SLOVENSKI STANDARD
oSIST prEN 17720:2023

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Rastlinski biostimulanti - Ugotavljanje prisotnosti in števila enterokokov (Enterococcaceae)

Plant biostimulants - Determination of Enterococcaceae

Pflanzen-Biostimulanzien - Bestimmung von Enterococcaceae

Biostimulants des végétaux - Détermination des Enterococcaceae

Ta slovenski standard je istoveten z: **prEN 17720**

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65.080 Gnojila Fertilizers

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English Version

Plant biostimulants - Determination of *Enterococcaceae*

Biostimulants des végétaux - Détermination des *Enterococcaceae*

Pflanzen-Biostimulanzien - Bestimmung von *Enterococcaceae*

This draft European Standard is submitted to CEN members for enquiry. It has been drawn up by the Technical Committee CEN/TC 455.

If this draft becomes a European Standard, 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.

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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 17720:2023) has been prepared by Technical Committee CEN/TC 455 “Plant Biostimulants”, the secretariat of which is held by AFNOR.

This document is currently submitted to the CEN enquiry.

This document will supersede CEN/TS 17720:2022.

This document has been prepared under a Standardization Request given to CEN by the European Commission and the European Free Trade Association, and supports essential requirements of EU Directive(s) / Regulation(s).

For relationship with EU Directive(s) / Regulation(s), see informative Annex ZA, which is an integral part of this document.

Introduction

This document was prepared by the experts of CEN/TC 455 “Plant Biostimulants”. The European Committee for Standardization (CEN) was requested by the European Commission (EC) to draft European standards or European standardization deliverables to support the implementation of Regulation (EU) 2019/1009 of 5 June 2019 laying down rules on the making available on the market of EU fertilising products (“FPR” or “Fertilising Products Regulation”). This standardization request, presented as SR M/564 and M/564/Amd1, also contributes to the Communication on “Innovating for Sustainable Growth: A Bio economy for Europe”. Working Group 5 “Labelling and denominations”, was created to develop a work program as part of this standardization request.

Technical committee CEN/TC 455 “Plant Biostimulants” was established to carry out the work program that will prepare a series of standards. The interest in biostimulants has increased significantly in Europe as a valuable tool to use in agriculture. Standardization was identified as having an important role in order to promote the use of biostimulants. The work of CEN/TC 455 seeks to improve the reliability of the supply chain, thereby improving the confidence of farmers, industry, and consumers in biostimulants, and will promote and support commercialization of the European biostimulant industry.

Biostimulants used in agriculture can be applied in multiple ways: to the soil, to plants, as seed treatments, etc. A microbial plant biostimulant consists of a microorganism or a consortium of microorganisms, as referred to in Component Material Category 7 of Annex II of the EU Fertilising Products Regulation.

This document is applicable to all microbial biostimulants in agriculture.

Table 1 summarizes many of the agro-ecological principles and the role played by biostimulants.

Table 1 — Agro-ecological principles and the role played by biostimulants [1]

Increase biodiversity
By improving soil microorganism quality/quantity
Reinforce biological regulation and interactions
By reinforcing plant- microorganism interactions
— symbiotic exchanges i.e. *mycorrhizae*
— symbiotic exchanges i.e. *rhizobiaceae/fabaceae*
— secretions mimicking plant hormones (i.e. *trichoderma*)
By regulating plant physiological processes
— e.g. growth, metabolism, plant development…
Improve biogeochemical cycles
— improve absorption of nutritional elements
— improve bioavailability of nutritional elements in the soil
— stimulate degradation of organic matter

This methodology has been developed to enumerate enterococci and enable the European Commission to control proper labelling of plant biostimulant products. It is mainly based on EN 15788:2009, *Animal feeding stuffs — Isolation and enumeration of Enterococcus (E. faecium)* spp. The method is based on an extensive screening of 12 pre-selected, commercially available media for the detection and enumeration of enterococci. The described methodology was validated in an interlaboratory study [2].

WARNING — Persons using this document should be familiar with normal laboratory practice. This document does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices and to ensure compliance with any national regulatory conditions.

IMPORTANT — It is absolutely essential that tests conducted in accordance with this document be carried out by suitably trained staff.

1 Scope

This methodology has been developed to determine enterococci in biostimulants as a single microorganism component or in a mixture with other microorganisms. This document is not applicable to mineral fertilizers that are defined as complementary feeding stuffs composed mainly of minerals and containing at least 40 % crude ash (Council Directive 79/373/EEC) [3].

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 7218:2007[1], *Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations (ISO 7218:2007)*

3 Terms and definitions

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

ISO and IEC maintain terminology 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
***Enterococcus* spp.**
bacteria forming colonies on the specified selective medium Slanetz Bartley agar after incubation of spread plates for 44 h at a temperature of 36 °C under aerobic conditions

Note 1 to entry: Colony description:

— circular;

— convex to dome-shaped;

— entire;

— glistening surface;

— pink, red, brown colour;

— opaque.

