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Community Reference Laboratory – Feed Additives Authorisation

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CRL Evaluation Report on the Analytical Methods submitted in connection with Section 2.5 (Control Methods) of the Application for Authorisation as a Feed Additive according to Regulation (EC) No 1831/2003

Dossier No.:	FAD-2005-0001
Name of Additive:	Biosaf ® Sc47 for horses
Active Substance:	*Saccharomyces cerevisiae* NCYC Sc 47
Rapporteur Laboratory:	National Reference Laboratory Rennes, France
Report prepared by:	J. Michard (NRL, Rennes, France) R. Leuschner (CRL-FAA)
Report checked by: Date:	A. M. Jensen (CRL-FAA) 02/09/2005
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1. EXECUTIVE SUMMARY

BIOSAF® Sc 47 is a zootechnical feed additive consisting of a strain of the yeast *Saccharomyces cerevisiae* NCYC Sc 47. The additive belongs to category 4 of zootechnical additives. The guaranteed number of viable yeast cells of the product is above 5 x 10^9 c.f.u./g (colony forming units / gram) additive.

The current dossier for BIOSAF® Sc 47 seeks an extension of its use in feeds for horses. The target level of supplementation is in the range of 10^6 - 10^7 c.f.u./g feed.

Concerning the enumeration of the active substances of BIOSAF® Sc 47 (*Saccharomyces cerevisiae*) in the additive, the applicant proposes a pour plate method, the Standard method ISO 7954. The ISO method is considered appropriate for the purpose of routine control, as well as for official controls.

Concerning the enumeration of the active substance in premixtures and feedingstuff, the applicant refers to a pour plate count method that has been published in a peer reviewed journal. This method is based on the principles of the above-mentioned ISO 7954 method and the authors of the article have validated the method in a full collaborative study applying an internationally accepted protocol for collaborative studies. Performance characteristics of this method obtained in the collaborative study were expressed in terms of relative standard deviations for repeatability (RSD_r) and reproducibility (RSD_R) which were around 5 % and 8 %, respectively. Both values are considered as acceptable. The limit of quantification (LOQ) of this method is 100 colony forming units (c.f.u) per gram (g) sample, i.e. well below the target application level in feedingstuffs. Alternatively, in cases where the species *S. cerevisiae* needs to be confirmed, a plate count method using an elective chromogenic agar was proposed, which has a limit of quantification of 1000 c.f.u. per gram (g) sample. This method was fully ring trial validated in parallel with the above mentioned pour plate method and resulted in acceptable method performance characteristics, because the RSD_r was about 3 % and the RSD_R was about 6%. The LOQ of this method is 1000 (c.f.u) per gram (g) sample, i.e. well below the target application level in feedingstuffs. Both methods are therefore considered appropriate for routine control and for official control purposes.

Concerning the unambiguous identification of the specific strain of *S. cerevisiae* NCYC Sc 47 in BIOSAF® Sc 47, in the additive, and if necessary in the premixture and feedingstuff, a polymerase chain reaction (PCR) method is used. This method was validated in a collaborative study which demonstrated a high level of correct identification between laboratories, and it is therefore considered suitable for routine and official control purposes.

Information on the composition of ingredients other than the active agents, including impurities, physical state of the product, toxins and virulence factors, antibiotic production and resistance, stability of the additive, other physico-chemical or biological properties and incompatibilities with other feed ingredients, was provided for the dossier. However, actual methods for determining these properties were not submitted for the extension of the authorisation for this dossier. *Saccaromyces cerevisiae* does not belong to a taxonomic group which includes members known to be capable of production of toxins or other virulence factors.

In the opinion of the CRL the methods for determination of the active substance in additives, premixtures and feedingstuff are considered fit for routine control and suitable for official control purposes. The CRL opinion is based on

(i) the low LOQ relative to the target level of application which ranges between 10^6 – 10^7 c.f.u./g of sample

(ii) the systematic and well performed validation studies for the quantitative microbiological enumeration method referenced in the BIOSAF® Sc 47 dossier, which have acceptable performance characteristics expressed in terms of RSD_r and reproducibility RSD_R and

(iii) the submitted identification methodology, which is a validated PCR method.

On the basis of the supplied documentation, no supplementary experimental work (testing or method validation) is required by the CRL.

