Enzyme Assays for Fresh Litter and Soil
Adapted from Bob Sinsabaugh Lab, 1994

Reagents:

1.0 M NaOH
- 4 g NaOH pellets
- 100 mL DI water

50 mM sodium acetate buffer, pH 5.0 (can make a 10X stock solution)
- 4.374 g sodium acetate trihydrate
- 1.1 ml glacial acetic acid (add more to make pH = 5)
- 1 L DI water

Assay	**Substrate**
acid phosphatase (AP)	5 mM pNP-phosphate
	185.6 mg/100 ml
cellobiohydrolase (CBH)	2 mM pNP-cellobioside
	92.7 mg/100 ml
β-glucosidase (BG)	5 mM pNP-β-glucopyranoside
	150.7 mg/100 ml
β-N-acetylglucosaminidase (NAG)	2 mM pNP-β-N-acetylglucosaminide
	68.5 mg/100 ml
glycine aminopeptidase (GAP)	5 mM glycine p-nitroanilide
	97.6 mg/100 ml
leucine aminopeptidase (LAP)	5 mM leucine p-nitroanilide
	125.7 mg/100 ml
polyphenol oxidase (PPO)	5 mM L-DOPA
	98.6 mg/100 ml
OR (in soils)	50 mM pyrogallol, 50 mM EDTA (for soil)
	631 mg/100 ml
	1.861 g EDTA (disodium, dihydrate)
peroxidase (POD)	5 mM L-DOPA
	98.6 mg/100 ml

Substrate solutions can be made up in 100 or 200 mL batches and stored in the refrigerator for up to a few weeks if uncontaminated.

Check the pH of the substrate solutions. Some may require pH adjustment after mixing. In particular, pNP-phosphate may depress pH slightly (add NaOH).

Protocol:

1) Obtain fresh soil or litter samples and split each into two parts.

2) Weigh one part, record mass, place in tared coin envelope or soil tin and dry at 60-105°C to constant weight (2 days). Record dry mass.

3) Weigh other part (1-2 g wet weight), record mass, and place in blender Mini-Jar. Add 60 ml acetate buffer and blend on highest speed for 2 minutes. Pour homogenate into labeled bottle.

4) Choose a microplate configuration. Use 6-8 replicates for all assays and controls. Each plate needs a set of wells for blanks (empty wells or 200 µL buffer only) and the substrate control (substrate+buffer). Each sample needs a set of wells for homogenate controls (homgenate+buffer) and the actual assay (homogenate+substrate).

5) Pour the homogenate into a petri dish or shallow container with a magnetic stir bar and stir vigorously. Be sure homogenates stay well mixed as you pipet homogenate into the wells. To prevent clogging of the pipet tips, snip off the ends to make an opening 1-2 mm in diameter.

6) Using the multichannel pipetter, add 50 µL homogenate to the homogenate control and assay wells. Add 50 µL buffer to the substrate control wells. Add 150 µL buffer to the homogenate control wells. Add 150 µL substrate to the substrate control and assay wells. For the peroxidase assay, add 10 µL 0.3% H_2O_2 to the substrate control and assay wells.

7) Place plates on a shaker. Oxidase assay plates should be shaken as rapidly as possible without spillage. Incubate AP assay for 45 min, PPO for 1-2 h, NAG for 3 h, CBH for 4 h, BG for 1-2 h, and peptidases for 4-6 h. Times can be varied based on the amount of activity in the sample.

8) Soil particles will sink to the bottom of the wells during the incubation. Using the multichannel pipetter, remove 100 µL from the wells, being careful not to suck up the soil on the bottom, and place in new microplate (tip: you can orient the pipetter so you don't have to change tips as often). Skip to (12) for PPO assay, and (14) for the peptidase reactions.

9) Add 5 µL 1.0 M NaOH to all wells to terminate the reaction and develop the color.

10) Read absorbance at 405 nm. In general, if sample absorbances exceed 2.000 the assay should be repeated using a shorter incubation time or less homogenate.

11) Activity is expressed in µmol of substrate hydrolyzed per hour per g dry organic matter as follows:

OD = Sample Abs – (Substrate control Abs + Sample control Abs)

Activity (µmol h^{-1} $gDOM^{-1}$) =

OD/ [(EC/µmol/ml)/(0.200 mL/assay) (incubation, hr) (gDOM/mL sample homogenate) (0.050 mL homogenate/assay)]

gDOM = (g wet litter mass) (oven dry mass/wet litter mass)

NOTE: The micromolar extinction coefficient for p-nitrophenol is ~4.2 under the conditions of this assay. To calculate, run a standard curve by making dilutions of a 1.00 µmol/mL solution of p-nitrophenol in buffer. Add 100 µL of each concentration and 5 µL 1 M NaOH to the wells and read absorbances. Do a linear regression of OD vs. concentration. The slope of the line is the extinction coefficient. Absorbance is linear with concentration up to an OD of about 2.000.

