



**SLOVENSKI STANDARD
SIST-TS CEN/TS 17720:2023**

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Rastlinski biostimulanti - Določanje Enterococcaceae

Plant biostimulants - Determination of Enterococcaceae

Pflanzen-Biostimulanzien - Bestimmung von Enterococcaceae

Biostimulants des végétaux - Détermination des Enterococcaceae

Ta slovenski standard je istoveten z: CEN/TS 17720:2022

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English Version

Plant biostimulants - Determination of *Enterococcaceae*

Biostimulants des végétaux - Détermination des
Enterococcaceae

Biostimulanzien für die pflanzliche Anwendung -
Bestimmung von *Enterococcaceae*

This Technical Specification (CEN/TS) was approved by CEN on 3 January 2022 for provisional application.

The period of validity of this CEN/TS is limited initially to three years. After two years the members of CEN will be requested to submit their comments, particularly on the question whether the CEN/TS can be converted into a European Standard.

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

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European foreword

This document (CEN/TS 17720:2022) has been prepared by Technical Committee CEN/TC 455 “Plant Biostimulants”, the secretariat of which is held by AFNOR.

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 has been prepared under a Standardization Request given to CEN by the European Commission and the European Free Trade Association.

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 announce this Technical Specification: 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.

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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 the European Parliament and of the Council 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 M/564, 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. <i>Mycorrhizae</i>
— symbiotic exchanges i.e. <i>Rhizobiaceae/Fava</i>
— secretions mimicking plant hormones (i.e. <i>Trichoderma</i>)
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].

This method can be applied to enumerate enterococci in additives, premixtures and biostimulants.

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.

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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 (Regulation (EC) No 767/2009) [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 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
***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: <https://standards.iteh.ai/catalog/standards/sist/c5caaed1-5915-4442-86b3-ee58b2992834/sist-ts-cen-ts-17720-2023>

- 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 Staphylococci. Incubate at $36\text{ °C} \pm 1\text{ °C}$ 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 $24\text{ h} \pm 2\text{ h}$ at $44\text{ °C} \pm 1\text{ °C}$.

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, see EN ISO 7218:2007¹ and EN ISO 11133:2014².

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 (1 000×) magnification. Verify up to 5 colonies.

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

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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 7218:2007¹ shall be used.

6.2 Incubator. Capable of maintaining a temperature of $44\text{ °C} \pm 1\text{ °C}$.

6.3 Water bath. Capable of maintaining a temperature of $47\text{ °C} \pm 2\text{ °C}$.

6.4 Mechanical stirrer. A mechanical stirrer, e.g. vortex mixer or equivalent.

6.5 Balance. Capable of weighing to two decimal places.

6.6 Sterile tubes, bottles, or flasks with caps of appropriate capacity. 25 ml universals, bottles, test tubes, flasks and 1 000 ml Duran[®] bottles³.

6.7 Pipette and sterile tips of the appropriate volume.

6.8 Sterile Petri dishes, 90 mm in diameter.

6.9 Microscope. Magnification 1 000 \times .

6.10 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) (see Annex A) maintained at room temperature and shake with a magnetic stirrer at half speed for 10 min or more, until the distribution is optimal [4].

³ Duran[®] 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.