SLOVENSKI STANDARD
SIST-TS CEN/TS 17709:2023

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Rastlinski biostimulanti - Določanje Azotobacter spp.

Plant biostimulants - Determination of Azotobacter spp.

Pflanzen-Biostimulanzien - Bestimmung von Azotobacter spp.

Biostimulants des végétaux - Détermination d'Azotobacter spp.

Ta slovenski standard je istoveten z: **CEN/TS 17709:2022**

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65.080 Gnojila Fertilizers

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CEN/TS 17709

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English Version

Plant biostimulants - Determination of *Azotobacter* spp.

Biostimulants des végétaux - Détermination d'*Azotobacter* spp.

Pflanzen-Biostimulanzien - Bestimmung von *Azotobacter* spp.

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

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EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
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Ref. No. CEN/TS 17709:2022 E

Contents

Page

European foreword

This document (CEN/TS 17709:2022) has been prepared by Technical Committee CEN/TC 455 “Plant biostimulants”, the secretariat of which is held by AFNOR.

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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 5 June 2019 laying down rules on the making available on the market of EU fertilizing 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". The Working Group 5 "Labelling and denominations", was created to develop a work program as part of this request. The 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 commercialisation of the European biostimulant industry.

Biostimulants used in agriculture can be applied in multiple ways: on soil, on plant, as seed treatment, 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 biostimulants in agriculture based on live microorganisms belonging to the genera *Azotobacter*.

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

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

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/Faba*
- secretions mimicking plant hormones (i.e. *Trichoderma*)
By regulating plant physiological processes
- for ex 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

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 document was developed to provide the methodology for the enumeration and determination of *Azotobacter* sp. in plant biostimulant products in accordance with the Regulation (EU) 2019/1009 of the European Parliament and of the Council [1].

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.

CEN/TS 17702-1, *Plant biostimulants — Sampling and sample preparation — Part 1: Sampling*

CEN/TS 17724, *Plant biostimulants — Terminology*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in CEN/TS 17724 apply.

4 Enumeration of *Azotobacter* spp.

4.1 General

This procedure is meant to determine the number of colony-forming units (CFU) of the above mentioned bacteria, per gram, per millilitre, per square centimetre, or per sampling device. The method, in order to be fast, cheap, repeatable, is based on serial dilutions and plating.

4.2 Sample preparation

4.2.1 General

A representative sample of the product to be analysed according to CEN/TS 17702-1 shall be prepared according to following procedure which takes into consideration the different formulations of biostimulants based products.

4.2.2 Liquid (based water) formulations

Dispense 25 ml of sample (or more for low concentrated products) in 225 ml of sterile Phosphate Buffer Solution (PBS) maintained at room temperature, in a flask and shake for 10 min or more until the distribution is optimal, with a magnetic stirrer at half speed [6].

4.2.3 Liquid - based oil, emulsifiable concentrate (EC) formulations

Dispense 25 ml of sample (or more for low concentrated products) in 225 ml of sterile Phosphate Buffer Solution (PBS) maintained at room temperature, in a flask and shake for 10 min or more until the distribution is optimal, with a magnetic stirrer at half speed [6].

4.2.4 Solid - Wettable Powder (WP) formulations

Dispense 25 g of sample (or more for low concentrated products) in 225 ml of sterile Phosphate Buffer Solution (PBS) maintained at room temperature, in a flask and shake for 20 min or more until the distribution is optimal, with a magnetic stirrer at half speed [6].

4.2.5 Solid - Water dispersible granules (WDG) formulations

Dispense 25 g of sample (or more for low concentrated products) in 225 g of sterile Phosphate Buffer Solution (PBS) maintained at room temperature, in a flask and shake for 40 min or more until the distribution is optimal, with a magnetic stirrer at half speed. If required help the

dispersion of the formulations with other apparatus such as a stomacher after having sieved (100 mesh sieve) the particles and resuspend them in the same suspension [6].

4.2.6 Solid – Pellets, granules, microgranules (slow release) formulations

Dispense 25 g of sample (or more for low concentrated products) in 225 g of sterile Phosphate Buffer Solution (PBS) maintained at room temperature, in a sterile bag and disperse them using a magnetic stirrer for 40 min at half speed and then sieve in a 100 mesh sieve and if material remain in the sieve repeat the process for a maximum of three times. Put attention to all the buffer used to make the exact final calculation [6].