----------PPO/POD----------

12) Measure the absorbance of the supernatant at 405 nm (Pyrogallol) or 450 nm (L-DOPA).

13) Compute activity as µmol substrate converted per hour per g dry organic matter of sample as follows:

OD = Sample Abs – (Substrate control Abs + Sample control Abs)

Activity (µmol h^{-1} $gDOM^{-1}$) =

OD/ [(EC/µmol/ml)/(0.200 ml/assay) (incubation, hr) (gDOM/ml sample homogenate) (0.050 ml homogenate/assay)]

Peroxidase activity is the difference in activity between the PPO and the peroxidase assay samples.

NOTE: To determine the extinction coefficient, I used a reaction mixture of 100 µl mushroom tyrosinase (1 mg/ml in 50 mM acetate buffer, pH 5.0), 3 ml acetate buffer, and 1 ml of 1 mM L-DOPA or pyrogallol in acetate buffer. The absorbance at 450 nm (L-DOPA) or 410 nm (pyrogallol) maxes out after about 6 hours at room temperature. Measure the absorbance of 100 µL aliquots of the reaction mixture after it maxes out, and divide by 0.25 µmol/ml to get absorbance/(µmol/ml). I got a micromolar extinction coefficient around 0.403.

----------Peptidases----------

14) Measure the absorbance at 405 nm.

15) Compute activity as µmol substrate converted per hour per g dry organic matter of sample as follows:

OD = Sample Abs – (Substrate control Abs + Sample control Abs)

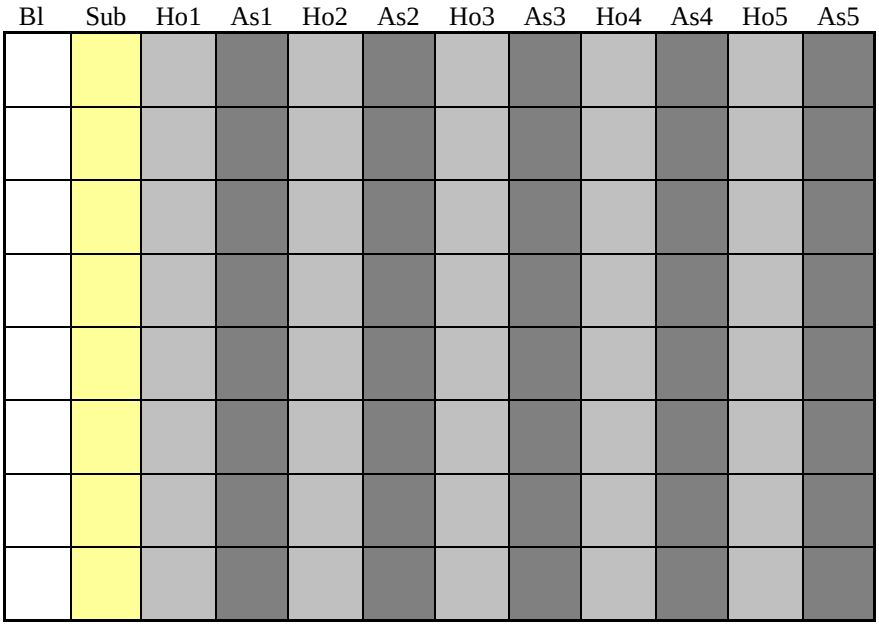
Activity (µmol h^{-1} $gDOM^{-1}$) =

OD/ [(EC/µmol/ml)/(0.200 ml/assay) (incubation, hr) (gDOM/ml sample homogenate) (0.050 ml homogenate/assay)]

NOTE: The micromolar extinction coefficient for p-nitroaniline is ~3.6 under the conditions of this assay. To calculate, run a standard curve by making dilutions of a 1.00 µmol/mL solution of p-nitroaniline in buffer. Add 100 µL of each concentration to the wells and read absorbances. Do a linear regression of OD vs. concentration. The slope of the line is the extinction coefficient. Absorbance is linear with concentration up to an OD of about 2.000.

Potential microplate configurations

5 samples:



7 samples