SLOVENSKI STANDARD
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Rastlinski biostimulanti - Določanje Escherichia coli

Plant biostimulants - Determination of Escherichia coli

Pflanzen-Biostimulanzien - Bestimmung von Escherichia coli

Biostimulants des végétaux - Détermination des Escherichia coli

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65.080 Gnojila Fertilizers

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Plant biostimulants - Determination of *Escherichia coli*

Biostimulants des végétaux - Détermination des *Escherichia coli*

Pflanzen-Biostimulanzien - Bestimmung von *Escherichia coli*

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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 (CEN/TS 17716:2022) has been prepared by Technical Committee CEN/TC 455 “Plant Biostimulants”, the secretariat of which is held by AFNOR.

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This document has been prepared under a Standardization Request given to CEN by the European Commission and the European Free Trade Association.

Any feedback and questions on this document should be directed to the users’ national standards body. A complete listing of these bodies can be found on the CEN website.

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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 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 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 Fertilising 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. *Mycorrhizae*
— symbiotic exchanges i.e. *Rhizobiaceae/Faba*
— secretions mimicking plant hormones (i.e. *Trichoderma*)
By regulating plant physiological processes
— e.g. 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.

CEN/TS 17708, *Plant Biostimulants — Preparation of sample for microbial analysis*

EN ISO 21148, *Cosmetics — Microbiology — General instructions for microbiological examination (ISO 21148)*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

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.

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 gram of sample, when test and calculations are carried out in accordance 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 non-exhaustive 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 used 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 used 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 CEN/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 (CEN/TS 17716): refer to CEN/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 25 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 8.2.1) is processed in a stomacher®[1] for 2 min at highest speed. Soon after, 10 ml of this suspension are incubated (see 8.2.4).

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

10 ml of the well mixed initial suspension (see 8.2.1) are sampled and incubated (see 8.2.4).

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 (8.2.4) 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 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).

[1] A stomacher® is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by CEN of this product.

8.3 Quantitative method

8.3.1 Test portion and initial suspension

8.3.1.1 General

A representative sample of the product is taken to prepare the initial suspension according to the following procedure which takes into consideration the different formulations of biostimulants based products.

8.3.1.2 Liquid - water based - formulations

Dispense 25 ml of sample in 225 ml of sterile Phosphate Buffer Solution (PBS) (see Annex C) 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.

8.3.1.3 Liquid – oil based (emulsifiable concentrate - EC) formulations

Dispense 25 ml or g of sample in 225 ml of sterile Phosphate Buffer Solution (PBS) (see Annex C) 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.

8.3.1.4 Solid - Wettable Powder (WP) formulations

Dispense 25 g of sample in 225 ml of sterile Phosphate Buffer Solution (PBS) (see Annex C) 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.

8.3.1.5 Solid - Water dispersible granules (WDG) formulations

Dispense 25 g of sample in 225 ml of sterile Phosphate Buffer Solution (PBS) (see Annex C) 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®[2] after having sieved (100 mesh sieve) the particles and resuspend them in the same suspension.

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

Dispense 25 g of sample in 225 ml of sterile Phosphate Buffer Solution (PBS) (see Annex C) maintained at room temperature in a sterile bag and disperse them using a stomacher®[2] for maximum 2 min and then repeat 3 times with 5 min interval where the bag is put in water with ice.

8.3.1.7 Solid - substrate

Dispense 25 g of sample in 225 ml of sterile Phosphate Buffer Solution (PBS) (see Annex C) 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.

8.3.2 Serial dilutions

Additional serial dilutions (e.g. 1:10 dilution) may be performed from the initial suspension using the same diluent (according to the expected level of contamination of the product). Mix the dilutions in order to avoid sedimentation of microorganism-containing particles.

Refer to CEN/TS 17708 for general rules in the preparation of the decimal dilutions.

[2] A stomacher® is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by CEN of this product.