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Animal feeding stuffs — Isolation and enumeration of yeast probiotic strains

ICS 65.120

BSI British Standards

National foreword

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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of an identical text or by endorsement, at the latest by March 2010, and conflicting national standards shall be withdrawn at the latest by March 2010.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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i) Opaque;

j) Vary in size (1 mm and 3 mm in diameter).

5.4 Biochemical characterisation

Presumptive yeast colonies can be biochemically characterised by classical or commercial test kits (e.g. API 20 C AUX or equivalent) for confirmation according to the manufacturers specifications.

6 Apparatus and glassware

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

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

According to EN ISO 7218.

6.2 Incubators

Capable of maintaining a temperature of 30 °C ± 1 °C and 35 °C ± 1 °C.

6.3 Water bath

Capable of maintaining a temperature of 48 °C ± 1 °C.

6.4 Blending equipment

Two-speed or variable adjustable blender (18 000 rotations per minute (rpm) and 22 000 rpm, with a one litre bowl which is sterilised in an oven for 1 h at 170 °C to 180 °C.

6.5 Mechanical stirrer

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

6.6 **Steamer,** or similar equipment for melting agar without autoclaving

A boiling water bath (or steamer) is used for the preparation of 500 ml volumes of agar, while a media preparator and plate pourer are used for more than 1 000 ml.

6.7 Balance

Capable of weighing to two decimal places.

6.8 Screw-cap bottles

Appropriate capacities, 25 ml universals, bottles, test tubes, flasks and 1 000 ml Duran bottles.

6.9 Pipettors

Total-delivery graduated pipettes or automatic pipettes and sterile tips to dispense 100 µl and 1 ml. Wide bore tips to pipette homogenised feed stuff for dilution.

6.10 Sterile spreaders

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

6.11 Sterile Petri dishes, triple vent, 90 mm in diameter

6.12 Laminar flow cabinet

6.13 Microscope

With phase-contrast-imaging (400x) and for use with oil immersion (1 000x)

7 Sampling

Carry out the sampling procedure in accordance with the specific standard appropriate to the product concerned. If such a specific standard is not available, it is recommended that agreement be reached on this subject among the parties concerned. Apply community rules [1] for official control sampling of animal feeds.

NOTE Sampling can be done according to ISO 6497 [5]. Although ISO 6497 is not applicable for micro organisms, due to the lack of other reference, it seems it is the most suitable protocol to be taken into account

WARNING — Take precautions to avoid potential cross-contamination of samples with yeast, particularly after sampling additives and premixtures supplemented with yeast.

8 Preparation of test samples

See the specific standard appropriate to the product concerned. If such a specific standard is not available, it is recommended that agreement be reached on this subject among the parties concerned. ISO 6498 gives general guidelines on test sample preparation and test sample preparation is recommended to be done in accordance with this ISO 6498.

9 Procedure

9.1 Preparation of agar plates

9.1.1 Preparation of yeast extract dextrose chloramphenicol (oxytetracycline) agar

Dissolve all components as described in 5.2.1 in water by boiling. If necessary adjust the pH so that after sterilisation it is pH 6,6 ± 0,4. Dispense the agar medium into suitable containers (bottles or flasks with non-toxic metal screw-caps may be used). Sterilise at 121 °C ± 1 °C for 15 min. For immediate use hold at 48 °C ± 1°C until use for preparation of pour plates.

NOTE If chloramphenicol is replaced by oxytetracycline the basic medium is prepared in the same way, and by omitting chloramphenicol. Dispense the medium in quantities of 100 ml and sterilise. Prepare also a 1 % mass concentration (m/m) solution of oxytetracycline hydrochloride in water and sterilise by filtration. Just prior to use, add 1 ml of this solution aseptically to 100 ml of the basic medium (in order to obtain a final concentration of 100 µg/ml of medium), that has been previously melted and maintained at 48 °C ± 1 °C.

