SLOVENSKI STANDARD
SIST EN 15784:2022

01-februar-2022

Nadomešča:
SIST EN 15784:2009

Krma: Metode vzorčenja in analize - Določanje in štetje prisotnih Bacillus spp. uporabljen kot krmni dodatek

Animal feeding stuffs: Methods of sampling and analysis - Detection and enumeration of Bacillus spp. used as feed additive

Futtermittel: Probenahme- und Untersuchungsverfahren - Nachweis und Zählung von Bacillus spp. als Futtermittelzusatzstoff

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

Ta slovenski standard je istoveten z: **EN 15784:2021**

ICS:

65.120	Krmila	Animal feeding stuffs

SIST EN 15784:2022 **en,fr,de**

EUROPEAN STANDARD

NORME EUROPÉENNE

EUROPÄISCHE NORM

EN 15784

November 2021

ICS 65.120

Supersedes EN 15784:2009

English Version

Animal feeding stuffs: Methods of sampling and analysis - Detection and enumeration of Bacillus spp. used as feed additive

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

Futtermittel: Probenahme- und Untersuchungsverfahren - Nachweis und Zählung von Bacillus spp. als Futtermittelzusatzstoff

This European Standard was approved by CEN on 2 August 2021.

CEN members are bound to comply with the CEN/CENELEC Internal Regulations which stipulate the conditions for giving this European Standard the status of a national standard without any alteration. Up-to-date lists and bibliographical references concerning such national standards may be obtained on application to the CEN-CENELEC Management Centre or to any CEN member.

This European Standard exists in three official versions (English, French, German). A version in any other language made by translation under the responsibility of a CEN member into its own language and notified to the CEN-CENELEC Management Centre has the same status as the official versions.

CEN members are the national standards bodies of Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Republic of North Macedonia, Romania, Serbia, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey and United Kingdom.

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

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

Contents

Page

European foreword

This document (EN 15784:2021) 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 15784:2009.

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

— Amendment of the title;

— Extension of the scope of application to all *Bacilli* used as feed additive and to mineral feeds;

— Updating of normative cross references;

— Addition of 0,2 % NaOH as diluent for initial suspension and serial dilutions;

— Removal of the necessity of a heating step;

— Unification of the treatment of all matrices;

— Replacement of the required laboratory mixer with a rotation speed of 18 000 min^{-1} to 22 000 min^{-1} by homogenization devices, for example according to EN ISO 7218, with a maximal requested rotation speed of 10 000 min^{-1};

— Addition of the option to use a spiral plater for plating;

— Addition of validation data derived from VDLUFA ring trials of different feeding stuff matrices including mineral feed;

— Adjustment of the range of accepted colony numbers for counting from '≥ 30 to ≤ 300' to '≥ 10 to ≤ 100' colonies per plate.

Any feedback and questions on this document should be directed to the users’ national standards body. A complete listing of these bodies can be found on the CEN website.

According to the CEN-CENELEC Internal Regulations, the national standards organisations of the following countries are bound to implement this European Standard: Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Republic of North Macedonia, Romania, Serbia, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey and the United Kingdom.

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 the EU project SMT4-CT98-2235 “Methods for the official control of probiotics used as feed additives” [1]. During the 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 [2]. The method is validated in this project for two strains of *Bacillus subtilis* (DSM 5750 and DSM 15544) and one strain of *Bacillus licheniformis* (DSM 5749). It can be assumed that the method is suitable also for other *Bacillus* strains used as feed additives. However, the applicability of the method to the determination of *Bacillus* spp. in specific feed additive preparations may need to be demonstrated based on a case by case decision. 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 treatment with 0,2 % sodium hydroxide solution and the *Bacillus* species characteristic colony morphology of the individually authorized strains is examined using the proposed method [3].

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

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

1 Scope

This document specifies general rules for the enumeration of bacilli in feeding stuffs (additives, premixtures and compound feeds including mineral feeds) [4] 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 about 10^{11} CFU/kg;

c) Compound feeds, meal or pellets containing 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.

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/

3.1
***Bacillus* strains**
genus of Gram-positive, rod-shaped bacteria

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

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 9.5.

4 Principle

a) Preparation of sterile and dry poured plates;

b) Drawing a representative test sample under aseptic conditions;

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

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

f) Incubation of inverted plates 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;

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

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

5 Diluent and culture medium

5.1 Diluent

This diluent, a sodium hydroxide solution, is used for the preparation of the initial suspension and for the preparation of further decimal dilutions. The composition of the diluent is given in Table 1.

Table 1 — Components of a 0,2 % sodium hydroxide solution

Sodium hydroxide	NaOH	2,0 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. 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.

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

5.2 Culture medium tryptone soy agar (TSA)[2]

5.2.1 Composition

The composition of the culture medium tryptone soy agar is given in Table 2. The resulting pH value at 25 °C is 7,3 ± 0,2.

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.

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

Table 2 — Composition of the tryptone soy agar (TSA)

Tryptone	15,0 g
Sodium chloride	5,0 g
Soja peptone	5,0 g
Agar agar	12 g to 15 g[a]
Water, distilled or deionized	1 000 ml
[a] Depending on the gel strength of the agar.	

5.2.2 Preparation

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

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 37 °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.

6.4 Blending equipment.

The following apparatus may be used according to EN ISO 7218 [5]:

— a rotary homogenizer (blender) with a notional variable speed of 3 000 min^{-1} to 10 000 min^{-1}, as well as aseptic glass or metals bowls equipped with covers; or

— a peristaltic homogenizer 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).

6.5 Mechanical stirrer.

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.

6.13 Laminar flow cabinet.

6.14 Microscope, capable of phase-contrast microscopy at a magnification of 600× to 1 000×.

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

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 the sampling equipment between each sample, particularly after the sampling of 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.

NOTE EN ISO 6498 gives general guidelines on test sample preparation.

9 Procedure

9.1 Preparation of poured agar plates for spread plate method

The culture medium is prepared as described in 5.2.2 or according to the manufacturer's directions. Cool after autoclaving in a water bath to a temperature between 44 °C and 47 °C. Pour portions of approximately 15 ml into each plate (6.12) under sterile conditions and spread to give a homogeneous layer.