SLOVENSKI STANDARD
SIST-TS CEN/TS 17711:2023

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Rastlinski biostimulanti - Ugotavljanje prisotnosti Vibrio spp.

Plant biostimulants - Detection of Vibrio spp

Pflanzen-Biostimulanzien - Nachweis von Vibrio spp.

Biostimulants des végétaux - Recherche des espèces de Vibrio

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65.080	Gnojila	Fertilizers

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Plant biostimulants - Detection of *Vibrio* spp.

Biostimulants des végétaux - Recherche des espèces de *Vibrio*

Pflanzen-Biostimulanzien - Nachweis von *Vibrio* spp.

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Contents

Page

European foreword

This document (CEN/TS 17711:2022) 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 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 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.

Because of the large variety of Plant Biostimulant products, the horizontal method described in this document may not be appropriate in every detail for certain products. In this case, different methods, which are specific to these products may be used if absolutely necessary for justified technical reasons. Nevertheless, every attempt will be made to apply this horizontal method as far as possible.

The harmonization of test methods cannot be immediate and, for certain groups of products, International Standards and/or national standards may already exist that do not comply with this horizontal method. It is hoped that when such standards are reviewed they will be changed to comply with this document so that eventually the only remaining departures from this horizontal method will be those necessary for well-established technical reasons.

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 specifies a horizontal method for the detection of enteropathogenic *Vibrio* spp., which causes human illness in or via the intestinal tract [1]. The species detectable by the methods specified include *Vibrio parahaemolyticus, Vibrio cholerae* and *Vibrio vulnificus*.

It is applicable to the following:

— microbial plant biostimulants.

NOTE 1 The World Health Organization (WHO) has identified that *V. parahaemolyticus, V. cholerae* and *V. vulnificus* are the major contaminants of *Vibrio* spp. [1].

NOTE 2 For confirmation, it is possible to use PCR tests; in this case the laboratory validates the procedure and data generated.

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

CEN/TS 17724, *Plant biostimulants — Terminology*

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

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

EN ISO 3696:1995, *Water for analytical laboratory use — Specification and test methods (ISO 3696:1987)*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in CEN/TS 17724 and the following apply.

3.1
potentially enteropathogenic *Vibrio* spp
microorganism which forms typical colonies on solid selective media and which possesses the described biochemical or molecular characteristics when the test is performed in accordance with this document

Note 1 to entry: This document describes specific procedures for *V. parahaemolyticus, V. cholerae* and *V. vulnificus.*

[1] As amended by EN ISO 7218:2007/A1:2013.
[2] As amended by EN ISO 11133:2014/A1:2018 and EN ISO 11133:2014/A2:2020.

3.2
detection of potentially enteropathogenic *Vibrio* spp
determination of the presence or absence of potentially enteropathogenic *Vibrio* spp. (3.1) *(V. parahaemolyticus, V. cholerae* and *V. vulnificus)* in a determined quantity of product, when the test is performed in accordance with this document

4 Principle

4.1 General

The detection of potentially enteropathogenic *Vibrio* spp. *(V. parahaemolyticus, V. cholerae* and *V. vulnificus)* requires four successive phases, as shown in the procedure diagram in Annex A.

Recovery of certain *Vibrio* spp. can be improved by the use of different incubation temperatures depending upon the target species or state of the matrix. In liquid products, recovery of *V. parahaemolyticus* and *V. cholerae* is enhanced by enrichment at 41,5 °C and the recovery of *V. vulnificus is* enhanced by enrichment at 37 °C. Whereas in solid products, for *V. vulnificus, V. parahaemolyticus* and *V. cholerae* recovery is enhanced by enrichment at 37 °C.

If detection of *V. parahaemolyticus, V. cholerae* and *V. vulnificus* is required, all specified incubation temperatures should be used. If detection of *V. parahaemolyticus, V. cholerae* and *V. vulnificus* together is not required, the specific procedure(s) may be selected according to the species being sought. Such a selection should be clearly specified in the test report.

V. parahaemolyticus, *V. cholerae* and *V. vulnificus* can be present in small numbers and are often accompanied by a much larger number of other microorganisms belonging to the *Vibrionaceae* family or to other families.

4.2 Primary enrichment in a liquid selective medium

Inoculation of the test portion in the primary enrichment medium alkaline saline peptone water (ASPW) (5.1) at ambient temperature, followed by incubation at 41,5 °C for 6 h and/or 37 °C for 6 h. The incubation conditions are determined by the target species and product state.

For detection of all target species in solid products, primary enrichment should be at 37 °C.

For detection of *V. vulnificus* in all products, primary enrichment should be at 37 °C.

