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Rastlinski biostimulansi - Določevanje Escherichia coli

Plant biostimulants - Determination of Escherichia coli

Biostimulanzien für die pflanzliche Anwendung - Bestimmung von Escherichia coli

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Plant biostimulants - Determination of Escherichia coli

Biostimulanzien für die pflanzliche Anwendung - Bestimmung von Escherichia coli

This draft Technical Specification is submitted to CEN members for Vote. It has been drawn up by the Technical Committee CEN/TC 455.

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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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European foreword

This document (FprCEN/TS 17716:2021) 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 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.

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. *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 gives general guidelines for the detection and identification of the specified microorganism *Escherichia coli* in technical and formulated biostimulant products, both in liquid and solid state, and also the horizontal method for the enumeration of ß–glucuronidase-positive *Escherichia coli* in plant biostimulants products (both in liquid and solid state).

The qualitative method described in this document is based on the detection of E*scherichia coli* in a non-selective liquid medium (enrichment broth), followed by isolation on a selective agar medium. Other methods can be appropriate, depending on the level of detection required.

NOTE For the detection of *Escherichia coli*, subcultures can be performed on non-selective culture media followed by suitable identification steps (e.g. using identification kits).

The quantitative method described in this document uses a colony-count technique at 44 °C on a solid medium containing a chromogenic ingredient for detection of the enzyme ß –glucuronidase.

WARNING — Strains of *Escherichia coli* which do not grow at 44 °C and, in particular, those that are ß -glucuronidase negative, such as *Escherichia coli* O157, will not be detected.

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*

EN ISO 11133:2014,[1] *Microbiology of food, animal feed and water - Preparation, production, storage and performance testing of culture media (ISO 11133:2014)*

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
escherichia coli
gram-negative rod, motile, smooth colonies, member of *Enterobacteriaceae*

Note 1 to entry: The main characteristics for identification are catalase positive, oxidase negative, fermentation of lactose, production of indole, growth on selective medium containing bile salts with characteristic colonies.

Note 2 to entry: *Escherichia coli* can be isolated from moist environmental sources (air, water, soil) and is a faecal contamination indicator.

[1] As amended EN ISO 11133:2014/A1:2018 and EN ISO 11133:2014/A2:2020

3.2
enrichment broth
non-selective liquid medium containing suitable neutralizers and/or dispersing agents and demonstrated to be suitable for the product under test

3.3
ß-glucuronidase-positive Escherichia coli
bacteria which at 44 °C form typical blue colony on tryptone-bile-glucuronide medium (TBX) under the conditions specified in the relative part of this document

3.4
enumeration of ß-glucuronidase-positive escherichia coli
determination of the number of colony-forming-unit (CFU) of ß-glucuronidase-positive Escherichia coli, per millilitre or per grams of sample, when test and calculations are carried out with the relative part of this document

4 Principle

4.1 Qualitative method

The first step of the qualitative procedure is to perform an enrichment by using a non-selective broth medium to increase the number of microorganisms without the risk of inhibition by the selective ingredients that are present in selective/differential growth media.

The second step of the test (isolation) is performed on a selective medium followed by identification tests.

The presence or absence of *Escherichia coli* per gram or per millilitre of sample is calculated (see Clause 9).

4.2 Quantitative method

In the quantitative method, duplicate plates of tryptone-bile-glucuronic medium (TBX) are inoculated with the specified quantity of the test sample (if the product is liquid) or the initial suspension (if the product is solid).

Under the same conditions, using decimal dilutions of the test sample or of the initial suspension, two plates per dilution are inoculated.

The dishes are incubated for 18 h to 24 h at 44 °C ± 1 °C then examined to detect the presence of colonies which, from their characteristics, are considered to be ß-glucuronidase-positive *Escherichia coli*.

The number of colony-forming units (CFU) of ß-glucuronidase-positive *Escherichia coli* per gram or per millilitre of sample is calculated (see Clause 9).

5 Diluent and culture media

5.1 General

The following diluents and culture media are suitable for the detection of *Escherichia coli* and enumeration of ß-glucuronidase-positive *Escherichia coli* according to the proper procedure. Other diluents and culture media may be used if they have been demonstrated to be suitable for use.

Diluents and culture media may be prepared using the descriptions provided or from reagents/dehydrated culture media, according to the instructions from the manufacturer. The instructions provided by the supplier of the media/reagents should be followed for storage conditions, expiry date and use.