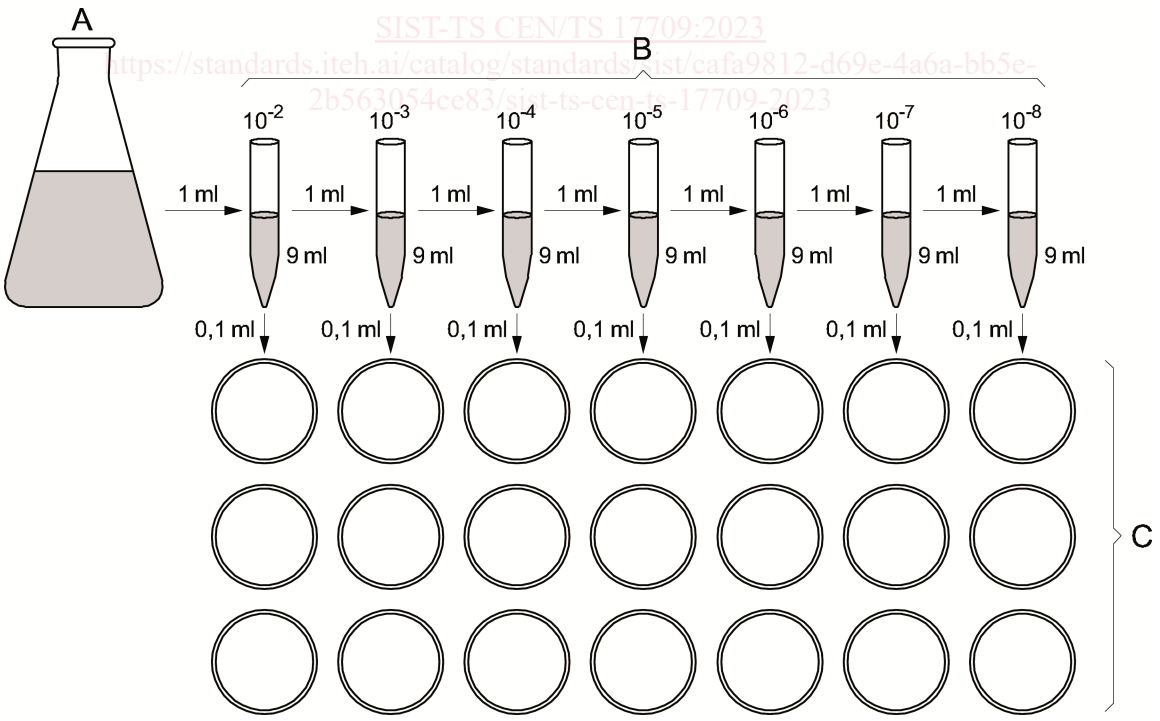
4.2.7 Solid - substrate

Dispense 25 g of sample (or more for low concentrated products) in 225 g of sterile Phosphate Buffer Solution (PBS) maintained at room temperature, in a flask and shake for 20 min or more until the distribution is optimal, with a magnetic stirrer at half speed [6].

4.3 Serial dilution

The principle in counting bacteria by dilution is to serially dilute them to reduce the bacterial density to the level where individual cells can be differentiated. This may be, for example, as live cells under the microscope, as colonies that grow on plates from single cells, or estimated in the plant-infection technique (with the principle that a single cell can multiply to initiate an infection). Serial dilution can be applied to all kind of formulations. A 10-fold serial dilution is most often used (Figure 1) but if the number of *Azotobacter* sp. cells is expected to be low then a lower number of dilutions can be adopted.

A sample of the product is shaken in a bulk diluent (PBS) which represents the first level of dilution. This is then serially diluted with a sample at each level of dilution directly plated.



Key

A product suspended in sterile diluent

B prepare dilution series at 10^{-8}

C 3 replicates of Petri dishes with media

Figure 1 — Scheme of a serial dilution series

4.4 Plate counts of *Azotobacter* sp in sterile diluent

The counting of microorganisms on plates, following dilution, is also called direct counting. Count only plates where there are between 30 to 300 colonies.

4.5 Spread-plate counting with ASHBY SUCROSE AGAR [6]

The steps of the procedure are the following:

1. Inoculate 0,1 ml of the serial dilutions desired (e.g. 10^{-5}, 10^{-6} and 10^{-7}) on the surface of the culture medium in Petri dishes (Figure 1);

2. Spread the 0,1 ml aliquot over the culture medium with a sterilized L-shaped glass spreader (or equivalent, e.g. a Drigalski loop);

3. There should be at least three separate replicate plates for each dilution;

4. After inoculation and absorption of the inoculum into the agar, the plates are placed in an incubator at approximately 30 °C, inverted and allowed to grow for a period of 4 days;

5. Count the number of colonies on plates where colonies are well separated. If colony numbers are low, variation between plates and errors may be large. If colony numbers are too high, overcrowding may result in an underestimation of numbers. Many texts recommend counting between 30 CFU (Colony Forming Units) and 300 CFU per plate to give statistical robustness.

4.6 Calculation

Multiply the average number of CFU on the three Petri dishes by the inverse of the dilution that gave the reading in the range 30 to 300 CFU by 10 (to correct for the 0,1 ml or gr used).

EXAMPLE Assuming that the average of three plates was 75 CFU and that the dilution that gave this reading was 10^{-6}, calculate:

Average = 75

Correction factor = 10

Dilution of the suspension = 10^{-6}

CFU = $75 \times 10 \times 10^{6} \Rightarrow 7{,}5 \times 10^{8}$ CFU/ml or g

5 Species determination of *Azotobacter* sp. via genetic analysis

5.1 General

This method aims to determine the genus and eventually the species of *Azotobacter* spp. using amplified rDNA restriction analysis (ARDRA) of the 16s rRNA genes [7] [8] or Whole-Genome Sequencing.

5.2 Preparation of the sample for the genomic DNA extraction

5.2.1 Isolation and preparation of the microorganism

— Put 1 g of the product in a 100 ml of sterile normal saline.

— Keep the suspension in agitation for 5 min under sterile conditions.

— Under sterile conditions, strike a loop of the suspension in a petri dish prepared with ASHBY SUCROSE AGAR, in order to isolate few single colonies.