2. KEYWORDS

BIOSAF® Sc 47, *Saccharomyces cerevisiae*, feed additive, horse, yeast

3. TABLE OF CONTENTS

4. BACKGROUND

The active substance of the zootechnical feed additive BIOSAF® Sc 47 is a strain (NCYC Sc 47) of the yeast *Saccharomyces cerevisiae*. The guaranteed number of viable yeast cells in the additive is above 5×10^9 c.f.u./g additive.

BIOSAF® Sc 47 is presently approved as a feed additive for cattle for fattening (Commission Regulation (EC) No 316/2003), for weaned piglets (Commission Regulation (EC) No 2148/2004), for sows (Commission Regulation (EC) No 1288/2004) and for rabbits for fattening (Commission Regulation (EC) No 600/2005). It is provisionally approved for milking cows (Commission Regulation (EC) No 937/2001, micro-organism no 3).

The current extension for BIOSAF® Sc 47 seeks authorisation for use as additive in feedingstuff for horses at a minimum level of 8×10^5 c.f.u./g and at a maximum level of 7×10^6 c.f.u./g of feedingstuff.

5. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005 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 tasks of the Community Reference Laboratory concerning applications for authorisations of feed additives, the CRL is required to submit a full evaluation report to the European Food Safety Authority for each application. For this particular dossier, the suitability of the control methods and validation studies submitted in connection with BIOSAF® Sc 47 (FAD-2005-0001) were evaluated.

6. EVALUATION

The numbering system under this point refers to the report of the Scientific committee on Animal Nutrition on the revision of the guidelines for the assessment of additives in animal nutrition, adopted on 22 October 1999 (Guidelines for the assessment of additives in feedingstuffs Part II: Enzymes and Section 2.5- Control Methods), in the following referred to as "the Guidelines".

General methods

Description of the methods used for the determination of the criteria listed under items 2.1.3, 2.1.4, 2.1.5, 2.2.5, 2.2.6, 2.3.1, 2.3.2 and 2.3.3 of the Guidelines.

Qualitative and quantitative composition of the additive BIOSAF® Sc 47

The methods for determination of the quantitative composition of the active substance in the additive are provided by the applicant. The applicant uses a pour plate method for enumeration of the active substance in the additive which is based on ISO 7954 [1]. The number of viable microorganisms is given in colony forming units (c.f.u.) per g and was provided by the applicant.

The active component is a strain of the yeast *Saccharomyces cerevisiae* (NCYC Sc 47). For identification of the authorised strain of *Saccharomyces cerevisiae* NCYC Sc 47 a published polymerase chain reaction (PCR) method was used [3] which was validated by a collaborative study [4]. This method is considered appropriate for routine and official control purposes.

The chemical analysis of the additive revealed a dry matter (DM) of 91 – 94 %, a protein content of 38.0 – 45.5 % DM and a P_2O_5 content of 1.8 – 2.3 % DM. Information on other ingredients of the additive and the related methods was not provided for the purpose of this extension. (*Cf.* the requirements listed in point 2.1.3 of the Guidelines.)

Qualitative and quantitative composition of any impurities in the additive

The applicant provided quality control results of analysis for bacteriological contaminants including pathogens and spoilage microorganisms and of heavy metals of the additive. The applicant was given official recognition that the Quality Assurance System conformed to the international standard ISO 9001. Quality Control is particularly concerned with the supply of raw materials, production process control, controls of trials during manufacture, final testing and trials, identification, traceability of products and management of documents. Information on other ingredients of the additive and the related methods were not provided for the purpose of this extension. (*Cf.* the requirements listed in point 2.1.4 of the Guidelines).

Physical state of each form of the additive

Screen analysis (granulometry) of the additive revealed a minimum particle size of below 0.5 mm for 1 % of the product and a maximum size below 2.7 mm for 100 % of the product. Other information and methods were not provided for the purpose of this extension. (*Cf.* the requirements listed in point 2.1.5 of the Guidelines).

Toxins and virulence factors of the active agent

Saccaromyces cerevisiae NCYC Sc 47 does not belong to a taxonomic group which includes members known to be capable of production of toxins or other virulence factors. (*Cf.* the requirements listed in point 2.2.5 of the Guidelines).

Antibiotic production and antibiotic resistance of the active agent

Not relevant for *S. cerevisiae.* Detailed information on studies concerning the safety of use of the additive are provided in ‘section IV’ of the dossier. (*Cf.* the requirements listed in point 2.2.6 of the Guidelines).