Note 2 to entry: Colony size varies between 0,5 mm and 2 mm in diameter.

Note 3 to entry: When using Bile Esculin Azide agar (BEA) the medium surrounding the colonies shows a dark brown to black coloration, due to the hydrolysis of esculin.

Note 4 to entry: Microscopic examination of selected colonies typically shows diplococci or short chains of cocci.

[1] As impacted by EN ISO 7218:2007/A1:2013.

4 Principle

An initial suspension of the sample is prepared in a diluent with suitable buffer capacity using a suitable homogenizer. Dilutions of the initial suspension (serial ten-fold dilutions) shall be immediately prepared before the suspension settles. Subsequently plate 0,1 ml of each dilution onto Slanetz-Bartley agar. This selective medium (as reported in EN ISO 7899-2:2000) inhibits the growth of Gram-negative bacteria and *Staphylococcus.* Incubate at 36 °C ± 1 °C for 44 h. The growing colonies show a pink-red or red-brown colour and should be further confirmed by subsequent inoculation onto BEA pre-heated plates (44 °C for 2 h).

If reaching a lower detection limit is required, 1 ml of the undiluted biostimulant product or 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).

BEA plates are inoculated with 0,1 ml of the chosen dilutions. The plates are incubated aerobically for 24 h ± 2 h at 44 °C ± 1 °C.

Presumptive enterococci colonies are counted and the number of colony-forming units (CFU) per g or ml is calculated.

To verify the colony count, a phenotypic characterization and confirmation of a selection of colonies may be done by means of an identification kit (widely available on the market).

5 Diluents, selective media and test kit for phenotypic characterization

5.1 General

For current laboratory practice, refer to EN ISO 7218:2007[1] and see EN ISO 11133:2014[2].

Composition of culture media and reagents and their preparation are described in Annex A (normative).

5.2 Diluent for initial suspension and for serial dilutions

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

Annex A gives several suitable diluents. Other diluents may be used if they have been demonstrated to be suitable for use.

5.3 Selective media

Slanetz-Bartley agar and BEA (commercially available from various suppliers) are used as selective media. For composition and preparation, see Annex A.

5.4 Phenotypic characterization and confirmation

Check selected colonies for typical morphology (3.1). To avoid false-positives, a catalase test can be carried out on doubtful colonies. Enterococci exhibit a negative reaction to the catalase test. If positive, consider the colony as non-enterococci.

NOTE Strains that could appear as false-positives on BEA such as *Listeria* spp. and *Enterobacter* spp. possess the enzyme and will show a positive reaction to the catalase test. Also, a microscopic examination can be performed, by wet-mount at (x1 000) magnification. Verify up to 5 colonies.

[2] As impacted by EN ISO 11133:2014/A1:2018 and EN ISO 11133:2014/A2:2020.

6 Apparatus and glassware

Usual microbiological laboratory equipment and, in particular, the following.

6.1 Equipment for dry sterilization (oven) or wet sterilization (autoclave).

The specifications in EN ISO 7218:2007[1] shall be used.

6.2 Incubator. Capable of maintaining a temperature of 44 °C ± 1 °C.

6.3 Water bath. Capable of maintaining a temperature of 47 °C ± 2 °C.

6.4 Mechanical stirrer. Vortex Mixer or equivalent.

6.5 Magnetic stirrer.

6.6 Balance. Capable of weighing to two decimal places.

6.7 Screw-cap sterile tubes, bottles, or flasks of appropriate capacity. Universals, bottles, test tubes and flasks of 25 ml; and Duran bottles of 1 000 ml.

6.8 Pipette and sterile tips of the appropriate volume.

6.9 Sterile Petri dishes, 90 mm in diameter.

6.10 Microscope. Magnification 1 000x.

6.11 Paddle blender to extract and wash intact microbes into a solution, which is then used for downstream analysis.

7 Sampling

Carry out the sampling procedure in accordance with the specific standard appropriate to the product concerned.

WARNING — Take precaution to avoid potential cross-contamination of samples.

8 Preparation of test sample

8.1 General

A representative sample of the product will be prepared according to the following procedures which takes into consideration the different formulations of biostimulants based products:

8.2 Liquid – water-based formulations

Dispense 25 g (or more for low concentration products) of the sample in a flask, add 225 g of sterile Phosphate Buffer Solution (PBS) maintained at room temperature and shake with a magnetic stirrer at half speed for 10 min or more, until the distribution is optimal [4].

8.3 Liquid – oil-based (emulsifiable concentrate – EC) formulations

Dispense 25 g (or more for low concentration products) of the sample in a flask, add 225 g of sterile PBS maintained at room temperature and shake with a magnetic stirrer at half speed for 10 min or more, until the distribution is optimal [4].