For detection of *V. parahaemolyticus* and/or *V. cholerae* only, in liquid products, primary enrichment should be at 41,5 °C.

4.3 Secondary enrichment in a liquid selective medium

Inoculation of the second enrichment medium (ASPW) with the cultures obtained in 4.2. Incubation of inoculated enrichment medium at 41,5 °C for 18 h and/or 37 °C for 18 h.

For detection of *V. vulnificus* in all products, secondary enrichment should be at 37 °C.

For detection of V. *parahaemolyticus* and/or *V. cholerae* only, in all products, secondary enrichment should be at 41,5 °C.

4.4 Isolation and identification

From the cultures obtained in 4.2 and in 4.3, inoculation of two solid selective media:

— thiosulfate citrate bile and sucrose agar (TCBS) medium (5.2.1);

— another appropriate solid selective medium (left to the choice of the laboratory), such as chromogenic agar, complementary to the TCBS medium (5.2.2).

Incubation of the TCBS medium at 37 °C, then examination after 24 h. Incubation of the second selective medium according to the manufacturer's recommendations.

4.5 Confirmation

Presumptive colonies of *V. parahaemolyticus, V. cholerae* and *V. vulnificus* isolated in 4.4 are subcultured and confirmed by means of appropriate biochemical test. The PCR test is also possible to use for confirmation; the PCR methods are suggested in Annexes C and D, but the laboratory must validate the procedure and data generated.

5 Culture media and reagents

For general laboratory practice, refer to EN ISO 7218:2007.

For clarity of the text, details of the composition of culture media and reagents and their preparation are described in Annex B.

For performance testing of culture media, refer to EN ISO 11133:2014.

5.1 Enrichment medium: alkaline saline peptone water (ASPW)

As specified in B.3.

5.2 Solid selective isolation media

5.2.1 First medium: thiosulphate, citrate, bile and sucrose agar medium (TCBS)

As specified in B.4. See Table 1 for performance testing data.

Table 1 — Performance testing of thiosulphate, citrate, bile and sucrose agar medium (TCBS)

Function	Incubation	Control strains	WDCM [a]	Method of control	Criteria [e]	Characteristic reactions
Productivity	37 °C ± 1 °C for 24 h ± 3 h	*Vibrio parahaemolyticus*	00185 [b]	Qualitative	Good growth (2)	Green colonies (sucrose negative)
	37 °C ± 1 °C for 24 h ± 3 h	*Vibrio furnissii*	00186 [b]	Qualitative	Good growth (2)	Yellow colonies (sucrose positive)
Selectivity	37 °C ± 1 °C for 24 h ± 3 h	*Escherichia coli* [c d]	00012, 00013 or 00090	Qualitative	Total inhibition (0)	—

[a] World Data Centre for Microorganisms (WDCM) strain catalogue available at http://refs.wdcm.org

[b] Strain to be used as a minimum (see EN ISO 11133:2014).

[c] Some national restrictions and directions can require the use of a different *E. coli* serovar. Make reference to national requirements relating to the choice of *E. coli* serovars.

[d] Strain free of choice; one of the strains shall be used as a minimum (see EN ISO 11133:2014).

[e] Growth is categorized as 0: no growth, 1: weak growth (partial inhibition), and 2: good growth (see EN ISO 11133:2014).

5.2.2 Second medium

The selection of the second medium is left to the choice of the test laboratory. Preparation of the medium should be strictly according to the manufacturer's instructions.

5.3 Saline nutrient agar (SNA)

As specified in B.5.

5.4 Reagent for detection of oxidase

As specified in B.6.

5.5 Biochemical tests

5.5.1 L-lysine decarboxylase saline medium (LDC)

As specified in B.7.

5.5.2 Arginine dihydrolase saline medium (ADH)

As specified in B.8.

5.5.3 Reagent for detection of β-galactosidase

As specified in B.9.

5.5.4 Saline medium for detection of indole

As specified in B.10.

5.5.5 Saline peptone water

As specified in B.11.

5.5.6 Sodium chloride solution

As specified in B.12.

6 Equipment and consumables

Disposable equipment is acceptable in the same way as reusable glassware, if the specifications are similar.

Ordinary microbiology laboratory equipment as specified in EN ISO 7218:2007, and in particular the following.