NOTE Ready-to-use diluents and media can be used when their composition and/or growth yields are comparable to those of the formulae given in the present document.

5.2 Broth and culture media in the qualitative method

5.2.1 Enrichment broth

The enrichment broth is used in the qualitative method to disperse the sample and to increase the initial microbial population. See Annex A for the list and recipes of the possible enrichment broth.

5.2.2 Selective culture media: Tryptone-bile-glucuronic medium (TBX) for isolation of *Escherichia coli*

The selective agar medium is used in the qualitative method for the isolation and identification of *Escherichia coli* See Annex B for the list and recipes of the selective agar medium.

5.3 Diluent and culture media in the quantitative method

5.3.1 Diluent

See Annex C for the list and recipes of the possible diluents to be use in the preparation of the initial suspension and further decimal dilutions.

5.3.2 Culture media: Tryptone-bile-glucuronic medium (TBX)

See Annex B for the list and recipes of the culture medium to be use in the inoculation by plating technique of the initial suspension and the further decimal dilutions.

6 Apparatus and glassware

The laboratory equipment, apparatus and glassware typical of microbiological laboratory according to FprCEN/TS 17708 shall be used.

7 Handling of plant biostimulants products and sampling

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

Sampling is not part of the method specified in this document (FprCEN/TS 17716): refer to FprCEN/TS 17702-1.

If necessary, the product to be tested may be equilibrated at room temperature before starting the analysis.

8 Procedure

8.1 General

According to the aim of the analysis one of the following described methods may be performed.

The qualitative method (see 8.2) allows to evaluate the presence or absence of *Escherichia coli* in at least 1 g or 1 mL of the product under test.

The quantitative method (see 8.3) allows to determine the number of ß-glucuronidase-positive *Escherichia coli* in terms of CFU per g or per mL of the product under test.

8.2 Qualitative method

8.2.1 General

Dispense 25 g or ml of sample in 225 ml of sterile enrichment broth. Note *S*, the exact weight or volume of the sample.

8.2.2 Solid formulations: Wettable Powder (WP), Water dispersible granules (WDG), Pellets, granules, microgranules (slow release) formulation

The initial suspension (see 8.2) is processed in a stomacher for 2 min at highest speed. Soon after, 10 ml of this suspension are incubated (see 8.2.3).

8.2.3 Liquid formulations: water based formulation and oil based (emulsifiable concentrate - EC) formulations

10 ml of the well mixed initial suspension (see 8.2) are sampled and incubated (see 8.2.3).

8.2.4 Incubation of the inoculated enrichment broth

Incubate the initial suspension prepared in broth (see 8.2.2 for solid formulations or 8.2.3 for liquid formulations) at 32,5 °C ± 2,5 °C for at least 20 h (maximum 72 h).

8.2.5 Detection and identification of Escherichia coli

8.2.5.1 Isolation

Using a sterile loop, streak an aliquot of the incubated enrichment broth (8.2.2) onto the surface of Tryptone-bile-glucuronic medium to obtain isolated colonies.

Invert the Petri dish and then incubate at 44 °C for 18 h to 24 h. The total incubation time shall not be longer than 24 h. Check for characteristic colonies (see Table 2).

WARNING — If the presence of stressed cells is suspected, incubate for an initial period of 4 h at 37 °C, and then raise the incubation temperature to 44 °C for 18 h to 24 h. The incubation temperature shall not exceed 45 °C.

Table 2 — Morphological characteristics of Escherichia coli on Tryptone-bile-glucuronic agar medium

Selective medium	Characteristic colonial morphology of *Escherichia coli*
Tryptone-bile-glucuronic medium	Blue to blue-green

8.2.5.2 Identification of Escherichia coli

8.2.5.2.1 General

In case of doubts about the morphological characteristic grown colonies, proceed to the following tests for these suspect colonies isolated on the selective agar medium. The presence of *Escherichia coli* may be confirmed by other suitable, cultural and biochemical tests.

8.2.5.2.2 Gram's stain

Perform the test specified in EN ISO 21148. Check for Gram-negative rods (bacilli).

8.2.5.2.3 Culture on levine eosin-methylene blue agar medium (EMB agar medium)

Inoculate the surface of the levine eosin-methylene blue agar medium (see Annex B for recipes) with suspect isolated colonies grown on TBX agar medium, so that isolated colonies develop. Invert the Petri dish and then incubate at 32,5 °C ± 2,5 °C for at least 24 h (maximum 48 h).

Check for characteristic colonies (see Annex B).