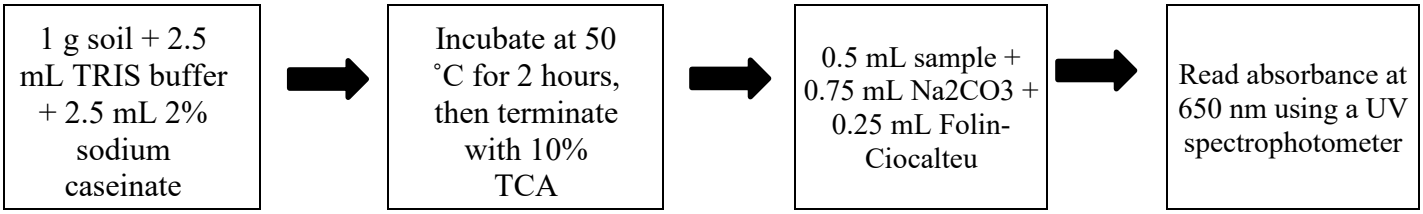
SOP: **Protease (casein) assay**

Overview:

This standard operating procedure (SOP) describes a protocol for determining the protease activity rates in soils using casein as a natural substrate. The method was adapted from Ladd and Butler (1972) by Nannipieri et al. (1979). Key instruments are a laboratory water bath (50˚C), micro-centrifuge machine and tubes, a hot plate stirrer, pipettes (1mL, 5mL) and an ultra-violet spectrophotometer. Key safety considerations are the use of nitrile gloves and laboratory coat. Soils that are ground to pass a 2 mm sieve are typically used.



Safety:

All standard safety protocols and online safety training via UIUC Division of Research Safety (DRS) are required.
Personal protection (PPE) for this procedure include:
Body Protection: Laboratory coat
Hand Protection: Nitrile gloves

Instrumentation & Consumables:

Sample preparation

- Analytical balance (up to three decimal places)
- 15 mL and 50 mL disposable polypropylene centrifuge tubes (Falcon tubes)
- Styrofoam centrifuge tube racks
- 1 mL and 5 mL pipettes and tips

Reagent preparation

- Tris buffer (0.2M, pH 8.0)
- Casein sodium salt from bovine milk (2%, CAS number: 9005-46-3)
- TCA (Trichloroacetic acid, 10%)
- Na_2CO_3 (1.4 M)
- Folin-Ciocalteu's phenol reagent (33%, CAS number: 12111-13-6)
- Tyrosine (1.0 mM)

- Deionized water or 18.2MΩ-cm water
- Hot plate stirrer and stir bar
- Laboratory glassware

Reaction

- Laboratory water bath capable of maintaining 50ºC for 2 hours
- Plastic wrap
- Metal tray
- Micro-centrifuge capable of maintaining 13000 rev min^{-1} for 1 minute
- Micro-centrifuge tubes

Spectrophotometry

- Ultra-violet spectrophotometer
- 96-well microplates
- 200-300µL pipette and tips

Detailed Procedure:

I. Reagent preparation

i. Tris buffer (0.2M, pH 8.0, 1L volume)

1. Dissolve 24.22 g TRIS (hydroxymethyl-amino-methane) buffer in 700 mL of 18.2MΩ-cm water.
2. Adjust the pH to 8.0 with 1M HCl and 1M NaOH.
3. At pH 8.0, bring up the volume to 1000 mL with 18.2MΩ-cm water.
   **Note that Tris buffer remains stable for months at room temperature and in a sterile location.*

ii. Sodium caseinate (2%, 100mL volume)

1. Suspend 2 g casein sodium salt from bovine milk in 50 mL warm water (not more than 37˚C) on a hot stirrer (use magnate stir bar).
2. Adjust pH 8.0 after dissolving casein sodium salt and bring it up to 100 mL in a volumetric flask.

