SLOVENSKI STANDARD
oSIST prEN 17709:2023

01-maj-2023

Rastlinski biostimulanti - Ugotavljanje prisotnosti in števila Azotobacter spp.

Plant biostimulants - Determination of Azobacter spp.

Pflanzen-Biostimulanzien - Bestimmung von Azotobacter spp.

Biostimulants des végétaux - Détermination d'Azotobacter spp.

Ta slovenski standard je istoveten z: **prEN 17709**

ICS:

65.080	Gnojila	Fertilizers

oSIST prEN 17709:2023 **en,fr,de**

EUROPEAN STANDARD

NORME EUROPÉENNE

EUROPÄISCHE NORM

DRAFT
prEN 17709

April 2023

ICS 65.080

Will supersede CEN/TS 17709:2022

English Version

Plant biostimulants - Determination of *Azobacter* spp.

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

Pflanzen-Biostimulanzien - Bestimmung von *Azotobacter* spp.

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.

This draft European Standard was established by CEN 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, Türkiye and United Kingdom.

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.

Warning : This document is not a European Standard. It is distributed for review and comments. It is subject to change without notice and shall not be referred to as a European Standard.

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 (prEN 17709:2023) 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 17709:2022.

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 fertilizing products (“FPR” or “Fertilising Products Regulation”).

This standardization request, presented as SR M/564and M/564 Amd1, 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 Plant 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 Plant 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 Plant Biostimulants, and will promote and support commercialisation of the European Plant 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 Plant 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 Plant Biostimulants.

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

Increase biodiversity
By improving soil microorganism quality/quantity
Reinforce biological regulation and interactions
By reinforcing plant-microorganism interactions
- symbiotic exchanges i.e. *mycorrhize*
- symbiotic exchanges i.e. *rhizobiaciae/fava*
- 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. [2] [3] in microbial plant biostimulants 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.

prEN 17702-1:—[1], *Plant biostimulants — Sampling and sample preparation — Part 1: Sampling*

prEN 17724:—[2], *Plant biostimulants —Terminology*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in prEN 17724:—[3] 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

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. 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 prEN 17702-1:—[4] shall be prepared according to following procedure which takes into consideration the different formulations of plant 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 [5].

[1] Under preparation.
[2] Under preparation.
[3] Under preparation.
[4] Under preparation.

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 [5].

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 [5].

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 [5].

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 [5].

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 [5].

4.3 Plate counts of *Azotobacter* sp. in sterile diluent with serial dilutions

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.

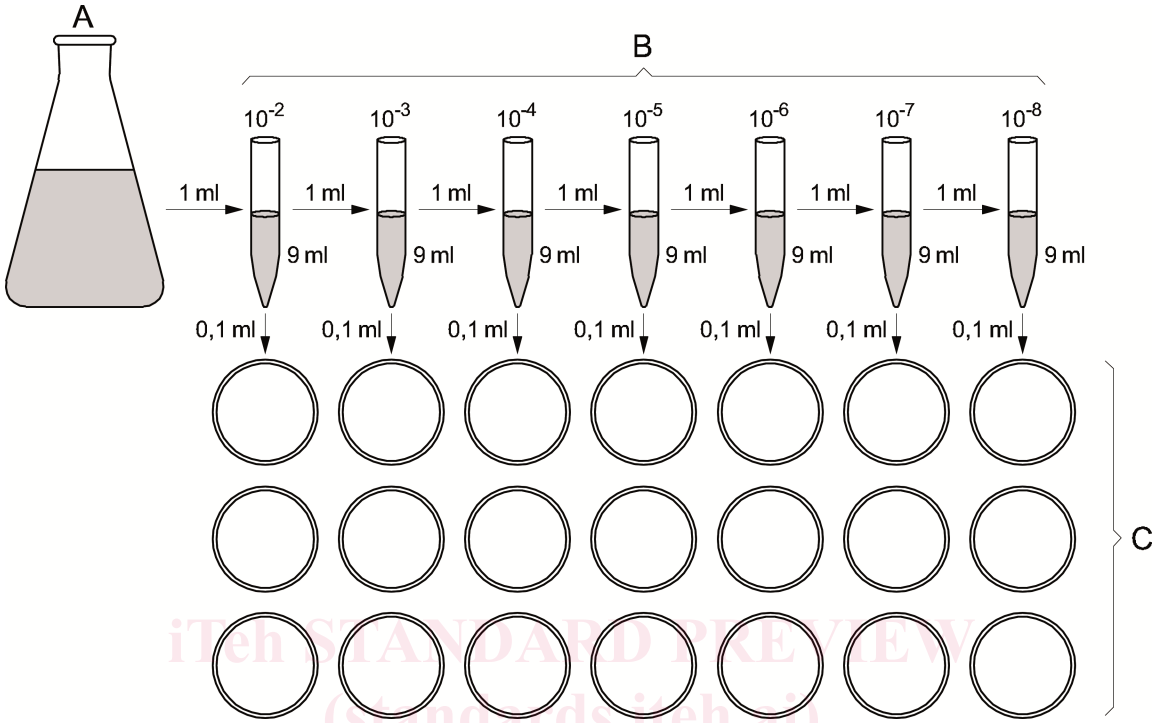
A sample of the product is shaken in a bulk diluent (PBS) which represents the first level of dilution. This is then serially diluted with a sample at each level of dilution directly plated.

4.4 Preparation of the culture media

The preparation and the composition of N-free Agar medium (Ashby sucrose agar) is described in Annex A. The preparation and performance of culture media is a fundamental step to ensure the integrity of microbiological examination. When ready-to-use media are used, the manufacturers of this available media should have a quality program that ensure the quality of the media they supply, according to EN ISO 11133:2014 [5]. Under these conditions, the user/laboratory does not need to run additional testing on such media but shall ensure the storage condition according to the ones recommended by the manufactures. For diluents and media prepared by the user/laboratory directly from commercially available dehydrated formulations and/or from basic

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

individual components, the performance of these diluents/media should be evaluated according to EN ISO 11133:2014 [5]



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

4.5 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.

4.6 Spread-plate counting with ASHBY SUCROSE AGAR [5] (for the different media, see Annex A)

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;