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Krma: metode vzorčenja in analize - Določanje in štetje Saccharomyces cerevisiae, uporabljenih kot krmni dodatek

Animal feeding stuffs: Methods of sampling and analysis - Detection and enumeration of Saccharomyces cerevisiae used as feed additive

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Futtermittel: Probenahme- und Untersuchungs-verfahren - Nachweis und Zählung von Saccharomyces cerevisiae als Futtermittelzusatzstoff

Aliments des animaux: Méthodes d'échantillonnage et d'analyse - Détection et dénombrement des souches de Saccharomyces cerevisiae utilisées comme additifs pour l'alimentation animale

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Animal feeding stuffs: Methods of sampling and analysis - Detection and enumeration of Saccharomyces cerevisiae used as feed additive

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European foreword

This document (EN 15789:2021) has been prepared by Technical Committee CEN/TC 327 “Animal feeding stuffs - Methods of sampling and analysis”, the secretariat of which is held by NEN.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by May 2022, and conflicting national standards shall be withdrawn at the latest by May 2022.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. CEN shall not be held responsible for identifying any or all such patent rights.

This document supersedes EN 15789:2009.

The main changes compared to the previous edition are as follows:

— Amendment of the title;

— Extension of the scope of application to all *Saccharomyces cerevisiae* strains used as feed additive;

— Updating of normative cross references;

— Supplement of phosphate buffered saline with Tween® 80;

— Addition of the option to use Tween® 80 supplemented phosphate buffered saline for the preparation of the initial suspension as well as diluent for serial dilutions;

— Removal of the chromogenic culture medium for the enumeration of *Saccharomyces cerevisiae*;

— Addition of the option to use spread plates as well as a spiral plater for enumeration;

— Preparation of initial suspensions generally conducted with tempered tPBS;

— Unification of the homogenization time for the preparation of initial suspensions to one minute for all feed matrices;

— Adjustment of the cultivation time and temperature to 48 h to 72 h at (30 ± 1) °C;

— Addition of a procedure for the investigation of feeding stuffs containing high amounts of copper in the informative Annex A;

— Adjustment of the range of accepted colony numbers for counting from '≥ 30 to ≤ 350' to '≥ 10 to ≤ 200' colonies per plate.

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Introduction

This methodology has been developed to enumerate yeasts (*Saccharomyces cerevisiae*) used as feed additives to enable the European Commission to control proper labelling of animal feeding products. It was compiled first during the EU project SMT4-CT98-2235 "Methods for the official control of probiotics used as feed additives" [1].

The procedure has been validated for one commercially used *Saccharomyces cerevisiae* strain [2]. As the method is not selective for this particular *Saccharomyces cerevisiae* strain, it can be assumed, that it can also be applied to enumerate other *Saccharomyces cerevisiae* strains in their respective dosage form in feed provided that the added yeast is present in far higher numbers than any other yeast.

The method has not been validated for other yeast species (e.g. *Kluyveromyces marxianus*).

This method is not applicable for the detection of any ubiquitous or pathogenic yeasts in food and animal feeding stuffs.

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1 Scope

This document specifies general rules for the enumeration of *Saccharomyces cerevisiae* in feeding stuffs (additives, premixtures and compound feeds excluding mineral feeds) that contain *Saccharomyces cerevisiae* as a single microorganism component or in a mixture with other microorganisms. Applying the method to premixtures and compound feeds with critical amounts of copper demands a special procedure (see Annex A). The document is not applicable to mineral feeds, which are defined as complementary feeding stuffs composed mainly of minerals and containing at least 40 % crude ash (Regulation (EC) 767/2009) [3].

There are different categories of feed samples:

a) Additives containing about 10^{10} colony forming units (CFU)/g;

b) Premixtures containing about 10^{11} CFU/kg;

c) Compound feeds, meal or pellets which contain about 10^{9} CFU/kg.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

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EN ISO 6498, *Animal feeding stuffs - Guidelines for sample preparation (ISO 6498)*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

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3.1
Saccharomyces cerevisiae
unicellular fungus which mostly reproduces vegetatively by budding

Note 1 to entry: This description is based on their characteristics as used for this document.

Note 2 to entry: Budding cells are broadly ellipsoidal with multilateral bud formation. They show no or simple pseudohyphae.

Note 3 to entry: *S. cerevisiae* forms colonies on the specified selective medium after incubation for 48 h to 72 h at 30 °C under aerobic conditions fitting the description in 9.6.

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4 Principle

— Preparation of sterile and dry poured agar plates or preparation of sterile liquid culture medium tempered at 44 °C to 47 °C;

— Drawing a representative test sample under aseptic conditions;

— Preparation of the initial suspension with a tempered diluent to obtain a homogeneous distribution of bacterial cells from the test portion;

— Preparation of further decimal dilutions of the initial suspension in order to reduce the number of microorganisms per unit volume to allow, after incubation, the counting of colonies;

— Inoculation of prepared poured plates with an aliquot of the optimum dilutions and dispersion of the inoculum using a sterile spreader or inoculation of blank plates with an aliquot of the optimum dilutions and pouring of the molten agar medium into each plate, mixing and solidification;

— Incubation of inverted plates for 48 h to 72 h at 30 °C ± 1 °C under aerobic conditions;

— Counting of typical colonies, considering the specific properties of *Saccharomyces cerevisiae*;

— Morphological verification of isolates by use of microscopy;

— Calculation of the colony forming units of *Saccharomyces cerevisiae* per g or kg of feed sample.

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5 Diluents and culture medium

5.1 Diluents

5.1.1 Diluent for initial suspension

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The diluent is used for the preparation of the initial suspension and may also be used for the preparation of further decimal dilutions. The composition of the diluent is given in Table 1.