Stability of the additive

According to the applicant, BIOSAF® Sc 47 maintains an activity greater than 5 x 10^9 c.f.u./g for a period of 1 year, when stored at about 26 ºC in its commercial packaging. A study design to test the stability of three different batches of BIOSAF® Sc 47 was presented in an earlier dossier for fattening bovines. The applicant uses a pour plate method for enumeration of the active substance which follows ISO 7954 [1]. This was used consistently for the study to investigate the stability of the additive and is appropriate for the purpose. (*Cf.* the requirements listed in point 2.3.1 of the Guidelines).

Other physico-chemical or biological properties

Information of mixing capacity of the additive (homogeneity) was provided for the integration in feedingstuff. Studies on dispersion of BIOSAF® Sc 47 in a mineral/vitamin horse premixture and a protein concentrate for horses was provided. The method used was a pour plate method applicable for this purpose. (*Cf.* the requirements listed in point 2.3.2 of the Guidelines).

Incompatibilities with other feed ingredients

The applicant states in the 'section V of the dossier entitled 'Biological properties' that no major incompatibilities with feedingstuffs, other approved additives or other medical products are reported in the literature. Other information and methods were not provided for the purpose of this extension. (*Cf.* the requirements listed in point 2.3.3 of the Guidelines).

Description of the qualitative and quantitative methods for routine control of the active agent in premixtures and feedingstuffs

For premixtures and feedingstuffs the applicant proposes a method that was published and validated by a full collaborative study [2] applying an internationally accepted protocol for collaborative studies [5]. A pour plate method following ISO 7954 using chloramphenicol glucose yeast extract agar and a plate count method using CHROMagar Candida, an agar where colonies of the species *S. cerevisiae* appear in a purple colour was used. The use of the chromogenic agar, as specified in the plate count method, is costly. Therefore, depending on the specific purpose of the analysis, the pour plate method would be the method of choice. Both methods are applicable for routine and official control purposes. The pour plate method

has a limit of quantification of 100 c.f.u./g and the plate count method a quantification limit of 1000 c.f.u./g both of which are well below the target application range.

(*Cf.* the requirements listed in point 2.5.2 of the Guidelines).

CHECK LIST

		Y	N	N/A	Comments
1.1	Is/Are the method(s) mentioned in Part I (1.- A. Premixtures) accompanied by information on:				
	- Sampling Method used		X		
	- Percentage Recovery			X	
	- Specificity	X			
	- Accuracy			X	
	- Precision			X	
	- Limits of detection			X	
	- Limits of quantification	X			
	- Validation procedure used	X			Full ring trial
1.2	Is/Are the method(s) mentioned in Part I (1.- A. Animal feed) accompanied by information on:				
	- Sampling Method used		X		
	- Percentage Recovery			X	
	- Specificity	X			
	- Accuracy			X	
	- Precision			X	
	- Limits of detection			X	
	- Limits of quantification	X			
	- Validation procedure used	X			Full ring trial
2.1	Is/Are the method(s) mentioned in Part I (2. – Target tissues) accompanied by information on:			X	
	- Sampling Method used				
	- Percentage Recovery				
	- Specificity				
	- Accuracy				
	- Precision				
	- Limits of detection				
	- Limits of quantification				
	- Validation procedure used				
2.2	Is/Are the method(s) mentioned in Part I (2. – Animal products) accompanied by information on:			X	
	- Sampling Method used				
	- Percentage Recovery				
	- Specificity				
	- Accuracy				
	- Precision				
	- Limits of detection				
	- Limits of quantification				
	- Validation procedure used				
3.	If the method(s) has/have been devised, consideration has been given to the fact that their limits of quantification must be below the MRLs.			X	

N/A: Not applicable

7. CONCLUSIONS AND RECOMMENDATIONS

Concerning the enumeration of the active substances of BIOSAF® Sc 47 (*Saccharomyces cerevisiae*) in the additive, the applicant proposes a pour plate method, the Standard method ISO 7954. The ISO method [1] is considered appropriate for the purpose of routine control, as well as for official controls.

