
**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*

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Foreword

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International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

In other circumstances, particularly when there is an urgent market requirement for such documents, a technical committee may decide to publish other types of document:

- an ISO Publicly Available Specification (ISO/PAS) represents an agreement between technical experts in an ISO working group and is accepted for publication if it is approved by more than 50 % of the members of the parent committee casting a vote;
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An ISO/PAS or ISO/TS is reviewed after three years in order to decide whether it will be confirmed for a further three years, revised to become an International Standard, or withdrawn. If the ISO/PAS or ISO/TS is confirmed, it is reviewed again after a further three years, at which time it must either be transformed into an International Standard or be withdrawn.

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.

ISO/TS 22939 was prepared by Technical Committee ISO/TC 190, *Soil quality*, Subcommittee SC 4, *Biological methods*.

Introduction

Micro-organisms are responsible for many key processes in the cycle of elements. Enzymes are responsible for the degradation of organic molecules and their mineralization. The main postulate is the microbial origin of soil enzymes, even if plant root exudates include enzymes. Extracellular enzymes in soil play key roles in the biodegradation of organic macromolecules. 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. 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*.

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

1 Scope

This Technical Specification specifies a method for the measurement of several enzyme activities simultaneously 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 referenced documents are indispensable for the application 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 10381-6, *Soil quality — Sampling — Part 6: Guidance on the collection, handling and storage of soil under aerobic conditions for the assessment of microbiological processes, biomass and diversity in the laboratory*

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ISO 10390, *Soil quality — Determination of pH*

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

3 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

4 Principle

This Technical Specification 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 $(30 \pm 2)^\circ\text{C}$ in multi-well plates. After the incubation the enzyme activities are measured as fluorescence with a plate-reading fluorometer (References [1] and [2] in the Bibliography). 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.

5 Reagents

5.1 Buffers

5.1.1 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 (Reference [3] in the Bibliography).

5.1.2 Sodium acetate buffer, 0,5 mol/l, pH 5,5

- sodium acetate trihydrate 68,04 g;
- deionized water, ad 1 000 ml;
- acetic acid > 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 $(121 \pm 3) ^\circ\text{C}$ for 20 min. Store in a refrigerator for a maximum of two weeks.

5.1.3 Modified universal buffer (MUB) (Reference [4])

5.1.3.1 Stock solution

- tris(hydroxymethyl)aminomethane 12,1 g;
- maleic acid 11,6 g;
- citric acid 14,0 g;
- boric acid 6,3 g;
- sodium hydroxide (1 mol/l) 488 ml;
- deionized water, ad 1 000 ml.

Dissolve the ingredients and store the solution in a refrigerator.

5.1.3.2 Final buffer

- hydrochloric acid (0,1 mol/l);
- sodium hydroxide (0,1 mol/l).

Place 200 ml of the stock solution (5.1.3.1) 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 $(121 \pm 3) ^\circ\text{C}$ for 20 min.

5.2 Substrates and standards

5.2.1 Preparation of standard solutions

5.2.1.1 4-Methylumbelliferone (MUF) solution

- 4-methylumbelliferone (MUF) 0,022 0 g;
- dimethylsulfoxide (DMSO), ad 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.

5.2.1.2 7-Amino-4-methylcoumarin (AMC) solution

- 7-amino-4-methylcoumarin (AMC) 0,021 9 g;
- dimethylsulfoxide (DMSO), ad 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.

5.2.2 Preparation of substrate solutions

Commercially available fluorogenic substrates are delivered as powders that can be stored deep-frozen at $(-20 \pm 2) ^\circ\text{C}$. On the day of use, weigh the amount required for a 1 000 $\mu\text{mol/l}$, 2 500 $\mu\text{mol/l}$ or 2 750 $\mu\text{mol/l}$ 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 $\mu\text{mol/l}$ solutions of the substrates should be prepared. However, for 4-MUF- β -D-glucopyranoside and for 4-MUF-phosphate substrates, a solution with the concentration of 2 750 $\mu\text{mol/l}$ is needed in order to produce the same final concentration of 500 $\mu\text{mol/l}$. 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 $\mu\text{mol/l}$ 4-MUF-N-acetyl- β -D-glucosaminide can be used to produce the final concentration of 200 $\mu\text{mol/l}$.

5.2.3 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 $(-20 \pm 2) ^\circ\text{C}$ for a year. Exposure to light shall be avoided during handling and storage of substrates and standards. A separate multi-well plate for substrates and standards has proved to be convenient.

5.2.4 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 ml) into a multi-well plate for concentrations of 0 µmol/l, 1,0 µmol/l, 5,0 µmol/l, 10 µmol/l, 25 µmol/l, 50 µmol/l, 100 µmol/l and 200 µmol/l, 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 ml) into a multi-well plate for concentrations of 0 µmol/l, 0,1 µmol/l, 0,5 µmol/l, 1,0 µmol/l, 5,0 µmol/l, 10 µmol/l, 25 µmol/l and 50 µmol/l, 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.

5.2.5 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.

5.2.6 Drying of multi-well plates

Handling depends on the instrument e.g. lyophilizator used. Exposure to light shall be avoided during processing of the multi-well plates.

5.2.7 Fluorogenic substrates

Table 1 gives a list of fluorogenic substrates and standards that are available commercially¹⁾.

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.

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

Enzyme	NC-IUBMB (see Clause 3)	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
Cellobiosidase	E.C. 3.2.1.91	4-MUF- β -cellobiopyranoside	Carbon	Cellulose
β -Xylosidase	E.C. 3.2.1.37	4-MUF- β -D-xylopyranoside	Carbon	Xylane, xylobiose
β -Glucosidase	E.C. 3.2.1.21	4-MUF- β -D-glucopyranoside	Carbon	Cellulose
Phosphodiesterase (PDE)	E.C. 3.1.4.1	bis-(4-MUF)-phosphate	Phosphorus	Hydrolysis of phosphate diesters
Chitinase	E.C. 3.2.1.30	4-MUF-N-acetyl- β -D-glucosaminide	Carbon	Breaking β -1-4-glycosidic bonds in N-acetyl-glucosaminide (chitin) and chitobiose
Phosphomonoesterase (PME)	E.C. 3.1.3.2	4-MUF-phosphate	Phosphorus	Hydrolysis of phosphate monoesters
Leucine-aminopeptidase	E.C. 3.4.11.1	L-leucine-AMC	Nitrogen	Hydrolysis of oligopeptides → aminoacids
Alanine-aminopeptidase	E.C. 3.4.11.12	L-alanine-AMC	Nitrogen	Hydrolysis of oligopeptides → aminoacids
MUF = 4-methylumbelliferone AMC = 7-amino-4-methylcoumarin				

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6 Apparatus and materials

6.1 Equipment for the homogenization of the soil samples.

6.1.1 Sieves, with grid size, e.g. 4 mm.

NOTE Other grid sizes can be used depending on the soil texture.

6.1.2 Mechanical homogenizer²⁾.

For some soil types, an ultrasonic disaggregator has been used.

6.2 Usual laboratory glassware.

6.3 Multi-well plates³⁾, with covers.

2) Bamix rod homogenizer and OmniMixer are examples of suitable homogenizers. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products. Equivalent products may be used if they can be shown to lead to the same results.

3) There are differences in background fluorescence between brands and MUF and AMC analyses may require different brands.