9.1.2 Preparation of poured agar plates for chromogenic agar[3]

The medium is prepared according to the manufacturer's directions. After steaming to dissolve all the agar, or after preparation in a media preparator and cooling to 48 °C ± 1 °C, 20 ml to 25 ml of medium is poured into each Petri dish (6.11) under sterile conditions.When the medium has solidified, spread the plates out in the laminar flow cabinet with the lids partially removed for about 30 min to dry the agar.

NOTE Dried plates, correctly protected from dehydration, can be stored in the dark in the fridge for seven days.

Bring plates to room temperature before their use. Check the dried plates for possible contamination by incubating uninoculated controls.

9.2 Preparation of the initial suspension and decimal dilutions

Weigh 20 g ± 0,1 g of plain additive or premixture, or 2 g ± 0,01 g of a microgranule (encapsulated) additive or 50 g ± 0,5 g of feed. Add 180 g ± 0,1 g of diluent to ordinary additives or premixtures, 198 g ± 0,1 g to microgranule additives or 450 g ± 0,1 g to feeding stuffs. Homogenise the mixture with a suitable homogeniser such as a blender.

NOTE 1 It is recommended to check the pH of the initial suspension and to correct it to a range of pH 7,3 – 8,1.

The numbers of capsules should be more than 25, to obtain a SD (Standard Deviation) of less than 20% for a good repeat of analysis.

Blend additives and premixtures for 3 min at high speed (see 6.4) and dilute immediately.

Blend feeding stuffs for 1 min at high speed (see 6.4); start to blend at low speed to avoid splashing, then turn the switch to high speed. Let the sample stand for 30 min. It is important for pelleted feeds to absorb the liquid. Blend for 2 min at high speed (see 6.4) and immediately dilute using a wide bore pipette.

NOTE 2 Intense foaming in initial suspensions during blending may be avoided by using a feasible antifoaming agent (e.g. silicon antifoaming agent or equivalent) in adequate concentrations.

In the case of encapsulated products the application of adequate additives has to be used (e.g. Polyoxyethylensorbitanmonooleat or equivalent) with adequate temperatures (e.g. 40 °C).

The weight of the withdrawn pipette volume has to be calibrated in order to receive a correction factor for the calculation of the counts of probiotic yeasts.

WARNING — Suspensions containing probiotics tend to settle quickly after blending. This shall be avoided by rapid pipetting subsamples of only properly homogenised samples for further dilutions.

Prepare a series of appropriate dilutions (serial dilutions) using a sterile pipette such as a microdispenser set at 1 ml. Transfer 1 ml of the initial suspension into a tube containing 9 ml of sterile peptone saline brought to room temperature, and mix with a mechanical stirrer. Repeat this procedure using the 10^{-2} dilution and all further dilutions until the appropriate estimate for the number of cells is obtained. After this dilution procedure, inoculate the plates immediately as described in section 9.3.

NOTE 3 It is recommended to examine the content of the copper in the initial suspension by a pretest. Copper contents exceeding 200 mg/kg need the application of chelating agents e.g. iminodiacetic acid in a feasible concentration with regard to the pH-value.

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[3] e.g. CHROMagar® Candida from CHROMagar
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NOTE 4 For samples with high yeast count (additives, premixes), better precision is obtained by using 1/100 and/or 1/1 000 dilutions instead of decimal dilutions. Change the pipette tip between each dilution.

9.3 Inoculation and incubation of the plates

9.3.1 General

The time elapsing between the end of the preparation of the initial suspension and the moment when the medium is poured into the dishes shall not exceed 15 min.

Prepare a control pour and spread plates to check sterility.

WARNING — Selected appropriate dilutions must be homogeneous prior to transferring a subsample onto plates.

9.3.2 Yeast extract dextrose chloramphenicol agar

Take two sterile Petri dishes. Transfer to each dish, by means of a pipettor, 1 ml of the relevant dilutions of the test sample. Pour about 15 ml of the yeast extract dextrose chloramphenicol agar previously melted and maintained at 48 ± 1 °C in a water bath.

