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Rastlinski biostimulansi - Določevanje koncentracije mikroorganizmov

Plant biostimulants - Determination of microorganisms' concentration

Biostimulanzien für die pflanzliche Anwendung - Bestimmung der Konzentration von Mikroorganismen

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Plant biostimulants - Determination of microorganisms' concentration

Biostimulanzien für die pflanzliche Anwendung - Bestimmung der Konzentration von Mikroorganismen

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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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Contents

Page

European foreword

This document (FprCEN/TS 17714:2021) 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 Vote on TS.

This document has been prepared under a mandate M/564 given to CEN by the European Commission and the European Free Trade Association, and supports essential requirements of EU Directive(s).

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 request, presented as SR 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 Fertilizing Products Regulation.

This document is applicable to all biostimulants in agriculture.

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. *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

This document defines the general rules for the determination of microorganism concentration in a sample of biostimulant product.

The specific concentrations of microorganisms required in specific standard methods take precedence over the general rules listed in this document.

WARNING — Person 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 provided to define general rules for determine microorganism concentration present in plant biostimulant products.

The method is applicable to microbial plant biostimulants for verifying that the concentration of microorganisms does not exceed the respective limits outlined in the EU Regulation on Fertilising Products [1].

This horizontal method might not be appropriate in very detail for certain products. In this case, it is necessary to refer to the methodology of specific determination and quantification of the microorganisms.

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.

FprCEN/TS 17708, *Plant biostimulants — Preparation of sample for microbial analysis*

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
plant biostimulant
product stimulating plant nutrition processes independently of the product's nutrient content with the sole aim of improving one or more of the following characteristics of the plant or the plant rhizosphere:

a) nutrient use efficiency;

b) tolerance to abiotic stress;

c) crop quality traits;

d) availability of confined nutrients in soil or rhizosphere

3.2
microorganism
entity of microscopic size, encompassing bacteria, fungi, protozoa and viruses

[SOURCE: EN ISO 11139:2018, 3.176]

3.3
colony
localized visible accumulation of microbial mass (such as prokaryotes, bacteria, micromycetes, yeast and fungi) or organisms (such as Dreissena species) developed on or in a solid nutrient medium from a viable particle or organism

NOTE 1 to entry: Frequently, micro colonies from nearby viable particles, before becoming visible, fuse into one macro colony. The number of visible colonies is, therefore, usually and underestimate of the number of viable particles.

[ISO 6107-6:2021, [3], modified]

3.4
product
portion of an identified plant biostimulant product received in the laboratory for testing

3.5
sample
portion of the **product** (3.4) (at least 1 g or 1 mL) that is used in the test to prepare the initial suspension

3.6
initial suspension
suspension (or solution) of the **sample** (3.5) in a defined volume of an appropriate diluent

4 Principe of the method

The method of determining the concentration of microorganisms has been developed to provide a general method for enumeration of microorganisms present in the plant biostimulant product. The results are expressed as the number of active units by volume or weight, or in any other way relevant to the microorganism, for example colony formation units per gram or the UPM for Mycorhize.

5 Sampling

Sampling is not part of the method specified in this document (see FprCEN/TS 17702-1). If there is no specific International or European Standard, it is recommended that the parties concerned come to an agreement on this subject.

It is important that the laboratory receives a sample which is representative and has not been damaged or changed during transport or storage.

6 Preparation of sample for microbial analysis

Prepare the test sample from the laboratory sample in accordance with FprCEN/TS 17708. If there is no specific International or European Standard, it is recommended that the parties concerned come to an agreement on this subject.

7 Method for enumeration of microorganism

7.1 General

When assessing the microbiological quality of biostimulants products, it is often not enough to know only which microorganisms are present. In most cases, the quantitative aspect is equally important, which brings about the need to enumerate microorganisms. This may be achieved in various ways: through direct examination (microscopy), by inoculating solid or liquid media. However, this document only covers enumeration using solid and liquid media.

Enumeration on solid media is based on the capacity of many microorganisms to produce colonies in or on agar media that can be recognized as such with the naked eye or with the aid of a simple magnifying glass.

If the level of bacteria is expected to be very low (less than 10 colonies in or on a plate at the lowest dilution), enumeration using liquid media is recommended (e.g. MPN) to improve statistical reliability of the results.

7.2 Enumeration using a solid media

The different steps for enumeration using a solid media (number of petri dishes/dilution, Pour plate techniques, surface inoculation, Incubation) are described in the different chapters below and/or specific standard for microorganism detection/determination.

7.2.1 General

Petri dishes should be labelled with the sample number, dilution, date and any other desired information.

Dilutions should be selected to ensure that plates containing the appropriate number of colonies are obtained (see 7.3.1) and to overcome any possible inhibitory properties.

Use a separate sterile pipette for transfers from each dilution, except if working from the highest dilution to the lowest dilution.

