SLOVENSKI STANDARD
kSIST-TS FprCEN/TS 17804:2022

01-januar-2022

Organska, organsko-mineralna in anorganska gnojila - Ugotavljanje prisotnosti enterokokov (Enterococcaceae)

Organic, organo-mineral and inorganic fertilizers - Detection of Enterococcaceae

Organische, organisch-mineralische und anorganische Düngemittel - Nachweis von Enterococcaceae

Engrais organiques, organo-minéraux et inorganiques - Recherche des Enterococaceae

Ta slovenski standard je istoveten z: **FprCEN/TS 17804**

ICS:

65.080 Gnojila Fertilizers

kSIST-TS FprCEN/TS 17804:2022 **en,fr,de**

TECHNICAL SPECIFICATION

SPÉCIFICATION TECHNIQUE

TECHNISCHE SPEZIFIKATION

FINAL DRAFT FprCEN/TS 17804

December 2021

ICS 65.080

English Version

Organic, organo-mineral and inorganic fertilizers - Detection of Enterococcaceae

Engrais organiques, organo-minéraux et inorganiques - Recherche des Enterococacaceae

Organische, organisch-mineralische und anorganische Düngemittel - Nachweis von Enterococcaceae

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EUROPEAN COMMITTEE FOR STANDARDIZATION
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Ref. No. FprCEN/TS 17804:2021 E

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European foreword

This document (FprCEN/TS 17804:2021) has been prepared by the Technical Committee CEN/TC 260 “Fertilizers and liming materials”, the secretariat of which is held by DIN.

This document is currently submitted to the Vote on TS.

This document has been prepared under a standardization request given to CEN by the European Commission and the European Free Trade Association.

Introduction

This methodology has been developed to detect and enumerate *Enterococcaceae* in organic, organo-mineral and inorganic fertilizers in order to be able to control certain hygienic requirements in Regulation (EU) 2019/1009 [1].

Enterococcaceae in the sense of this document include several species of the genus *Enterococcus* (3.7, 3.8) with a faecal origin. Consequently, it can be used as an indicator of faecal contamination. It can also be used to monitor the effectiveness of pasteurization or disinfection treatments. Compared to *E.coli*, they have a higher tenacity and can therefore better reflect the behaviour of all pathogens in fertilizers.

Because of the large variety of fertilizers, this method is not appropriate in every detail for certain products. In this case, different methods which are specific to these products can be used if absolutely necessary for justified technical reasons. Nevertheless, every attempt should be made to apply this method as far as possible.

Mineral components in fertilizers can have a negative impact on the survivability of microorganisms when they go into solution. In addition to an unfavourable shift in the pH value, the products can have a strong osmotic effect or be toxic to cells themselves (e.g. copper). Therefore, it can be necessary to test the inhibitory effect of the fertilizers to be investigated in a pre-test.

The method is validated in an interlaboratory study for the following products (*Enterococcaceae* were investigated in both native and spiked test material):

Table 1 — Product groups and matrices for which the methods described in this method are applicable and tested in a validation trial

Product group	matrix
Organic fertilizers	to be determined at an international ring trial
Organo-mineral fertilizers	to be determined at an international ring trial
Inorganic fertilizers	to be determined at an international ring trial

International ring trials will be conducted on the basis of this document.

1 Scope

This document specifies a method for the detection and enumeration of *Enterococcaceae* in fertilizers of the following Product Function Categories (PFCs) of EU fertilizing products, as described in Regulation (EU) 2019/1009 [1]:

- PFC 1(A): Organic fertilizer;
- PFC 1(B): Organo-mineral fertilizer;
- PFC 1(C): Inorganic fertilizer, which contains more than 1 % by mass of organic carbon, other than organic carbon from chelating or complexing agents, nitrification inhibitors, denitrification inhibitors or urease inhibitors, coating agents, urea or calcium cyanamide. The present method was validated on products known as present on the market in April 2021 and conform to Regulation (EU) 2019/1009 [1] that are inorganic fertilizers with more than 1 % of organic carbon such as poultry manure and struvite with low level of organic matter. In case that other products would be developed having other physical and chemical characteristics, it might become necessary to develop different methods to correctly account for pathogens they might contain.

This document specifies a colony-count technique on selective media, Slanetz Bartley agar or Bile Esculin Azide agar, respectively. The method is based on EN ISO 7899-2:2000.

2 Normative references

There are no normative references in this document.

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 https://www.electropedia.org/
- ISO Online browsing platform: available at https://www.iso.org/obp

3.1
laboratory sample
sample intended for laboratory inspection or testing

3.2
test sample
sample prepared from the laboratory sample (3.1) and from which test portions (3.3) will be taken

3.3
test portion
quantity of material taken from the test sample (or if both are the same, from the laboratory sample) and on which the test is carried out

3.4
initial suspension
primary dilution obtained after a weighed or measured quantity of the product under examination (or of a test sample prepared from the product) has been mixed with, normally, a nine-fold quantity of diluent

Note 1 to entry: A closer ratio between the diluent and the quantity of product is often not recommended because of possible inhibiting influences of the matrix.

3.5
further dilution
suspension or solution obtained by mixing a measured volume of the initial suspension (3.4) with an x-fold volume of diluent and by repeating this operation with further dilutions until a dilution series, suitable for the inoculation of culture media, is obtained

Note 1 to entry: Ten-fold dilutions are normally used to produce a decimal dilution series, but other ratios can be required for specific purposes.

