TECHNICAL SPECIFICATION

ISO/TS 22939

Second edition
2019-08

Soil quality — Measurement of enzyme activity patterns in soil samples using fluorogenic substrates in micro-well plates

Qualité du sol — Mesure en microplaques de l'activité enzymatique dans des échantillons de sol en utilisant des substrats fluorogènes

Reference number
ISO/TS 22939:2019(E)

ISO copyright office
CP 401 • Ch. de Blandonnet 8
CH-1214 Vernier, Geneva
Phone: +41 22 749 01 11
Fax: +41 22 749 09 47
Email: copyright@iso.org
Website: www.iso.org

Published in Switzerland

Contents

Page

Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 190, *Soil quality*, Subcommittee SC 4, *Biological characterization*.

This second edition cancels and replaces the first edition (ISO/TS 22939:2010), which has been technically revised. The main changes compared to the previous edition are as follows:

— Clause 3 “Terms and definitions” added;

— 6.2.4: unit corrected in (40 ml to 40 µl);

— 6.2.6, Table 1 (Chitinase change E.C. 3.2.1.30 to E.C.3.2.1.52 and Alanin-aminopeptidase E.C. 3.4.11.12 to E.C. 3.4.11.2).

Any feedback or questions on this document should be directed to the user’s national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

Micro-organisms are responsible for many key processes in the cycle of elements. Enzymes play key roles in the degradation and mineralization of organic macromolecules. The main postulate is the microbial origin of soil enzymes, even if plant root exudates include enzymes. The simultaneous monitoring of several enzyme activities important in the biodegradation of organic compounds and mineralization of C, N, P and S in soil may reveal harmful effects caused by chemicals and other anthropogenic impacts (e.g. acidification, compaction). However, the measurements carried out under selected laboratory conditions using artificial substrates cannot be a substitute for the actual rate of enzymatic processes in soil in situ.

Soil quality — Measurement of enzyme activity patterns in soil samples using fluorogenic substrates in micro-well plates

1 Scope

This document specifies a method for the measurement of several enzyme activities (arylsulfatase, α –glucosidase, β -glucosidase, Cellubisidase, β -Xylosidase, phosphodiesterase (PDE), chitinase, phosphomonoesterase (PME), leucine-aminopeptidase, Alanine-aminopeptidase) simultaneously (or not) using fluorigenic substrates in soil samples. Enzyme activities of soil vary seasonally and depend on the chemical, physical and biological characteristics of soil. Its application for the detection of harmful effects of toxic chemicals or other anthropogenic impacts depends on the simultaneous comparison of enzyme activities in a control soil similar to the test soil, or on exposure tests with chemicals or treatments.

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.

ISO 18400-206, *Soil quality — Sampling — Part 206: Collection, handling and storage of soil under aerobic conditions for the assessment of microbiological processes, biomass and diversity in the laboratory*

ISO 10390, *Soil quality — Determination of pH*

ISO 10694, *Soil quality — Determination of organic and total carbon after dry combustion (elementary analysis)*

3 Terms and definitions

No terms and definitions are listed in this document.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

4 Abbreviated terms

E.C. Enzyme code number defined by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB)

SOM Soil organic matter content

MUB Modified universal buffer

5 Principle

This document describes a method for the simultaneous measurements of several enzymes in soil samples. It is based on the use of soil samples diluted in buffer containing fluorogenic substrates, which

are incubated for 3 h at (30 ± 2) °C in multi-well plates. After the incubation the enzyme activities are measured as fluorescence with a plate-reading fluorometer [1][2]. The method described is based on dried standard and substrate plates enabling storage and limiting bias due to differences between reagent batches, and also enabling comparison between reagent batches. Annex A describes a method utilizing freshly prepared reagents, which has a clearly defined and exact incubation period. The advantage of the use of freshly prepared substrates is that an instrument for lyophilization is not required.

6 Reagents

6.1 Buffers

6.1.1 General

The selection of the buffer depends on the soil sample because the pH strongly affects enzyme activities. Sodium acetate buffer, 0,5 mol/l, at pH 5,5 has been used for acid soils with a high organic matter content. The use of the modified universal buffer (MUB) at the pH of the soil sample gives the flexibility necessary for coverage of a broad spectrum of different soils. Adequate stability of substrates at different buffers needs to be ensured. Good stability has been observed in 0,5 mol/l sodium acetate buffer at pH 5,5[3].