Carefully mix the inoculum with the medium and allow the mixture to solidify, by leaving the Petri dishes to stand on a cool horizontal surface.

Invert dishes and place in an incubator 35 ± 1 °C for 2 days.

9.3.3 Chromogenic agar[2)]

From each of the chosen dilutions, 100 µl is dispensed with a pipettor in duplicate plates and evenly spread using a sterile spreader.

When the inoculum is absorbed (within 20 min), the plates are inverted in piles of four and incubated aerobically at 30 °C for 3 days to 5 days or at 35 °C for 2 days to 3 days with a beaker of distilled water in the incubator to increase the humidity. Incubate up to 5 days depending on temperature and growth.

NOTE CHROMagar® Candida plates can be incubated at 35 °C ± 1 °C for 2 days, but the colonies are smaller and the colour is not fully developed. Therefore 30 °C ± 1 °C and 3 days incubation is recommended.

9.4 Counting of colonies

9.4.1 General

Depending on medium, count colonies on each plate after 2 days, 3 days and 4 days of incubation. In the case that no Petri dishes with colony counts between ≥ 30 and < 300 up to 350 are available the analysis has to be repeated with appropriate dilutions.

9.4.2 Yeast extract dextrose chloramphenicol (oxytetracycline) agar

Count dishes containing more than 30 and not more than 300 colonies are used for the enumeration of colony-forming units (CFU). The yeast often adheres to the plastic bottom of the dishes, particularly at the inoculation point, giving a swarm of colonies. Each of these colonies should be counted. If possible, count 4 Petri dishes (two successive dilutions).

9.4.3 Chromogenic agar[4]

After incubating for 2 days to 3 days under above specified conditions, all plates containing more than 30 and not more than 300 colonies are used for the enumeration of colony-forming units (CFU).

Count presumptive yeast colonies, and calculate the number of colony-forming units per gram feed according to the weighted arithmetical mean value in accordance with EN ISO 7218. *Saccharomyces cerevisiae* colonies appear as mauve/purple colonies whereas other yeast species can be distinguished (e.g. *Candida spp.* varies from white to dark pink).

9.5 Phenotypic characterisation - confirmation

Depending on the number of colonies appearing on the plate after incubation, select 1-5 typical colonies and use them for the phenotypic characterisation test. This will verify the findings of the colony count for *Saccharomyces cerevisiae* only. Washing of the cells 2x in Phosphate Buffered Saline before suspension in saline at McFarland standard 2 can enhance the differentiation of strains of *S. cerevisiae*.

Yeasts that produce typical colonies on the specified medium (see 5.3) and exhibit the typical phenotypic profile are counted as probiotic yeast.

These results are the basis for calculating the number of colony-forming units (CFU) per gram of feed or supplement.

10 Expression of results

The number of colony forming units (CFU) per gram of feed is calculated according to EN ISO 7218.

The number of yeasts (N) per g or per ml is equal to:

$$N = \frac{\sum C}{V(n_1 + 0{,}1 n_2)d} \qquad (1)$$

ΣC is the sum of the colonies counted on all the plates;

V is the volume of inoculum applied to each dish, in ml;

n_1 is the number of plates counted at the first dilution;

n_2 is the number of plates counted at the second dilution;

d is the dilution from which the first counts were obtained.

Round off the result obtained to two significant figures. For a three-figure number, round off the third to the nearest 0. If the third is 5, round off to the figure below if the first two figures are even numbers, and to the figure above if the first two figures are odd numbers.

The result is the number of micro organisms per gram product, expressed as a number between 1,0 and 9,9 multiplied by the appropriate power of 10.

[4] e.g. CHROMagar® Candida from CHROMagar
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11 Precision

11.1 General

The precision of the method using different media in terms of repeatability and reproducibility was determined in an interlaboratory study [3].