   **Note that sodium caseinate has to be made just before every experiment.*

iii. TCA (10%, 100mL volume)

1. Add 10 g of TCA (Trichloroacetic acid) in 60 mL 18.2MΩ-cm water and swirl until dissolved.
2. Transfer the solution in a 100 mL volumetric flask and volume it to 100 mL with 18.2MΩ-cm water.
   **Note that 10% TCA is unstable and needs to be made just before use.*

iv. Na_2CO_3 (1.4 M)

1. Dissolve 74.19 g of Na_2CO_3 in 500 mL of 18.2MΩ-cm water on a stirrer (use magnate stir bar).
   **Note that Na_2CO_3 remains stable for months at room temperature and in a sterile location.*

v. Folin-Ciocalteu's phenol reagent (33%, 100mL volume)

1. Transfer 33 mL of Folin-Ciocalteu reagent to a 100 mL volumetric flask and bring the volume up to 100 mL with 18.2MΩ-cm water.
   **Note that diluted Folin-Ciocalteu reagent is unstable and needs to be prepared just before use.*

vi. D or L- Tyrosine (1.0 mM, 100mL)

1. Dissolve 0.018 g in 60 mL TRIS buffer (0.2M, pH 8.0) and stir it at 50˚C.
2. Transfer the dissolved tyrosine solution to a 100 mL volumetric flask and bring the volume up to 100 mL with TRIS buffer (0.2M, pH 8.0).
   **Note that tyrosine solution remains stable for up to 1 month if stored at 4ºC and in a sterile location.*

II. Sample preparation

1. Prepare and label two sets of 50mL centrifuge tubes (one will serve as a sample, and the other as a control).
2. Add 1g (±0.01g) of air-dry (or oven-dried equivalent) soil in each tube (1g sample, 1g control, separately).

III. Standard preparation

1. In 15 mL centrifuge tubes, prepare a series of standard solutions by adding Tyrosine (1000.0 µM), TRIS buffer (0.2 M, pH 8.0) and TCA (10%). Do not add TCA until samples are ready for termination. See standard dilutions below:

Standard solutions preparation								
1000 µM Tyrosine solution (mL)	**0.000**	**0.025**	**0.050**	**0.250**	**0.500**	**1.000**	**2.500**	**5.000**
TRIS buffer (mL)	5.000	4.975	4.950	4.750	4.500	4.000	2.500	0.000
TCA (mL)	5	5	5	5	5	5	5	5
Total volume (mL)	10	10	10	10	10	10	10	10

2. At the same time as your terminated samples (e.g. after adding TCA), transfer 0.5 mL of the standard solutions into micro-centrifuge tubes, then add 0.75 mL of Na_2CO_3 (1.4 M) and 0.25 mL of 33% Folin-Ciocalteu reagent exactly 5 minutes before the color measurement.

IV. Reaction

1. Add 2.5 mL of TRIS buffer (0.2 M, pH 8.0) into samples and controls.
2. Add 2.5 mL of 2% sodium caseinate into samples (DO NOT add sodium caseinate to the controls before incubation).
3. Swirl the centrifuge tubes for 1-2 min.
4. Place plastic wrap on top of the tubes to avoid evaporation during incubation.
5. Incubate the tubes at 50 °C for 2 hours using a water bath. (To keep the tubes inside the water bath, place weight – e.g., tray + flasks/bottles filled with water – on top of the tubes.)
6. Take out the tubes from the water bath and add 2.5 mL of 2% sodium caseinate into the controls.

V. Termination and centrifugation

1. Add 5 mL of 10% TCA into samples, controls, and standards to precipitate the remaining casein (termination).
2. Transfer 1.5 mL of the solution into micro-centrifuge tubes, and centrifuge them at 13000 rev min^{-1} for ~1 minute.

VI. Colorimetry

1. Transfer 0.5 mL of clear supernatant into a new micro-centrifuge tube and add 0.75 mL of Na_2CO_3 (1.4 M).
    i. Add 0.25 mL of Folin-Ciocalteu reagent to the samples, controls, and standards exactly 5 minutes before the color measurement. **Note: You may not be able to add reagent to all of your samples within five minutes. We recommend dividing samples for multiple 5-minute durations. Include a new standard curve with each new duration.*
2. Transfer 300 µL of the samples, controls, and standards into a 96-well microplate, and measure their absorbance at 650 nm using an ultra-violet spectrophotometer.