6.1.2 **Sodium acetate buffer**, 0,5 mol/l, pH 5,5.

— sodium acetate trihydrate (CAS N°: 6131-90-4 – 136,08 g/mol): 68,04 g;

— deionized water 1 000 ml;

— acetic acid (CAS N°: 64-19-7 – 60,05 g/mol): >99,8 %.

Dissolve sodium acetate trihydrate in water (e.g. 800 ml) and adjust the pH to 5,5 with concentrated acetic acid (>99,8 %; pro-analysis). Fill up to 1 000 ml. Sterilize in an autoclave at (121 ± 3) °C for 20 min. Store in a refrigerator for a maximum of two weeks.

6.1.3 **Modified universal buffer (MUB)**[4].

6.1.3.1 Stock solution

— tris(hydroxymethyl)aminomethane (CAS N°: 77-86-01 – 121,14 g/mol): 12,1 g;

— maleic acid (CAS N°: 110-16-7 – 116,07 g/mol): 11,6 g;

— citric acid (CAS N°: 77-92-9 – 192,12 g/mol): 14,0 g;

— boric acid (CAS N°: 10043-35-3 -61,83 mol/l): 6,3 g;

— sodium hydroxide (CAS N°: 1310-73-2 – 40,00 g/mol): (1 mol/l) 488 ml;

— deionized water 1 000 ml.

Dissolve the ingredients and store the solution in a refrigerator.

6.1.3.2 Final buffer

— hydrochloric acid (CAS N°: 7647-01-0 -36,46 g/mol): (0,1 mol/l);

— sodium hydroxide (CAS N°: 1310-73-2 – 40,00 g/mol): (0,1 mol/l).

Place 200 ml of the stock solution (6.1.3.1) in a 500 ml beaker containing a magnetic stirring bar and place the beaker on a magnetic stirrer. Set the required pH with hydrochloric acid or with sodium

hydroxide. Adjust the volume to 1 000 ml with deionized water. Sterilize in an autoclave at (121 ± 3) °C for 20 min.

6.2 Substrates and standards

6.2.1 Preparation of standard solutions

6.2.1.1 4-Methylumbelliferone (MUF) solution

— 4-methylumbelliferone (MUF) (CAS N°: 90-33-5 - 176,17 g/mol): 0,022 g;

— dimethylsulfoxide (DMSO) (CAS N°: 67-68-5 – 78,13 g/mol): add 25 ml.

MUF in powder form can be stored at room temperature but protected from light. Weigh MUF carefully and dissolve it in DMSO in a brown volumetric flask, avoiding exposure to daylight. The solution cannot be stored.

6.2.1.2 7-Amino-4-methylcoumarin (AMC) solution

— 7-amino-4-methylcoumarin (AMC) (CAS N°: 26093-31-2 – 175,18 g/mol): 0,021 9 g;

— dimethylsulfoxide (DMSO) (CAS N°: 67-68-5 – 78,13 g/mol): add 25 ml.

AMC as powder can be stored in the refrigerator. Weigh AMC carefully and dissolve it in DMSO in a brown volumetric flask, avoiding exposure to daylight. The solution cannot be stored.

6.2.2 Preparation of substrate solutions

Commercially available fluorogenic substrates are delivered as powders that can be stored deep-frozen at (–20 ± 2) °C. On the day of use, weigh the amount required for a 1 000 µmol/l, 2 500 µmol/l or 2 750 µmol/l concentration in a volume of, for example, 50 ml, avoiding exposure to light. Weigh the powder into a brown volumetric flask and fill to the required volume with DMSO.

The volume should be big enough for reliable weighing and measurement of volumes. It also depends on the number of plates needed.

The commonly used dispensers are able to distribute simultaneously just one volume (e.g. 40 µl) to eight rows. To facilitate the use of these instruments enabling good volumetric precision, 2 500 µmol/l solutions of the substrates should be prepared. However, for 4-MUF-β-D-glucopyranoside and for 4-MUF-phosphate substrates, a solution with the centration of 2 750 µmol/l is needed in order to produce the same final concentration of 500 µmol/l. These two solutions are further diluted simultaneously with the addition of the sample; 20 µl dimethylsulfoxide is added to the wells of these two substrates to facilitate dissolution. For chitinase activity measurement, a lower concentration is needed in order to avoid substrate inhibition, and the preparation of a solution with a concentration of 1 000 µmol/l 4-MUF-*N*-acetyl-β-D-glucosaminide can be used to produce the final concentration of 200 µmol/l.

