SLOVENSKI STANDARD
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Krma: metode vzorčenja in analize - Izolacija in štetje prisotnih kvasovk probiotičnih sevov (Saccharomyces cerevisiae)

Animal feeding stuffs: Methods of sampling and analysis - Isolation and enumeration of yeast probiotic strains (Saccharomyces cerevisiae)

Futtermittel: Probenahme- und Untersuchungsverfahren - Trennung und Zählung von Hefestämmen (Saccharomyces cerevisiae)

Aliments des animaux - Méthodes d'échantillonnage et d'analyse - Isolement et dénombrement de souches probiotiques de levures (Saccharomyces cerevisiae)

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65.120	Krmila	Animal feeding stuffs

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Animal feeding stuffs: Methods of sampling and analysis - Isolation and enumeration of yeast probiotic strains (Saccharomyces cerevisiae)

Aliments des animaux - Méthodes d'échantillonnage et d'analyse - Isolement et dénombrement de souches probiotiques de levures (Saccharomyces cerevisiae)

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EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
EUROPÄISCHES KOMITEE FÜR NORMUNG

CEN-CENELEC Management Centre: Rue de la Science 23, B-1040 Brussels

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European foreword

This document (prEN 15789:2019) 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 document is currently submitted to the CEN Enquiry.

This document will supersede EN 15789:2009.

Introduction

This method 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 (EU project SMT4-CT98-2235 “Methods for the official control of probiotics (microorganisms) used as animal feeds” [1].

The procedure has been validated for one commercially used *Saccharomyces cerevisiae* strain [1]. As the method is not selective for this particular *Saccharomyces cerevisiae* strain, 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*).

1 Scope

This document defines 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 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 R767/2009) [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.

The detection limit is defined in EN ISO 7218.

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.

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

EN ISO 7218, *Microbiology of food and animal feeding stuffs – General requirements and guidance for microbiological examinations (ISO 7218)*

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:

- IEC Electropedia: available at http://www.electropedia.org/
- ISO Online browsing platform: available at http://www.iso.org/obp

3.1
saccharomyces cerevisiae
unicellular fungus which mostly reproduces vegetatively by budding

Note1 to entry This description is based on their characteristics as used for this standard

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

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

4 Principle

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

b) A representative test sample is taken under aseptic conditions;

c) An initial suspension is prepared with a tempered diluent to obtain a homogeneous distribution of yeast cells from the test portion;

d) The number of microorganisms per unit volume is reduced by the preparation of further decimal dilutions from the initial suspension to obtain a countable number of colonies on the selective enumeration media;

e) 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 petri dishes with an aliquot of the optimum dilutions and pouring of the molten agar medium into each Petri dish, mixing and solidification;

f) The inoculated plates are incubated for 48 h to 72 h at 30 °C ± 1 °C under aerobic conditions;

g) Counting of typical colonies, considering the specific properties of *Saccharomyces cerevisiae* as listed in 3.1;

h) Morphological verification of isolates by use of microscopy;

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

5 Diluents and selective media

5.1 Diluents

5.1.1 Diluent for initial suspension

This diluent is used for the preparation of the initial suspension and may also be used for the preparation of further decimal dilutions.

Table 1 — Phosphate buffered saline supplemented with Tween®[1] 80 (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
Polyoxyethylensorbitanmonooleate (Tween® 80)		1 ml
Water, distilled or deionized		1 000 ml

[1] Tween® 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

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

Temper to 40 ± 1°C in a water bath or incubator immediately before usage.

NOTE The use of commercially available PBS buffer tablets is acceptable. However, 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 medium as specified in this International Standard.

5.1.2 Diluents for serial dilutions

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

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

Enzymatic digest casein		1.0g
Sodium chloride	NaCl	8.5g
Water, distilled or deionized		1000 ml

Dissolve the components in the water in flasks, bottles or test tubes. 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 weight loss during autoclaving.

Sterilize at 121 °C ± 1 °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.

5.2 Enumeration media

5.2.1 Yeast extract dextrose chloramphenicol (oxytetracycline) agar (YGC agar)

Table 3 — Composition of the YGC agar

Yeast extract	5 g
D(+)-Glucose	20 g
Chloramphenicol	0,1 g
Agar agar	12–15 g[a]
Water, distilled or deionized	1 000 ml
pH 6,6 ± 0,2 at 25 °C	
a Depending on the gel strength of the agar.	

The base agar without antibiotic can be purchased and the chloramphenicol supplement has to be added or it 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)

5.2.2 Preparation

Dissolve all components 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 ± 1 °C for 15 min. Excessive heating shall be avoided.

NOTE If chloramphenicol is replaced by oxytetracycline the basic medium is prepared in the same way but without chloramphenicol. 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 – 47 °C.

6 Apparatus and glassware

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

6.1 Equipment for dry sterilization (oven) and wet sterilization (autoclave)

According to EN ISO 7218.

6.2 Incubator

Capable of maintaining a temperature of 30 °C ± 1 °C. Optionally also capable of maintaining a temperature of 40 ± 1°C and/or 44 °C to 47 °C.

6.3 Water bath

Capable of maintaining a temperature of 44 °C to 47 °C and 40 °C ± 1 °C.

6.4 Blending equipment

A rotary homogenizer (blender), with a fixed or variable speed of minimum 22 000 r/min, with aseptic glass or metals bowls equipped with covers (see EN ISO 7218).

6.5 Mechanical stirrer

A mechanical stirrer e.g. Vortex Mixer (see EN ISO 7218), or equivalent.

6.6 Balance

Balances of the required range and accuracy according ISO 7218 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 5 ml graduated pipettes

For full outlet with wide (approx. 3 mm) tips (e.g. serological pipette; alternatively: 5 ml-graduated pipettes without tips**).**

6.11 Bacterial Cell spreaders

Sterile L- or triangular-shaped spreaders from glass or metal or sterile disposable plastic spreaders.

Alternatively a spiral plater with a sanitized dispensing system or disposable one–way microsyringes can be used.