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Djurfoder – Isolering och kvantifiering av probiotiska jäststammar

Animal feeding stuffs – Isolation and enumeration of yeast probiotic strains

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EUROPEAN STANDARD

NORME EUROPÉENNE

EUROPÄISCHE NORM

EN 15789

September 2009

ICS 65.120

English Version

Animal feeding stuffs - Isolation and enumeration of yeast probiotic strains

Aliments des animaux - Isolation et dénombrement de souches probiotiques de levures (saccharomyces cerevisiae)

Futtermittel - Keimzählung von Hefestämmen

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

Management Centre: Avenue Marnix 17, B-1000 Brussels

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Foreword

This document (EN 15789:2009) has been prepared by Technical Committee CEN/TC 327 “Animal feeding stuffs”, 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of an identical text or by endorsement, at the latest by March 2010, and conflicting national standards shall be withdrawn at the latest by March 2010.

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Introduction

This method has been developed to enumerate probiotic yeasts in additives, premixtures and feeding stuffs. 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]. It is based on ISO 7954, a pour plate method using extract dextrose chloramphenicol (CGYE) (alternatively oxytetracycline) agar, a selective agar for yeasts [1]. This method is not selective for probiotic yeast but can be applied to enumerate yeast in feed assuming that the probiotic yeast is present in far higher numbers than any other yeast.

In addition or alternatively a spread plate method and a chromogenic[1] agar can be used allowing an elective enumeration of the probiotic yeast species for example *Saccharomyces cerevisiae*, which forms distinct mauve/purple colonies. The presence of other yeasts will be identified on the elective agar by different colouration.

The application of both agars (CGYE and Chromagar® Candida) have been validated for four probiotic commercially used yeast strains, belonging to *Saccharomyces cerevisiae,* in premixtures and feeding stuffs [3].

[1] e.g. CHROMagar® Candida from CHROMagar
This is a suitable product available commercially. This information does not constitute an endorsement by CEN of this product. Equivalent products may be used if they can be shown to lead to the same results.

1 Scope

This European Standard defines general rules for the enumeration of probiotic yeasts in feed samples (additives, premixtures and feeding stuffs) that contain yeast as a single microorganism component or in a mixture with other microorganisms. The standard is not applicable to mineral feeds which are defined as complementary feedingstuffs composed mainly of minerals and containing at least 40% crude ash (Council Directive 79/373/EEC) [4].

There are different categories of feed samples:

a) Additives which contain about 10^9 CFU/g to 10^{10} CFU/g (CFU = colony forming units):

b) Premixtures which contain about 10^8 CFU/g,

c) Feeds, meal or pellets, which contain about 10^6 CFU/g and include complete feedingstuffs, and milk replacers.

The detection limit is as defined in EN ISO 7218.

2 Normative references

The following referenced documents are indispensable for the application 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 6887-1, *Microbiology of food and animal feeding stuffs - Preparation of test samples, initial suspension and decimal dilutions for microbiological examination - Part 1: General rules for the preparation of the initial suspension and decimal dilutions (ISO 6887-1:1999)*

EN ISO 7218*, Microbiology of food and animal feeding stuffs - General requirements and guidance for microbiological examinations (ISO 7218:2007)*

ISO 6498, *Animal feeding stuffs – Preparation of test samples*

3 Terms and definitions

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

3.1
yeasts (described by their characteristics as used for this standard)
microorganisms which form colonies on the selective yeast extract dextrose chloramphenicol (oxytetracycline) agar according to the method specified in this Standard

4 Principle

a) Preparation of sterile and dry poured agar plates, and sterile molten agar at 48 °C ± 1 °C for poured plates.

b) Drawing a representative test sample under sterile conditions.

c) Preparation of the initial suspension to obtain a homogeneous distribution of yeast-like cells from the test portion.

d) 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.

e) Inoculation of the prepared plates with an aliquot of the optimum dilutions and dispersion of the inoculum by using a sterile spreader, or poured plate.

f) Aerobic incubation of inverted spread plates for 3 days at 30 °C ± 1 °C, or 35 °C ± 1 °C for 2 days for poured plates.

g) Counting of typical colonies, considering the specific properties of yeast as listed above.

h) Morphological verification of isolates of yeast through the use of microscope analysis.

i) Calculation of the colony count per g or kg of feed sample.

5 Diluent, selective media and test kit for phenotypic characterisation

5.1 Diluents

5.1.1 Diluent for initial suspension of premixtures, additives and feeding stuffs

This diluent is used to decimally dilute the sample to prepare an initial decimally diluted sample suspension (10^{-1}) in appropriate containers (e.g. universals, bottles or flasks).

Phosphate buffered saline (PBS):

Dissolve 8 g sodium chloride, 0,2 g potassium chloride, 1,15 g disodium hydrogen phosphate, 0,2 g potassium dihydrogen phosphate, pH 7,3 ± 0,2 in 1 l of distilled water. Aliquote this saline into appropriate containers (e.g. universals, bottles or flasks). Autoclave all capped containers with the initial diluent at 121 °C ± 1 °C for 10 min. To avoid loss during autoclaving, screw cap bottles are recommended.

Bring the diluent to room temperature before use.

Measure the pH of the diluent to ensure the suitable buffer capacity.

5.1.2 Diluent for serial dilutions

This diluent is used to decimally dilute the initial sample suspension and subsequent dilutions.

Peptone salt solution:

A peptone salt solution is made complying with EN ISO 6887-1.

Compose the solution of enzymatic digest of 1 g casein such as pancreatic peptone of casein (or peptone of same quality) and 8,5 g sodium chloride) per liter (l) distilled water. Dissolve the ingredients in water. Adjust the pH to 7,0 ± 0,2 at 25 °C ± 1 °C. For decimal dilutions, prepare test tubes containing 9,0 ml ± 0,1 ml after sterilisation or use screw cap bottles to avoid weight loss during autoclaving.

Sterilise in the autoclave for 15 min at 121 °C ± 1 °C. Bring the diluent to room temperature before use.

5.2 Media

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

Yeast extract dextrose chloramphenicol agar (CGYE) is composed of:

a) yeast extract 5 g;

b) dextrose 20 g;

c) chloramphenicol 0,1 g;

d) agar 12 g to 15 g;

e) made up to 1 000 ml with distilled water.

The base without antibiotic can be purchased and the chloramphenicol supplement has to be added or it can be purchased as a complete medium

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

5.2.2 Chromogenic agar[2)]

5.3 Phenotypic characterisation

Selected colonies are checked microscopically by suspension in a drop of 0,85% sterile saline with a coverslip and a feasible magnification (e.g. oil immersion) for morphology. Yeast obtains large cells of varying shape and size, possibly with 'budding'.

Typical microscopic and phenotypic profile for *Saccharomyces cerevisiae*:

On yeast extract dextrose chloramphenicol (oxytetracycline) agar:

a) Cream and opaque;

b) Irregular shaped;

c) Vary in size (1 mm to 6 mm in diameter).

On chromogenic agar[2]:

d) Circular;

e) Convex to dome-shaped;

f) Entire;

g) Mauve/purple ;

h) Matt or shiny surface;

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[2] e.g. CHROMagar® Candida from CHROMagar
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