EUROPEAN COMMISSION
DIRECTORATE-GENERAL
JOINT RESEARCH CENTRE
Institute for Reference Materials and Measurements
Community Reference Laboratory for Feed Additives Authorisation

D08/FSQ/CVH/(2006) D5583

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

Dossier related to:	EFSA-Q-2005-234
Name of Additive:	LEVUCELL®SC for horses
Active Subtance(s):	*Saccharomyces cerevisiae* CNCM I-1077
Rapporteur Laboratory:	Community Reference Laboratory for Feed Additive Authorisation, IRMM, Geel, Belgium (CRL-FAA)
Report prepared by:	Renata Leuschner
Report checked by:	Giuseppe Simone (CRL-FAA)
Date:	13/02/2006
Report approved by:	Christoph von Holst (CRL-FAA)
Date:	03/03/2006

EXECUTIVE SUMMARY

In the current application authorisation is sought for LEVUCELL® SC under the category 'zootechnical additives', according to Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought to use LEVUCELL® SC in its two forms LEVUCELL® SC 20 and LEVUCELL® SC 10ME for horses. LEVUCELL® SC 20 is a light brown powdery uncoated product whereby LEVUCELL® SC 10ME is micro-encapsulated. The feed additive is proposed for use in premixtures and feedingstuffs for horses at a concentration of 3 x 10^9 colony forming units (c.f.u.) per kg complete feedingstuff.

For the determination of the active agent (*Saccharomyces cerevisiae* CNCM I-1077) in the *feed additive* LEVUCELL® SC, a pour plate method for enumeration and a polymerase chain reaction (PCR) method for identification are proposed which are considered appropriate for the intended purpose.

For the determination of the active agent *S. cerevisiae* CNCM I-1077 in *premixtures* and *feedingstuffs*, the same methods as for the feed additive are proposed. The method's performance characteristics of the enumeration method include relative standard deviations for repeatability (RSD_r) and between-laboratory reproducibility (RSD_R) of around 5 % and 8 %, respectively [System. Appl. Microbiol. 2003, 26, 147-153]. The limit of quantification (LOQ) of the method is 10^5 c.f.u./kg. These performance characteristics are considered acceptable. The PCR method for strain identification was ring trial validated and performed appropriately [System. Appl. Microbiol. 2004, 27, 492-500]. Both methods are considered suitable for official control for the field of application that is sought.

Official and/or standard methods are proposed by the applicant for the determination of impurities (heavy metals, mycotoxins, microbiological quality) in the feed additive. The methods are therefore considered suitable for official control purposes.

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

KEYWORDS

LEVUCELL® SC, feed additive, yeast, horses, zootechnical, *Saccharomyces cerevisiae*

TABLE OF CONTENTS

1. BACKGROUND

LEVUCELL® SC is a feed additive for which authorisation is sought under the category 'zootechnical additives' according to Annex I of Regulation (EC) No 1831/2003. It contains viable cells of the yeast strain *Saccharomyces cerevisiae* CNCM I-1077 as the active agent. The yeast strain is deposited at the Collection Nationale de Cultures de Microorganismes (C.N.C.M.) at the Institut Pasteur, Paris, France. LEVUCELL® SC is presented in two different forms:

- LEVUCELL® SC 20 containing 20 x 10^9 c.f.u. revivable dried cells of *S. cerevisiae* CNCM I-1077 per gram of additive
- LEVUCELL® SC 10ME containing 10 x 10^9 c.f.u. revivable dried cells of *S. cerevisiae* CNCM I-1077 per gram microencapsulated product (50 % LEVUCELL® SC 20 and 50 % fatty acid blend)

LEVUCELL® SC is already authorised as feed additive for dairy cows and cattle for fattening (Regulation EC No.1200/2005). The intended use (*cf.* EFSA-Q-2005-234) of the current application is for horses, by mixing the feed additive into premixtures and feedingstuffs at a proposed final concentration of 3 x 10^9 c.f.u./kg complete feedingstuff.

2. 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 requested 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, *cf*. EFSA-Q-2005-234 was evaluated.

3. EVALUATION

The numbering system under this point refers to that of the Guidelines for the assessment of additives in feedingtuffs, PART II: Enzymes and Micro-organisms. The method protocols and corresponding validation data – if applicable – are given in Section II of the dossier.

Description of some of the methods listed under items 2.5.1 of the Guidelines.

Qualitative and quantitative composition of the additive LEVUCELL® SC

The additive LEVUCELL® SC 20 is a fine, granulated, light brown powder which flows easily and has an odour similar to baker's yeast. It is composed of approximately 80 % revivable *Saccharomyces cerevisiae* CNCM I-1077 dried yeast cells and approximately 14 % non-viable yeast cells which lost their viability during the drying process. About 1 % represents sorbitane monostearate (E 491) and a rest moisture content of 5 % remains in the additive. LEVUCELL® SC 10ME is composed of 50 % LEVUCELL® SC 20 and 50 % stearic/palmitic acids blend. The concentrations of the active agents in colony forming units (c.f.u.) per gram in both forms of the additive LEVUCELL® SC are described above (background section).

