode of action" according to Annex I of Regulation (EC) No 1831/2003 [1,2]. The authorisation is sought for the use of the *feed additive* for all pigs (piglets, pigs for fattening, sows and minor growing and reproductive porcine species) [1,3].

According to the Applicant, the *feed additive* contains as *active substance fumonisin B1 esterase (EC 3.1.1.87)* which is produced by the genetically modified strain *Komagataella phaffii NCAIM Y001485* [4].

The activity of *fumonisin B1 esterase* is expressed in fumonisin esterase units (U), where “one U unit is the amount of enzyme that releases one µmol of hydrolyzed fumonisin B1 (HFB1) per minute from 100 µM of fumonisin B1 (FB1) in 50 mM phosphate buffer pH 6.0 at 37 °C” [5].

The *feed additive* is intended to be marketed as a solid pelleted preparation (*Free Yeast® F*) containing 3.0 to 8.0 % (w/w) of the *active substance* supplemented up to 100 % with inactivated Saccharomyces cerevisiae [6]. The *feed additive* has a guaranteed minimum activity of the *fumonisin B1 esterase* corresponding to 1200 U/g [6].

*Free Yeast® F* is intended to be incorporated through *premixtures* or directly into *feedingstuff*s [3]. The Applicant recommended to include the *feed additive* in *feedingstuffs* in order to obtain a content ranging from 50 to 300 g/ton *feedingstuffs* [3].

Note: The EURL has already evaluated analytical methods for the determination of *fumonisin B1 esterase* (EC 3.1.1.87) in the frame of other dossiers [7].

## 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 *fumonisin B1 esterase (3.1.1.87) produced by Komagataella phaffii NCAIM (P) Y001485* 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 *fumonisin B1 esterase* activity in the *feed additive*, *premixtures* and *feedingstuffs* the Applicant submitted a single-laboratory validated and further verified method based on the selective quantification of the reaction product HFB1 by High Performance Liquid Chromatography coupled with fluorescence detection (HPLC-FLD) [5,8,9].

According to the method, 1.0 g of milled and homogenised sample is mixed with the sample buffer and shaken at 37 °C for 1 h (specific dilution is applied to the different matrices). The extract is filtered and an aliquot of 200 µl of the diluted extract from the previous step and 100 µM of *fumonisin B1* solution are further mixed in a 50 mM phosphate buffer (pH 6.0), adjusted to 1000 µl final volume and incubated at 37 °C for 1 h. The reaction is then stopped by boiling the mixture for 5 min. An aliquot of 100 µl of the reaction mixture is diluted with

900 µl of methanol. The mixture is derivatised by addition of o-phthalaldehyde (OPA) reagent and filtered. The derivatives are analysed by HPLC-FLD using excitation wavelength of 335 nm and emission wavelength of 440 nm. The amount of HFB1 is quantified using a standard calibration. The *fumonisin B1 esterase* activity is calculated from the HFB1 concentration in the sample using a specific equation [8]. The performance characteristics obtained for the quantification of the *fumonisin B1 esterase* activity in the *feed additive*, *premixture* and feedingstuffs in the frame of the validation and verification studies are presented in Table 1 [8,9]. Furthermore, a limit of detection (LOD) and a limit of quantification (LOQ) of 0.21 and 0.67 U of *fumonisin B1 esterase* /kg of *feedingstuffs*, respectively, were reported by the Applicant [8].

However, in the frame of the verification studies for *feedingstuffs*, the second laboratory introduced a modification in the standard operating procedure that caused, in correspondence of the retention time of the peak of the target analyte, a co-elution of an interfering compound. Therefore, according to the Applicant, it was not possible to collect reliable results and the verification study for demonstrating the applicability of the analytical method to *feedingstuffs* is to be considered unsuccessful [9]. Nevertheless, the results obtained in the frame of the validation study or in the other supporting studies within the dossier (i.e. stability and homogeneity) showed the fitness-for-purpose of the analytical method proposed for the analysis of *fumonisin B1 esterase* activity in *feedingstuffs* [10,11].

Based on the performance characteristics available, the EURL recommends for official control the single-laboratory validated and further verified HPLC-FLD method, based on the selective quantification of the reaction product HFB1, for the quantification of *fumonisin B1 esterase* activity in the *feed additive*, *premixtures* and *feedingstuffs*.

**Table 1:** Performance characteristics for the quantification of *fumonisin B1 esterase* activity in the *feed additive*, *premixture* and *feedingstuffs*

| | Feed Additive | | Premixture | | Feedingstuffs | |
|---|---|---|---|---|---|---|
| | Validation [8] | Verification [9] | Validation [8] | Verification [9] | Validation [8] | Verification [9] |
| **Activity** | 167-1667 U/g | | 417-4167 U/g | | 21-208 U/kg | |
| **$RSD_r$, %** | 3.0-5.1 | 1.8 | 2.9-7.6 | 4.6 | 3.1-4.1 | n.a. |
| **$RSD_{ip}$, %** | 4.1-9.4 | 4.4 | 5.8-9.8 | 4.7 | 3.4-4.4 | n.a. |
| **$R_{rec}$, %** | 88-102 | 97 | 89-109 | 99 | 90-101 | n.a. |

$RSD_r$, $RSD_{ip}$: relative standard deviation for *repeatability* and *intermediate precision; $R_{Rec}$: recovery rate*; *n.a.: not available.*

***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 HPLC-FLD method, based on the selective quantification of the reaction product HFB1 produced at pH 6.0 and 37 °C by the action of *fumonisin B1 esterase (3.1.1.87)* to fumonisin B1, for the quantification of the *fumonisin B1 esterase* activity in the *feed additive*, *premixtures* and *feedingstuffs*.

***Recommended text for the register entry (analytical method)***

For the determination of *fumonisin B1 esterase* activity (U) in the *feed additive*, *premixtures* and *feedingstuffs*:

- High Performance Liquid Chromatography coupled to fluorescence detection (HPLC-FLD) based on the quantification of hydrolyzed fumonisin B1 released from the action of the enzyme on fumonisin B1 at pH 6,0 and 37 °C.

One U unit is the amount of enzyme that releases one µmol of hydrolyzed fumonisin B1 per minute from 100 µM of fumonisin B1 in 50 mM phosphate buffer pH 6.0 at 37 °C.

## 5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of the *fumonisin B1 esterase (3.1.1.87) produced by Komagataella phaffii NCAIM (P) Y001485* 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. 1831-0030-2021 & Annex I – submission number 1615458620246-2870

[2] *Technical dossier, Section II: 2.1.2 Proposal for classification

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

[4] *Technical dossier, Section II: 2.2.1.2 Microorganisms

[5] *Technical dossier, Section II: 2.6.1 Methods of analysis for the active substance

[6] *Technical dossier, Section II: 2.1.3 Qualitative and quantitative composition

[7] EURL evaluation Reports: FAD-2013-0002 and FAD-2017-0005 https://ec.europa.eu/jrc/en/eurl/feed-additives/evaluation-reports

[8] *Technical dossier, Section II: Annex_2.6.1_1.pdf

[9] *Technical dossier, Section II: Annex_2.6.1_2.pdf

[10] *Technical dossier, Section II: Annex 2.4.1.2 Stability in premixtures and feedingstuff

[11] *Technical dossier, Section II: Annex 2.4.2. Homogeneity

*Refers to Dossier no: FAD-2021-0055

## 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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