Concerning the enumeration of the active substance in premixtures and feedingstuff, the applicant refers to a pour plate count method that has been published in a peer reviewed journal. This method is based on the principles of the above-mentioned ISO 7954 method and the authors of the article have validated the method in a full collaborative study applying an internationally accepted protocol for collaborative studies. Performance characteristics of this method obtained in the collaborative study were expressed in terms of relative standard deviations for repeatability (RSD_r) and reproducibility (RSD_R) which were around 5 % and 8 %, respectively. Both values are considered as acceptable. The limit of quantification (LOQ) of this method is 100 colony forming units (c.f.u) per gram (g) sample, i.e. well below the target application level in feedingstuffs. Alternatively, in cases where the species *S. cerevisiae* needs to be confirmed, a plate count method using an elective chromogenic agar was proposed, which has a limit of quantification of 1000 c.f.u. per gram (g) sample. This method was fully ring trial validated in parallel with the above mentioned pour plate method and resulted in acceptable method performance characteristics, because the RSD_r was about 3 % and the RSD_R was about 6%. The LOQ of this method is 1000 (c.f.u) per gram (g) sample, i.e. well below the target application level in feedingstuffs. Both methods are therefore considered appropriate for routine control and for official control purposes.

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Information on the composition of ingredients other than the active agents, including impurities, physical state of the product, toxins and virulence factors, antibiotic production and resistance, stability of the additive, other physico-chemical or biological properties and incompatibilities with other feed ingredients, was provided for the dossier. However, actual

methods for determining these properties were not submitted for the extension of the authorisation for this dossier. *Saccaromyces cerevisiae* does not belong to a taxonomic group which includes members known to be capable of production of toxins or other virulence factors.

In the opinion of the CRL the methods for determination of the active substance in additives, premixtures and feedingstuff are considered fit for routine control and suitable for official control purposes. The CRL opinion is based on

(i) the low LOQ relative to the target level of application which ranges between 10^6 – 10^7 c.f.u./g of sample

(ii) the systematic and well performed validation studies for the quantitative microbiological enumeration method referenced in the BIOSAF® Sc 47 dossier, which have acceptable performance characteristics of RSD values, and

(iii) the submitted identification methodology, which is a validated PCR method.

On the basis of the supplied documentation, no supplementary experimental work (testing or method validation) is required by the CRL.

8. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

Concerning section II of the dossier entitled 'Identity, characterisation and conditions of use of the additive – Methods of control', a complementary dossier for horse feedingstuff for section II (39 pages), including appendices regarding stability, storage and homogeneity was provided.

Additional information provided by applicant:

- Answers to the questions of the CRL (BIOHORSE0705, 6 page document) containing:
  - Appendix 1 - the former method to enumerate the active substance in the additive
  - Appendix 2 - the former method to enumerate the active substance in the premixture and feedingstuff
  - Appendix 3 – validation of two methods for enumeration of *Saccharomyces cerevisiae* as pure additive and active substance in feedingstuff
  - Appendix A – stating that the protocols correspond to the protocols added in appendix 1 and 2 of this document
  - Appendix B – validation data from 3 laboratory collaborative study in feedingstuff comprising the pure additive and the additive in mashed and pelleted feedingstuff

- Appendix 4 – literature reference for *S. cerevisiae* identification PCR method (Ness et al., 1993, J. Sci. Food Agric. 62, 89-94)

Samples have been made available to the CRL on 17/02/2005..

The dossier has been made available to the CRL by EFSA.

9. REFERENCES

1) Anonymous: ISO 7954, 1987. General guidance for enumeration of yeasts and moulds - Colony count technique at 25 °C.
2) Leuschner R. G. K., Bew J., Bertin G. (2003) Validation of an official control method for enumeration of authorised probiotic yeast in animal feedingstuff. System. Appl. Microbiol., 26, 147-153.
3) Ness F., Lavallée F., Dubourdieu D., Aigle M., Dulau L. (1993) Identification of yeast strains using the polymerase chain reaction. J. Sci. Food Agric., 62, 89-94
4) Leuschner R.G.K., Bew J., Fourcassier P., Bertin G. (2004) Validation of the Official Control Methods based on polymerase chain reaction (PCR) for identification of authorised probiotic yeast in animal feedingstuff. System. Appl. Microbiol. 27, 492-500.
5) Horwitz, W., (1995) Protocol for the design, conduct and interpretation of method-performance studies, Pure and Applied Chemistry, 67, 331-343

10. RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was the National Reference Laboratory, Rennes, France.