3.6
Enterococcaceae
include among other genera and species several species of the genus *Enterococcus* (3.7, 3.8) with a faecal origin

3.7
genus *Enterococcus*
gram-positive, catalase-negative facultative-anaerobic cocci of the family *Enterococcaceae*, which often occur in pairs (diplococci) or short chains, unable of forming spores, tolerant of a wide range of environmental conditions (extreme temperature (10 °C to 45 °C), pH (4,5 to 10,0), high sodium chloride concentrations) and occur ubiquitously in the environment (water, soil), in animals and in humans (in the normal intestinal flora) and for which they are considered indicator germs for faecal contamination (intestinal *enterococci*)

3.8
enterococci
gram-positive, catalase-negative cocci, able to reduce 2,3,5-triphenyl tetrazolium chloride to formazan on Slanetz Bartley agar and to hydrolyze esculin at 44 °C on Bile Esculin Azide agar

3.9
presumptive *enterococci*
gram-positive, catalase-negative cocci, able to reduce 2,3,5-triphenyl tetrazolium chloride to formazan on Slanetz Bartley agar or to hydrolyze aesculin at 44 °C on Bile Esculin Azide agar

4 Principle

a) Preparation of Slanetz Bartley agar plates and/or Bile Esculin Azide agar.

b) Drawing a representative test sample under aseptic conditions.

c) Preparation of the initial suspension with a tempered diluent to obtain a homogeneous distribution of bacterial 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 or to reduce the cell inhibitory properties of the initial suspension to allow, after incubation, the counting of colonies.

e) Inoculation of plates with Slanetz Bartley agar or Bile Esculin Azide agar with an aliquot of the optimum dilutions by plating method.

f) Incubation of inverted plates at 37 °C ± 1 °C for 44 h to 48 h (Slanez Bartley agar) or at 44 °C ± 1 °C for 24 h ± 2 h (Bile Esculin Azide agar).

g) Counting of typical colonies showing pink-red or red-brown colour, considering the specific properties of *enterococci.*

h) Confirmation of typical colonies from Slanez Bartley agar by inoculation on pre-heated plates with Bile Esculin Azide agar at 44 °C ± 1 °C for 2 h or typical colonies from Bile Esculine Azide agar by inoculation on Slanez Bartley agar at 37 °C ± 1 °C for 24 h.

i) If a lower detection limit is required, 1 ml of the first dilution (10^{-1}) can be spread over the surface of agar plates (140 mm diameter or 3 smaller plates having a diameter of 90 mm).

j) Calculation of the number of colony-forming units (CFU) of *enterococci* per gram or per millilitre of sample.

5 Reagents

5.1 General

For standard laboratory practice, EN ISO 7218 and EN ISO 11133 can be used.

Composition of culture media and reagents and their preparation are specified in Annex B.

For uniformity of results, in the preparation of media, either use a dehydrated complete medium or use constituents of uniform quality and chemicals of recognized analytical grade.

5.2 Diluents

5.2.1 General

Fertilizers with a high mineral content can significantly change the pH value of the initial suspension, which can negatively affect the viability of the microorganisms under investigation. In general (organic fertilizers), a basic phosphate buffer is sufficient to prepare the initial suspension. When testing organo-mineral or inorganic fertilizers, the pH value of the substrate in solution should be determined in a preliminary test. The general use of a double-buffered phosphate buffer is recommended. If the substrate is simultaneously tested for the presence of *Salmonella* (FprCEN/TS 17780) the initial suspension for enrichment can be used with buffered or double- buffered peptone water (with 10 g peptone). In this case, rapid processing of the initial suspension is necessary.

5.2.2 Basic phosphate buffer

See B.2 and Table B.1.

5.2.3 Double-buffered phosphate buffer

See B.3 and Table B.2.

5.3 Selective media

5.3.1 Slanetz Bartley agar

See B.4 and Table B.3.

5.3.2 Bile Esculin Azide agar

See B.5 and Table B.4.

6 Equipment and consumables

6.1 General

Disposable equipment is an acceptable alternative to reusable glassware if it has suitable specifications.

Usual microbiological laboratory equipment (EN ISO 7218 can be used) and, in particular, the following.

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

EN ISO 7218 can be used.

6.3 Incubator

Capable of maintaining a temperature of 44 °C ± 1 °C. Optionally also capable of maintaining a temperature of 37 °C ± 1 °C and/ and/or 44 °C to 47 °C.

6.4 Blending equipment

The following apparatus can be used:

- a peristaltic homogenizer with sterile bags (paddle homogenizer), possibly with the option to adjust blending speed and time, or
- a laboratory shaker with sterile bags.

6.5 Mechanical stirrer

A mechanical stirrer e.g. Vortex Mixer facilitates the homogenous mixing of decimal dilutions.

6.6 Scale

Scales of the required range and accuracy comparable to EN ISO 7218 for the different products to be weighed.

6.7 Water bath

Capable of maintaining temperatures of 44 °C to 47 °C.

6.8 Cooling unit, adjustable at 5 °C ± 3 °C.

6.9 pH meter, capable of reading to the nearest 0,1 pH unit at 20 °C to 25 °C.

6.10 Sterile loops of approximate diameter, 3 mm (10 µl volume).

6.11 Sterile tubes, bottles, or flasks with caps of appropriate capacity.

6.12 Pipettes or pipettor and sterile tips of nominal capacities of 10 ml and 1 ml.

6.13 Sterile Petri dishes with a diameter of approximately 90 mm and (optional) large size (diameter approximately 140 mm).

7 Sampling

Sampling should be performed carefully, following the principles described in EN 1482 (all parts) with appropriate adaptations, required to account for specificities of organic and organo-mineral fertilizers and to microbiological quality of the samples.