Table 1 — Phosphate buffered saline with Polysorbate 80 (Tween® 80)[1] (tPBS)

Sodium chloride	NaCl	8,00 g
Potassium chloride	KCl	0,20 g
Disodium hydrogen phosphate anhydrous	Na_2HPO_4	1,15 g
Potassium dihydrogen phosphate anhydrous	KH_2PO_4	0,20 g
Polyoxyethylene (20) sorbitan monooleate (Tween® 80)[1]	$C_{64}H_{124}O_{26}$	1 ml
Water, distilled or deionized	H_2O	1 000 ml

Dissolve the components (see Table 1) in water. If necessary, adjust to a final pH of 7,3 ± 0,2 at 25 °C after sterilization. Fill the solution into appropriate containers (e.g. bottles, flasks, or test tubes) and sterilize at 121 °C ± 3 °C for 15 min. To avoid loss during autoclaving, screw cap bottles are recommended.

[1] Tween® 80 is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by CEN of this product.

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For immediate use hold at 40 °C ± 1 °C in a water bath or incubator.

NOTE When using commercially available PBS buffer tablets, please take note that variations in composition and pH can occur between products from different manufacturers and could therefore give results different from the ones obtained with the buffer as specified in this document.

5.1.2 Diluents for serial dilutions

For serial dilutions, the diluent for initial suspension (5.1.1) or alternatively a peptone salt solution (PSS) according to EN ISO 6887-1 [4] can be used.

Table 2 — Peptone salt solution (PSS) according to EN ISO 6887-1

Enzymatic digest casein		1,0 g
Sodium chloride	NaCl	8,5 g
Water, distilled or deionized	H_2O	1 000 ml

Dissolve the components (see Table 2) in water in flasks or bottles. Adjust the pH if necessary so that, after sterilization, it is 7,0 ± 0,2 at 25 °C. For decimal dilutions, prepare test tubes containing 9,0 ml ± 0,1 ml after sterilization or use screw cap bottles to avoid volume loss during autoclaving.

Sterilize at 121 °C ± 3 °C for 15 min. Bring the diluent to room temperature before use.

NOTE Commercially available, ready-to-use PSS tubes of 9 ml are suitable.

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5.2 Culture medium

5.2.1 Yeast extract dextrose chloramphenicol (YGC) agar

The composition of the yeast extract dextrose chloramphenicol (YGC) agar is given in Table 3. The resulting pH value at 25 °C is 6,6 ± 0,2.

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Table 3 — Composition of the YGC agar

Yeast extract	5 g
D(+)-glucose	20 g
Chloramphenicol	0,1 g
Agar	12 g to 15 g[a]
Water, distilled or deionized	1 000 ml

[a] Depending on the gel strength of the agar.

The base agar (see Table 3) without the antibiotic can be purchased and the chloramphenicol supplement has to be added, or the YGC agar can be purchased as a complete medium.

NOTE 1 Chloramphenicol can be replaced by oxytetracycline ($C_{22}H_{24}N_2O_9$) at a final concentration of 100 µg/ml of medium.

NOTE 2 Any other medium leading to comparable results can be used (e.g. Sabouraud dextrose agar (SDA) or Wort agar supplemented with chloramphenicol).

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5.2.2 Preparation

Dissolve all components (see Table 2) in water under heating and fill into appropriate containers (e.g. bottles or flasks with non-toxic metal screw-caps). If necessary, adjust to a final pH of 6,6 ± 0,2 at 25 °C after sterilization. Sterilize at 121 °C ± 3 °C for 15 min. Excessive heating during sterilization shall be avoided.

If chloramphenicol is replaced by oxytetracycline, the basic medium is prepared in the same way. Prepare a 1 % mass concentration (m/m) solution of oxytetracycline hydrochloride in water and sterilize by filtration. Just prior to use, add 10 ml of this solution aseptically to 1 000 ml of the basic medium after sterilization (in order to obtain a final concentration of 0,1 g/l of medium), that has been maintained at 44 °C to 47 °C.

6 Apparatus

Usual microbiological laboratory equipment and, in particular, the following:

6.1 Equipment for dry sterilization (oven) and wet sterilization (autoclave), for example according to EN ISO 7218 [5].

6.2 Incubator, capable of maintaining a temperature of 30 °C ± 1 °C. Optionally also capable of maintaining a temperature of 40 °C ± 1 °C and/or between 44 °C and 47 °C.

6.3 Water bath, capable of maintaining a temperature of 40 °C ± 1 °C and between 44 °C and 47 °C.

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6.4 Blending equipment, e.g. a rotary homogenizer (blender), with a fixed or variable speed of minimum 22 000 min^{-1}, with aseptic glass or metals bowls equipped with covers according to EN ISO 7218 [5].

6.5 Mechanical stirrer.

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A mechanical stirrer (e.g. Vortex Mixer) facilitates the homogenous mixing of decimal dilutions, as described in e.g. EN ISO 7218 [5].

6.6 Balances, of the required range and accuracy, for example according to EN ISO 7218 [5], for the different products to be weighed.

6.7 Flasks or screw-cap bottles, of appropriate capacities.

6.8 Test tubes, of appropriate capacities.

6.9 Pipettes or pipettor and sterile tips, to dispense 0,1 ml to 1 ml.

6.10 Sterile pipettes, to dispense 5 ml, for full outlet with wide (approx. 3 mm) tips (e.g. serological pipette).

NOTE As alternative, 5 ml graduated pipettes without tips can be used.

6.11 Spreading spatula, sterile L- or triangular-shaped spreaders from glass or metal or sterile disposable plastic spreaders.

NOTE As alternatives, a spiral plater with a sanitized dispensing system or disposable one–way micro syringes can be used.

6.12 Sterile Petri dishes, with triple vents (plates), 90 mm in diameter.