6.2.3 Preparation of multi-well plates

The substrate and standard solutions are added to multi-well plates as solutions and dried (e.g. freeze-dried) on the multi-well plates directly after dispensing. Dry plates can be stored at (–20 ± 2) °C for a year. Exposure to light shall be avoided during handling and storage of substrates, standards and multiwell plates. A separate multi-well plate for substrates and standards has proved to be convenient.

6.2.4 Preparation of standard plates

Adequate replicate measurements, e.g. three to four replicates, are necessary due to the small sample volume. Standardization requires several concentrations of MUF or AMC, in replicate. Exposure to light shall be avoided during the dilution of standards. Calculate the required volume that depends on the number of samples and multi-well plates prepared. One example for the preparation of standards

covering a wide range of enzyme activities is given below, but modifications can be made depending on the range of enzyme activities in the samples studied.

The stock solution of MUF with a concentration of 5 mmol/l is used to produce the dilutions containing 1 000 µmol/l, 500 µmol/l, 250 µmol/l, 125 µmol/l, 50 µmol/l, 25 µmol/l and 5 µmol/l MUF. Distribute the volumes needed (e.g. 40 µl) into a multi-well plate for concentrations of 0 nmol/well, 0,2 nmol/well, 1,0 nmol/well, 2,0 nmol/well, 5,0 nmol/well, 10 nmol/well, 20 nmol/well and 40 nmol/well, in replicate. This step is critical for the measurement uncertainty.

NOTE 1 This set of stock solutions enables the use of automatic dispensers, which yield a significantly better precision than manual pipetting.

The stock solution of AMC with a concentration of 5 mmol/l is used to produce the dilutions containing 250 µmol/l, 125 µmol/l, 50 µmol/l, 25 µmol/l, 5 µmol/l, 2,5 µmol/l and 0,5 µmol/l AMC. Distribute the volumes needed (e.g. 40 µl) into a multi-well plate for concentrations of 0 nmol/well, 0,2 nmol/well, 1,0 nmol/well, 2,0 nmol/well, 5,0 nmol/well, 10 nmol/well, 20 nmol/well and 40 nmol/well, in replicate. This step is critical for the measurement uncertainty.

NOTE 2 This set of stock solutions enables the use of automatic dispensers, which yield a significantly better precision than manual pipetting.

6.2.5 Preparation of substrate plates

Exposure to light shall be avoided during dilution of substrates. When using multi-well plates with dry substrates with the final substrate concentration of 500 µmol/l and the sample volume of 200 µl, a volume of 40 µl of the 2 500 µmol/l solution for the substrates is added, in replicate, to the wells. For 4-MUF-β-D-glucopyranoside and for 4-MUF-phosphate, a 2 750 µmol/l substrate solution is added, in replicate, to the wells. For chitinase activity measurement, 40 µl of the substrate 4-MUF-*N*-acetyl-β-D-glucosaminide is added as a 1 000 µmol/l solution to reach the final concentration of 200 µmol/l.

This is the concentration that has been used for several different soils with the assumption of an approximate saturation level. In validation tests for a broad spectrum of soils, appropriate substrate concentrations should be checked and/or an enzyme kinetic approach considered.

If a plate with 96 wells and an automatic dispenser are used for eight substrates, 12 replicates are conveniently available. When using four replicates, it is possible to analyse three different samples or dilution levels on one plate.

6.2.6 Fluorogenic substrates

Table 1 gives a list of fluorogenic substrates and standards that are available commercially[1)].

Table 1 — Fluorogenic artificial substrates available commercially for the enzyme activity measurements

Enzyme	NC-IUBMB (see Clause 4)	Substrate	Element	Macromolecule degraded
Arylsulfatase	E.C. 3.1.6.1	4-MUF-sulfate	Sulfur	Mineralization of organic sulfur
α-Glucosidase	E.C. 3.2.1.20	4-MUF-α-D-glucopyranoside	Carbon	Starch and glycogen
MUF = 4-methylumbelliferone AMC = 7-amino-4-methylcoumarin				

1) Glycosynth and Sigma are examples of producers of fluorogenic molecules. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the producer named. Equivalent products may be used if they can be shown to lead to the same results.