11.2 Interlaboratory study

Details of the interlaboratory study on the precision of the method are published (see [1], [3]) and are summarized in Annex A. Repeatability and reproducibility limits were determined using three types of feed samples contaminated at two levels of contamination. The values derived from the interlaboratory study may not be applicable to concentration ranges and matrices other than those given.

11.3 Repeatability

The absolute difference between two independent single ($\log_{10}$-transformed) test results (number of yeasts per g or per ml) or the ratio of the higher to the lower of the two test results on the normal scale, obtained using the same method on identical test material in the same laboratory by the same operator using the same apparatus within the shortest feasible time interval, will in not more than 5% of the cases exceed the repeatability limit *r*.

11.4 Reproducibility

The absolute difference between two single ($\log_{10}$-transformed) test results (number of yeasts per g or per ml) or the ratio of the higher to the lower of the two test results on the normal scale, obtained using the same method on identical test material in different laboratories with different operators using different equipment, will in not more than 5% of cases exceed the reproducibility limit *R*.

12 Test report

The test report shall specify:

a) information necessary for the complete identification of the sample;

b) the sampling method used, if known;

c) the test method and medium used, with reference to this European Standard;

d) the temperature of incubation;

e) operating details not specified in this European Standard, or regarded as optional, together with

f) details of any incidents which may have influenced the test result(s);

g) the test result(s) obtained, or, if the repeatability has been checked, the final quoted result obtained.

Annex A
(informative)

Results of the interlaboratory study

An interlaboratory study involving 20 laboratories in 12 European countries was carried out using feed samples with two different concentrations and a blank. The sample type with the lower yeast concentration ($2x10^7$ CFU/g) contained lactobacilli and enterococci at approximately double the yeast concentration. The sample with the higher yeast concentration ($1x10^8$ CFU/g) contained yeast only. The blank samples contained lactobacilli and pediococci however no yeast. The precision data derived from the study are presented in Table A.1.
The study was organized in 2002 and coordinated by Central Science Laboratory (UK) [1], [3].

Table A.1 — Precision data obtained from the collaborative study [1], [3].

parameters	**Sample (contamination level)**			
	CGYE*		**Chromogenic agar[5]**	
	low level	**high level**	**low level**	**high level**
Number of samples	3	3	3	3
Number of laboratories retained after eliminating outliers	11	15	6	9
Mean value (log_{10} CFU/g)	7,13	7,48	7,04	7,50
Repeatability standard deviation, s_r (log_{10} CFU/g)	0,17	0,36	0,14	0,21
Repeatability relative standard deviation, RSD_r (%)	2,38	4,86	1,93	2,81
Repeatability limit *r*:				
- as difference on log_{10} scale (log_{10} CFU/g)	0,47	1,02	0,38	0,59
Reproducibility standard deviation, s_R (log_{10} CFU/g)	0,55	0,60	0,14	0,44
Reproducibility relative standard deviation, RSD_R (%)	7,68	7,98	1,93	5,91
Reproducibility limit R:				
- as difference on log_{10} scale (log_{10} CFU/g)	1,53	1,67	0,38	1,24
* media reference see clause 5				

[5] e.g. CHROMagar® Candida from CHROMagar
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[1] European Community Project SMT4-CT98-2235. 'Methods for the official control of probiotics used as feed additives (vol. 1-3). 2002. Report EUR 20873/1-3. Office for Official Publications of the European Communities. ISBN 92-894-6249-3 (set)'

[2] ISO 7954:1987, *Microbiology – General guidance for enumeration of yeasts and moulds — Colony count technique at 25 °C*

[3] Leuschner R.G.K., J. Bew, G. Bertin. 2003. A collaborative study of a method for the enumeration of probiotic yeast in animal feed. Sys. Appl. Microbiol. 26, 147- 153

[4] Council Directive (79/373/EEC) of 2 April 1979 on the marketing of compound feedingstuffs (OJ No L 86, 6.4.1979, p.30)

[5] ISO 6497, *Animal feeding stuffs – Sampling*

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