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[Not translated]

Plant biostimulants - Determination of the anaerobic plate count

Biostimulanzien für die pflanzliche Anwendung - Bestimmung der anaeroben Keimzahl

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ICS:

65.080 Gnojila Fertilizers

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Plant biostimulants - Determination of the anaerobic plate count

Biostimulanzien für die pflanzliche Anwendung - Bestimmung der anaeroben Keimzahl

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

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European foreword

This document (FprCEN/TS 17719:2021) 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 Standardization Request given to CEN by the European Commission and the European Free Trade Association.

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 plants, 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 2019/1009 [1].

The method is applicable to microbial plant biostimulants except those composed of aerobic bacterium to verify that the concentration of anaerobes does not exceed the respective limits described in the EU Fertilizers Regulation.

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

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 provides a horizontal method for enumeration of microorganisms that are able to grow and form colonies in a solid medium after anaerobic incubation at 30 °C.

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

This method does not apply to the microbiological monitoring of the environment in which microbial plant biostimulants are manufactured.

No information about potential human pathogens can be inferred from anaerobic plate counts.

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 17724, *Plant Biostimulants – Terminology*

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

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

EN ISO 11133:2014, *Microbiology of food, animal feed and water – Preparation, production, storage and performance testing of culture media*[2]

3 Terms and definitions

For the purposes of this document, the terms and definitions given in FprCEN/TS 17724 and the following 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
microorganism
any microbiological entity, including lower fungi, bacteria and viruses, cellular or non-cellular, capable of replication or of transferring genetic material

[SOURCE: Regulation (EC) No 1107/2009, Article 3, point 15]

3.2
facultative organism
microorganism capable of both aerobic and anaerobic metabolism

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

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

[SOURCE: EN ISO 11139:2018, 3.114]

3.3
obligate anaerobe
organism that only lives and grows in the absence of molecular oxygen

[SOURCE: EN ISO 11139:2018, 3.186]

3.4
viable count
value established from enumeration of recoverable colony-forming units

[SOURCE: EN ISO 11139:2018, 3.186]

4 Principle

4.1 General

Viable anaerobic bacteria are enumerated by the plate count technique under an anaerobic atmosphere [2]. Caution shall be exercised when applying the method since isolates may be pathogenic.

4.2 Brief description

A fixed amount of a dilution of the test sample is placed in an empty Petri dish and mixed in a specified melted agar culture medium to create a pour plate.

The plates are incubated at 30 °C ± 1 °C under anaerobic conditions for 48-72 h.

The number of microorganisms per gram or millilitre of test sample is calculated as specified in Clause 8.

5 Culture media and reagents

For current laboratory practices, FprCEN/TS 17708 and EN ISO 11133:2014[2] shall be used.

Composition of Anaerobic Agar (ANA) [3] and reagents and their preparation are described in Annex B.

6 Equipment and consumables

6.1 Special apparatus, anaerobic incubator, BBL GasPak or equivalent, equipped with GasPak hydrogen and CO_2 generator envelopes with an anaerobic indicator.

6.2 Apparatus for dry sterilization (oven) or wet sterilization (autoclave), according to EN ISO 7218:2007[1] shall be used.

6.3 Drying cabinet or incubator, capable of operating at 30-35 °C ± 1 °C.

6.4 Water bath, capable of operating at 47°C ± 2°C.

6.5 pH-meter, having an accuracy of calibration of ± 0,1 pH unit at 25 °C.

6.6 Sterile graduated pipettes or automatic pipettes, of nominal capacities of 0,1 mL, 0,5 mL, 1 mL, and 10 mL.

6.7 Sterile Petri dishes, with a diameter of approximately 90 mm and (optional) large size (diameter approximately 140 mm).

6.8 Refrigerator, capable of operating at 5 °C ± 3 °C.

6.9 Peristaltic blender (stomacher) with 400 mL sterile bags.

6.10 Blender motor and jars or vortex.

7 Preparation of test sample

7.1 General

To ensure a truly representative analytical unit, agitate liquids or free flowing materials until the contents are homogeneous. If the sample unit is a solid, obtain the analytical unit by taking a portion from several locations within the sample unit. To reduce the workload, the analytical units may be combined for analysis. It is recommended that a composite contains no more than five analytical units.

General rules for the preparation of the initial suspension for microbiological examination are described in FprCEN/TS 17708.

A representative sample of the product should be prepared taking into consideration the different formulations of the plant biostimulants.