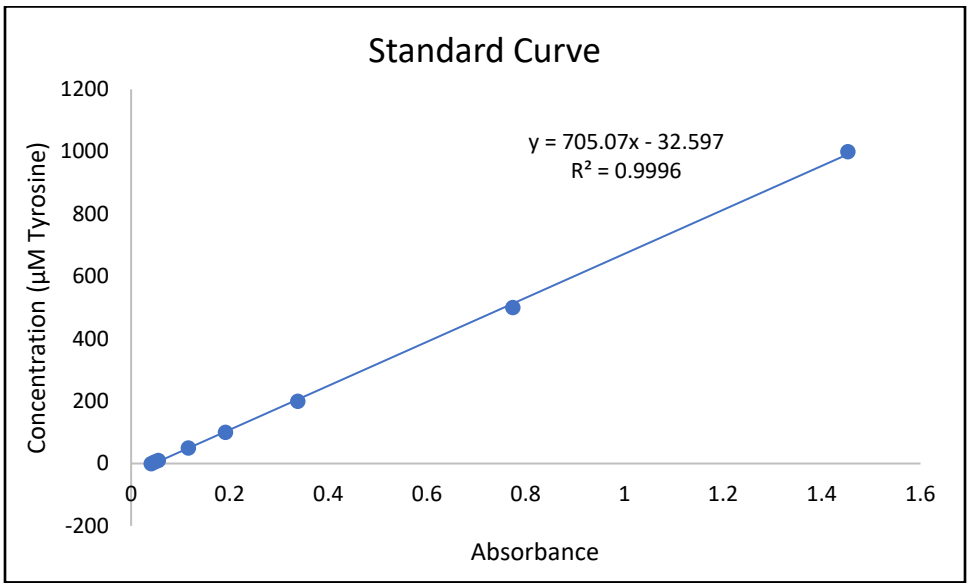
VII. Clean up

1. Waste from Falcon tubes, micro-centrifuge tubes, and microplates should be consolidated in a capped and labeled bottle.
2. Used but emptied Falcon tubes, micro-centrifuge tubes, and microplates may be thrown away in regular trash bins.

VIII. Calculations

Measurement of protease activity is usually expressed in units of µmol tyrosine g^{-1} soil h^{-1}. A standard curve ($R^2 \geq 0.99$) is needed to calculate tyrosine concentration from absorbance. See example standard curve below:

Absorbance	Standard (µM)
0.041	0
0.047	5
0.055	10
0.116	50
0.191	100
0.338	200
0.774	500
1.453	1000



$$\text{Protease activity (µmol tyrosine } g^{-1} \text{ soil } h^{-1}) = \frac{C \times V}{W \times H}$$

Where:

C = tyrosine concentration (µmol/L)

V = final volume (L)

W = sample weight (1 g)

H = incubation period (2 hours)

Example calculation:

Sample Absorbance = 0.314
Control Absorbance = 0.148
Dilution = 1
Standard curve y = 440.16x – 44.106

1) **Convert absorbance to concentration (µmol/L)**

Sample

$$\mu\text{mol/L tyrosine} = 440.16(0.314) - 44.106$$

$$\mu\text{mol/L tyrosine} = 94.10$$

Control

$$\mu\text{mol/L tyrosine} = 440.16(0.148) - 44.106$$

$$\mu\text{mol/L tyrosine} = 21.03$$

2) **Subtract control from sample**

$$\mu\text{mol/L tyrosine} = 94.10 - 21.03$$

$$\mu\text{mol/L tyrosine} = 73.07$$

3) **Multiply by final volume (0.01 L) and divide by mass of oven-dried equivalent of soil (1g) and incubation period (2h)**

$$\mu\text{mol tyrosine g}^{-1}\text{soil h}^{-1} = \frac{(73.07)(0.01)}{(1)(2)} = \mathbf{0.3654}$$

References:

1. Ladd, J. N., & Butler, J. H. A. (1972). Short-term assays of soil proteolytic enzyme activities using proteins and dipeptide derivatives as substrates. *Soil Biology and Biochemistry, 4*(1), 19-30. doi:https://doi.org/10.1016/0038-0717(72)90038-7
2. Nannipieri, P., Pedrazzini, F., Arcara, P. G., & Piovanelli, C. (1979). Changes in amino acids, enzyme activities, and biomasses during soil microbial growth. *Soil Science*, *127*(1), 26-34.

Suggested reading:

1. Greenfield, L. M., Puissant, J., & Jones, D. L. (2021). Synthesis of methods used to assess soil protease activity. *Soil Biology and Biochemistry, 158, 108277.* doi:10.1016/j.soilbio.2021.108277
2. Rejsek, K., Formanek, P., & Pavelka, M. (2008). Estimation of protease activity in soils at low temperatures by casein amendment and with substitution of buffer by demineralized water. *Amino Acids*, *35*(2), 411-417. doi:10.1007/s00726-007-0601-5

Citation:

SOP: Protease (casein) assay. 2021. Soils Lab, University of Illinois Urbana-Champaign. Urbana, IL. Accessed at: https://margenot.cropsciences.illinois.edu/methods-sops/

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