The method for quantifying the active agent in the additive as provided by the applicant represents a pour plate method using chloramphenicol glucose yeast extract (CGYE) agar which is considered acceptable for official control purposes [1].

The active agent is a strain of the yeast *Saccharomyces cerevisiae* (CNCM I-1077). The physiological and biochemical properties of the strain are appropriately characterised by using an accepted commercially available API 20C gallery. For identification of the authorised

strain of *Saccharomyces cerevisiae* CNCM I-1077, a range of DNA based techniques are used such as polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) and pulsed field gel electrophoresis (PFGE). For a RFLP method based on mitochondrial polymorphism a rapid minimal-preparation method is used according to the applicant, however the method protocol or performance data are not provided. A ring-trial validated and published polymerase chain reaction (PCR) method was also used [2, 3]. This method is considered appropriate for official control purposes.

Qualitative and quantitative composition of any impurities in the additive

The applicant provided quality control results and copies of a short description of the method of analysis for heavy metals including arsenic (As), cadmium (Cd), chromium (Cr), mercury (Hg), nickel (Ni), tin (Sn) and selenium (Se) using an atomic adsorption method as described in the European Pharmacopeia, 2nd Edition, V. 6.17, 2.2.22. The method is considered suitable for official control purposes.

For aflatoxins B_1, B_2, G_1 and G_2 the applicant proposed an EU method (Directive 76/372/EEC) which contains a validation for analysis of aflatoxin B_1. It may be considered equally appropriate for aflatoxins B_2, G_1 and G_2. Zearalenone was analysed by a standard method (AFNOR standard, NF V 18-201; ISO 6870). For the analysis of ochratoxin A the method described by the Association of the Analytical Community International (AOAC, 1984, 26, 494) was used. The methods described are considered fit for the purpose of official control.

Microbiological quality was ensured by investigating aerobic mesophilic bacteria, coliforms, pathogenic staphylococci, sulphite reducing bacteria, *Clostridium perfringens* and *Salmonella* species. For the determination of mesophilic bacteria tryptone agar was used (NF ISO 4833). Coliforms were determined at 44 ºC using crystal violet red bile lactose medium (NF ISO 4832). Staphylococci were enumerated on an appropriate selective agar (NF ISO 6888). Sulphite reducing anaerobic bacteria were determined using tryptone sulphite cycloserine (TSC) medium. *Clostridium perfringens* was determined using tryptone sulphite cycloserine (TSC) medium (NF ISO 7937). The detection of *Salmonella* species was carried out in accordance with the standard method NF ISO 6579.

Copies of the protocols for the standard methods were provided by the applicant without any corresponding method performance data for the field of application. The standard methods are

however considered appropriate for the purpose of official control for the field of application sought.

Description of the qualitative and quantitative methods for routine control of the active agent in premixtures and feedingstuffs (Cf. requirements in guideline section 2.5.2)

To analyse premixtures and feedingstuffs for the active agent of LEVUCELL® SC, the applicant proposes a method that was published and validated by a full collaborative study [1]. The pour plate method using chloramphenicol glucose yeast extract (CGYE) agar would be recommended for analysis. 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 and would be considered acceptable for official control purposes. The pour plate method has a limit of quantification (LOQ) of 10^5 c.f.u./kg which is well below the target application range.

Concerning the unambiguous identification of the specific strain of *S. cerevisiae* CNCM I-1077 in the additive LEVUCELL® SC a polymerase chain reaction (PCR) method is used [2], which can also be applied to premixtures and feedingstuffs. This method was validated in a collaborative study using feed samples which demonstrated a high level of correct identification between laboratories [3]. The PCR method is considered suitable for official control purposes for the field of application that is sought.

4. CONCLUSIONS AND RECOMMENDATIONS

Concerning the enumeration of the active agents of LEVUCELL® SC (*Saccharomyces cerevisiae* CNCM I-1077) in the *additive*, in *premixtures* and *feedingstuffs*, the applicant proposes a ring-trial validated pour plate method using chloramphenicol glucose yeast extract (CGYE) agar [1]. 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 acceptable. The limit of quantification (LOQ) of this method is 10^5colony forming units (c.f.u) per kilogram (kg) sample, i.e. well below the target application level in feedingstuffs. The method is considered appropriate for official control purposes.

Concerning the unambiguous identification of the specific strain of *S. cerevisiae* CNCM I-1077 in the additive LEVUCELL® SC a polymerase chain reaction (PCR) method is used [2],

which can also be applied to premixtures and feedingstuffs. 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 official control purposes [3].
Official and/or standard methods are proposed by the applicant for the determination of impurities (heavy metals, mycotoxins, microbiological quality) in the feed additive. The methods are therefore suitable for official control purposes.

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

5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, samples of LEVUCELL® SC 20 and LEVUCELL® SC ME10 have been sent to the Community Reference Laboratory for feed additives authorisation. The dossier has been made available to the CRL by EFSA.

6. REFERENCES

[1] 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

[2] Nes, 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

[3] 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

7. RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was the Community Reference Laboratory for Feed Additive Authorisation (CRL-FAA), Geel, Belgium.