7.2 Liquid - water based- formulations

Aseptically add 25 mL of the product (the analytical unit) in a 400 mL sterile stomacher bag (6.9) or in a blender jar (6.10) containing 225 mL of an appropriate sterile diluent (i.e. buffered peptone water) maintained at room temperature. Blend, stomach or vortex as required for thorough mixing.

7.3 Liquid - oil based (emulsifiable concentrate - EC) formulations

Aseptically add 25 mL of the product (the analytical unit) in a 400 mL sterile stomacher bag (6.9) or in a blender jar (6.10) containing 225 mL of an appropriate sterile diluent (i.e. buffered peptone water) maintained at room temperature. Blend, stomach or vortex as required for thorough mixing.

7.4 Solid - Wettable Powder (WP) formulations

Aseptically add 25 g of the product (the analytical unit) in a 400 mL sterile stomacher bag (6.9) containing 225 mL of an appropriate sterile diluent (i.e. buffered peptone water) maintained at room temperature. Homogenize the mixture 2 min at higher speed with a stomacher (6.9).

7.5 Solid - Water dispersible granules (WDG) formulations

Aseptically add 25 g of the product (the analytical unit) in a 400 mL sterile stomacher bag (6.9) containing 225 mL of an appropriate sterile diluent (i.e. buffered peptone water) maintained at room temperature. Homogenize the mixture 2 min at higher speed with a stomacher (6.9).

7.6 Solid - Pellets, granules, microgranules (slow release) formulations

Aseptically add 25 g of the product (the analytical unit) in a 400 mL sterile stomacher bag (6.9) containing 225 mL of an appropriate sterile diluent (i.e. buffered peptone water) maintained at room temperature. Homogenize the mixture 2 min at higher speed with a stomacher (6.9).

7.7 Solid substrates

Aseptically add 25 g of the product (the analytical unit) in a 400 mL sterile stomacher bag (6.9) containing 225 mL of an appropriate sterile diluent (i.e. buffered peptone water) maintained at room temperature. Homogenize the mixture 2 min at higher speed with a stomacher (6.9).

8 Procedure

8.1 Test portion, initial suspension and dilutions

For the preparation of the initial suspension, use an appropriate sterile diluent (i.e. buffered peptone water; described in B.1.4). Aseptically weigh 25 g or mL of sample into 225 mL of the diluent to yield a tenfold dilution (see FprCEN/TS 17708). 1 mL of the primary dilution is aseptically transferred into 9 mL of the diluent, obtaining the dilution 10^{-2}. Further ten-fold serial dilutions can be prepared (adding 1 mL of the previously diluted sample into 9 mL of the diluent) according to the expected level of contamination of the product (e.g. until reach the dilution 10^{-5}).

8.2 Inoculation and incubation

Pipet 1,0 mL of each sample dilution into two Petri dishes. In case of liquid biostimulant products, 1 mL of the undiluted product can be inoculated (in duplicate). At least 2 successive dilutions shall be inoculated. Pour approximately 15 mL of ANA agar which has been cooled to 47 °C ± 2 °C into each dish. Swirl plates and allow to solidify. Immediately after solidification invert the plates and place them in an anaerobe jar. Following manufacturer's directions, generate the anaerobic atmosphere.

Incubate the anaerobe jar at 30-35 °C ± 1 °C (6.3) for 48-72 h. Count the number of colonies for each plate (if present) and record the numbers.

Appropriate negative controls (diluent-only) should be run concurrently with the sample serial dilutions.

8.3 Critical Control Point

For preparation of microbial suspensions, serial dilutions, and plating, refers to FprCEN/TS 17708.

9 Calculation

For the calculation of CFU/g or CFU/mL of anaerobes refers to FprCEN/TS 17708.

$$N = \frac{\Sigma C}{V \times \left[\left(1 \times n_1\right) + \left(0{,}1 \times n_2\right) \right] \times d}$$

where

- ΣC sum of the colonies counted on all the dishes retained from two successive dilutions, countable plates (in the established range);
- V volume of inoculum (in millilitres);
- n_1 number of plates retained with the highest number of countable colonies;
- n_2 number of plates retained with the lowest number of countable colonies;
- d retained dilution with the highest number of countable colonies.

10 Expression of results

Report the result as the number of anaerobes per millilitre (liquid products) or per gram (other products), expressed as a number between 1,0 and 9,9 inclusive multiplied by 10x where x is the appropriate power of 10.