6.1 Refrigerator, adjustable to 5 °C ± 3,0 °C.

6.2 Incubator, adjustable to 37 °C ± 1,0 °C.

6.3 Incubator, adjustable to 41,5 °C ± 1,0 °C.

6.4 Freezer, adjustable to < −15 °C.

6.5 Micro-centrifuge tubes, with a capacity of 1,5 ml and 2,0 ml.

6.6 Micro-centrifuge, for reaction tubes with a capacity of 1,5 ml and 2,0 ml and capable of running at 10 000 g.

6.7 Heating block capable of operating at 95 °C ± 2,0 °C or equivalent.

6.8 Vortex.

6.9 Graduated pipettes and pipette filter tips, for volumes between 1 µl and 1 000 µl.

6.10 Magnetic stirrer with a magnetic stirring bar.

6.11 Stomacher apparatus.

7 Sampling

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

Sampling does not form part of the method specified in this document. See the International Standard specific to the relevant product. If a specific document does not exist, it is recommended that the relevant parties reach agreement on this subject.

8 Preparation of the test sample

Prepare the test sample in accordance with CEN/TS 17702-1 and/or the document concerning the product to be examined. If a specific document does not exist, it is recommended that the relevant parties reach agreement on this subject.

9 Procedure (see Figure A.1)

9.1 Test portion and initial suspension

This document has been validated for test portions of up to 25 g or 25 ml. A smaller test portion may be used without the need for additional validation/verification provided that the same ratio between (pre-) enrichment broth and test portion is maintained. A larger test portion than that initially validated may be used if a validation/verification study has shown that there are no adverse effects on the detection of *Vibrio* spp.

NOTE Validation can be conducted in accordance with the appropriate document of EN ISO 16140 (all parts). Verification for pooling samples can be conducted in accordance with the protocol described in EN ISO 6887-1:2017, Annex C.

For the preparation of the initial suspensions, use the first enrichment medium (ASPW) specified in 5.1.

Prepare initial suspension with a representative test portion in according to the following procedure taking into consideration the different formulations of biostimulants based products.

a) Liquid – water based and liquid – oil based formulations:

   Take test portions (25 g or 25 ml) and homogenize in 225 ml of enrichment medium at room temperature in a flask and homogenize with a magnetic stirrer at half speed for 10 minutes or more, until the distribution is optimal.

b) Solid (WP or WDG) formulations:

   Take test portions (25 g or 25 ml) and homogenize in 225 ml of enrichment medium at room temperature in a sterile bag using stomacher apparatus for 2 minutes at highest speed.

c) Solid –pellets, granules, microgranules (slow release) formulations:

Take test portions (25 g or 25 ml) and homogenize in 225 ml of enrichment medium at room temperature in a sterile bag with stomacher apparatus for 2 minutes at highest speed, until the dispersion is optimal. If required, repeat this operation twice.

In the case of large quantities (greater than 25 g or 25 ml), the ASPW should be warmed to 37 °C ± 1 °C and/or 41,5 °C ± 1 °C (4.2) before inoculation with the test portion.

In order to reduce the amount of examination work, where more than one 25 g or 25 ml test portion stemming from a determined batch is to be examined, and where proof is available indicating that a mixture (gathering together the test portions) does not modify the results concerning this product in particular, the test portions may be mixed. For example, if 10 test portions of 25 g or 25 ml are to be examined, it is possible to combine these 10 units in order to obtain a composite sample of 250 g or 250 ml and to add 2,25 l of enrichment medium.

Cell counts of *Vibrio* spp. potentially decline significantly on storage at refrigeration temperatures. Storage of samples and, to a lesser extent, of suspensions at such temperatures should be avoided where possible and should otherwise be kept to a minimum.

9.2 Primary selective enrichment

Incubate the initial suspensions (9.1) at 41,5 °C ± 1 °C and/or 37 °C ± 1 °C for 6 h ± 1 h according to Table 2.

Table 2 — Primary incubation and target species/product state

Target *Vibrio* spp. in liquid product formulations			
Incubation temperature	***Vibrio parahaemolyticus***	***Vibrio cholerae***	***Vibrio vulnificus***
41,5 °C ± 1 °C	✓	✓	
37 °C ± 1 °C			✓
Target *Vibrio* spp. in solid dried product formulations			
Incubation temperature	***Vibrio parahaemolyticus***	***Vibrio cholerae***	***Vibrio vulnificus***
41,5 °C ± 1 °C			
37 °C ± 1 °C	✓	✓	✓

9.3 Secondary selective enrichment

Transfer 1 ml of the culture obtained in 9.2 taken from the surface into a tube containing 10 ml of ASPW (5.1). It is recommended that the sample is not agitated before taking the aliquot.

Incubate the ASPW at 41,5 °C ± 1 °C and/or 37 °C ± 1 °C for 18 h ± 1 h according to Table 3.

NOTE Cultures and/or boiled broths obtained in 9.3 can be screened using PCR also.