SLOVENSKI STANDARD
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Krma: metode vzorčenja in analize - Izolacija in štetje domnevno prisotnih Bacillus spp

Animal feeding stuffs: Methods of sampling and analysis - Isolation and enumeration of presumptive Bacillus spp.

Futtermittel: Probenahme- und Untersuchungsverfahren - Trennung und Zählung von mutmaßlichen Bacillus spp.

Aliments des animaux - Méthodes d'échantillonnage et d'analyse - Isolement et dénombrement des souches de Bacillus spp. présumées

Ta slovenski standard je istoveten z: **prEN 15784**

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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 presumptive Bacillus spp.

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Recipients of this draft are invited to submit, with their comments, notification of any relevant patent rights of which they are aware and to provide supporting documentation.

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

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European foreword

This document (prEN 15784:2019) 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 15784:2009.

Introduction

This methodology has been developed to enumerate bacilli spores used as feed additives capable of germinating, to enable the European Commission to control proper labelling of animal feeding products. It was compiled first during EU project SMT4-CT98-2235 - "Methods for the official control of probiotics (microorganisms) used in animals feeds") [1]. During revision of the method it was adjusted to VDLUFA method 28.2.2 Enumeration of Bacillus licheniformis and Bacillus subtilis and completed with validation data from interlaboratory studies with commercial feed products [1]. The method is validated in this project for two strains of *Bacillus subtilis* and one strain of *Bacillus licheniformis.* It may be assumed that the method is suitable also for other Bacillus spp. used as feed additives. Vegetative cells are not taken into account in this method, as all approved *Bacillus* species products at present are spores.

Spores of *Bacillus* species survive a suspension with 0,2 % sodiumhydroxid solution and the *Bacillus* species characteristic colony morphology of the individually authorized strains is examined using the proposed method [2].

This method is not selective for bacilli used as feed additives but can be applied to enumerate bacilli in feeding stuffs assuming that the bacilli used as feed additives are present in far higher numbers than other bacilli.

1 Scope

This documentdefines general rules for the enumeration of bacilli in feeding stuffs (additives, premixtures and compound feeds including mineral feeds) that contain bacilli as a single microorganism component or in a mixture with other microorganisms. There are different categories of feed samples:

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

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

c) Compound feeds, meal or pellets which contain about 10^{9} to 10^{10} 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 7218, *Microbiology of food and animal feeding stuffs - General requirements and guidance for microbiological examinations (ISO 7218)*

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:

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

3.1
bacillus
genus of Gram-positive, rod-shaped bacteria

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

Note 2 to entry Bacillus species can be either obligate aerobes or facultative anaerobes. Cultured Bacillus species are catalase-positive if cultivated in the presence of oxygen

Note 3 to entry Bacilli can form oval endospores

Note 4 to entry Bacilli form colonies on the surface of Tryptone Soy agar (TSA) after incubation at a temperature of 37 °C under aerobic conditions for 16 h to 24 h fitting the description given in 9.3

4 Principle

a) Preparation of sterile and dry poured agar plates;

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

c) An initial suspension is prepared with a tempered 0,2 % sodiumhydroxid diluent to obtain a homogeneous distribution of bacterial cells from the test portion and to reduce the vegetative bacterial flora in the suspension;

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 enumeration media;

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

f) The inoculated plates are incubated for 16 h to 24 h at 37 °C ± 1 °C under aerobic conditions;

g) Counting of typical colonies, considering the specific properties of bacilli as listed in item 3;

h) Confirmation of exemplary isolates by microscopy or biochemical properties if necessary;

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

5 Diluent and selective medium

5.1 Diluent

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

Table 1 — 0,2 % sodium hydroxide solution

Sodium hydroxide (NaOH)	2,0 g
Polyoxyethylensorbitanmonooleate (Tween®[1] 80)	1 ml
Water, distilled or deionized	1 000 ml

Dissolve the components in the water, fill the solution into appropriate containers (e.g. bottles or flasks, test tubes) and sterilize at 121 °C ± 1 °C for 15 min. To avoid loss during autoclaving, screw cap bottles are recommended.

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

[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.

5.2 Enumeration Medium Tryptone soy agar (TSA)[2)]

5.2.1 Composition

Table 2 — Composition of the TSA agar

Tryptone	15,0 g
Sodium chloride (NaCl)	5,0 g
Soja peptone	5,0 g
Agar agar	12–15 g [a]
Water, distilled or deionized	1 000 ml
pH 7,3 ± 0,2 at 25 °C	
a Depending on the gel strength of the agar.	

5.2.2 Preparation

Dissolve all components (described in 5.2.1) 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 7,3 ± 0,2 at 25 °C after sterilization. Sterilize at 121 °C ± 1 °C for 15 min.

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

The following apparatus may be used (EN ISO 7218):

— a rotary homogenizer (blender) with a notional variable speed of 3 000 rpm to 10 000 rpm, as well as aseptic glass or metals bowls equipped with covers; or

— a peristaltic blender with sterile bags (paddle homogenizer), possibly with the option to adjust blending speed and time; or

— a vibrational mixer with sterile bags; or

— any other homogenizing system with equivalent efficiency (e.g. a hand blender with aseptic beaker).

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2) The medium is commercially ready made available from various suppliers.

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 EN 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 100 µl to 1 ml

6.10 Sterile 5 ml graduated

For full outlet with wide (approx. 3 mm) tips (e.g. serological pipette; alternative: 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.

6.12 Sterile Petri dishes with triple vents, 90 mm in diameter

6.13 Laminar flow cabinet

6.14 Microscope

Capable of phase-contrast microscopy at a magnification of 600x to 1 000x.

6.15 pH meter

Having an accuracy of calibration of ± 0,1 pH unit at 20 °C to 25 °C.

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 EN ISO 6497 [7]. Although EN ISO 6497 is not applicable for microorganisms, due to the lack of other references, it seems to be the most suitable protocol to be taken into account for microorganisms as feed additives.

WARNING — Take precautions to avoid potential cross-contamination of samples with microorganisms. Particularly after sampling additives and premixtures supplemented with microorganisms. When required, clean and disinfect sampling equipment between each sample, particularly after sampling additives and premixtures containing microorganisms. Put the sample in a sterile container.

8 Preparation of test sample

The test sample preparation shall be done in accordance with EN ISO 6498 and the congruent product standard. EN ISO 6498 gives general guidelines on test sample preparation.