7.2.2 Number of Petri dishes per dilution

For enumeration techniques in biostimulant(s) product, one plate per dilution shall be used with at least two successive dilutions. Two plates per dilution may also be used to improve reliability.

If only one dilution is used, then two plates of this dilution shall be used to improve reliability of the results.

For laboratories that do not operate under quality assurance principles, two plates per dilution shall be used to improve reliability of the results.

7.2.3 Pour plate techniques

Withdraw the defined volumes of the dilution to be examined, touching the tip of the pipette against the side of the tube to remove excess liquid adhering to the outside. Lift the sterile Petri dish lid just high enough to insert the pipette, then dispense the contents.

After removing tempered agar medium from the water bath, blot the bottle dry with a clean towel to prevent water from contaminating the plates. Avoid spilling the medium on the outside of the container or on the inside of the plate lid when pouring. To avoid contamination of the media, hold the bottle in a near horizontal position.

Also avoid setting down the bottle between pouring steps. Pour molten agar medium at 44 °C to 47 °C into each Petri dish (generally 18 ml to 20 ml of agar in 90 mm Petri dishes and 45 ml to 50 ml in 140 mm Petri dishes, to obtain at least 3 mm thickness) within 15 min of inoculation (to avoid aggregation of colonies). Avoid pouring the molten medium directly on to the inoculum. Immediately mix the molten medium and the inoculum carefully so as to obtain a homogeneous distribution of the microorganisms within the medium, e.g. by gently moving the dish backwards and forwards, from side to side and in a circular direction. Allow to cool and solidify by placing the Petri dish on a cool horizontal surface (the solidification time of the agar shall not exceed 10 min).

7.2.4 Surface inoculation

7.2.4.1 General

Methods of plating designed to produce only surface colonies on agar plates have certain advantages. The morphology of surface colonies is easily observed, improving the analyst's ability to distinguish between different types of colony.

Use pre-poured plates, of at least 3 mm thickness of the agar medium, that are level and free from air bubbles and surface moisture.

To facilitate uniform spreading, the surface of solidified agar should be dried in accordance with EN ISO 11133:2014 or as specified in the relevant International Standard so that the inoculum is absorbed within 15 min.

7.2.4.2 Spreading-spatula method

Using a sterile pipette, transfer the inoculum (usually 0,1 ml or 0,5 ml) of the liquid test sample or of the initial suspension in the case of other samples to the agar plate (90 mm or 140 mm in diameter, respectively). Repeat this step for the next decimal dilution (the colonies to be counted will then be present in a dilution step of 10^{-1} in the case of liquid sample material and 10^{-2} in the case of other sample material) and, if necessary, repeat for further decimal dilutions.

The limit of enumeration can be lowered by a factor of 10 by inoculating 1,0 ml of the test sample if liquid, or 1,0 ml of the initial suspension for other products, either on the surface of one large agar plate (140 mm) or on the surface of three small agar plates (90 mm). In both cases, if only one dilution is used, prepare duplicates by using two large plates or six small ones.

Using a spreading spatula made of glass, plastic or steel (for example made from a glass rod and shaped like a hockey stick about 3,5 mm in diameter and 20 cm long, bent at right angles at about 3 cm from one end and flattened at the ends by heating), spread the inoculum as quickly as possible evenly over the agar surface without touching the side walls of the Petri dish. Allow the inoculum to absorb with the lids in place for about 15 min at room temperature.

In certain cases (as stated in the relevant International Standard), the inoculum may be deposited on a membrane then spread as described previously.

7.2.5 Incubation

Unless otherwise stated in specific standards, invert dishes once they have been inoculated, and place them quickly in the incubator set at the appropriate temperature. If excessive dehydration occurs (e.g. at 55 °C or in the event of strong air circulation), wrap the dishes loosely in plastic bags prior to incubation or use any similar system of equivalent efficiency.

During the incubation period, minor variations in the incubation temperature may be unavoidable and acceptable, for example during the usual operations of loading or unloading the incubator, but it is important that these periods are kept to a minimum. The duration of these variations should be monitored to ensure that they do not have a significant effect on the result.

It may sometimes be useful to laboratory operations to refrigerate inoculated dishes before incubation for no more than 24 h. If this is done, the laboratory shall ensure that this practice does not affect the resulting counts.

Generally, Petri dishes should be stacked no more than six high for aerobic incubation and should be separated from each other and from the incubator walls by at least 25 mm. However, higher stacks with less spacing may be acceptable in incubators fitted with air circulation systems; in this case, the temperature distribution should be verified. After incubation, the dishes should normally be examined immediately. They may, however, be stored, unless otherwise specified in specific standards, for up to 48 h in a refrigerator. Refrigerated storage is only acceptable if it has been shown to have no effect on the numbers, appearance or the subsequent confirmation of the colonies. With certain media containing indicator dyes, refrigerated plates should be allowed to equilibrate at room temperature before examining, to ensure that the correct colour is regained.