Enzyme Assays for Fresh Litter and Soil
Adapted from Bob Sinsabaugh Lab, 1994
Steven Allison, 2001

1) Remove litterbags from freezer or soil from cold room and split sample in two parts.

2) Weigh one part, record mass, place in coin envelope or soil tin and dry at 70-105°C to constant weight (2 days). Record dry mass.

3) Weigh other part (one half of sample or approx 2g wet weight), record mass, and place in blender Mini-Jar. Add 60 ml acetate buffer and blend on 'whip' for 1 minute (2 mintues for stems).
Pour homogenate into labeled bottle.

4) Prepare substrate solutions.

1.0 N NaOH
    40 g NaOH pellets
    1 L DI water

Dissolve substrates in 50 mM, pH 5, acetate buffer (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

NOTE: The goal is to conduct assays under conditions of substrate saturation. In general, 5 mM solutions are sufficient. However, when analyzing unfamiliar samples or under conditions of insufficient particle homogenization or insufficient mixing during incubation it may be necessary to use higher substrate concentrations to assure zero order kinetics.

Assay	Substrate
phosphatase	5 mM pNP-phosphate
	185.6 mg/100 ml
cellobiohydrolase (CBH)	2 mM pNP-cellobioside
	92.7 mg/100 ml
β-glucosidase	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	5 mM glycine p-nitroanilide
	97.6 mg/100 ml
leucine aminopeptidase	5 mM leucine p-nitroanilide

	125.7 mg/100 ml
polyphenol oxidase	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	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).

5) Mix 0.750 ml homogenate with 0.750 ml substrate in a 2 ml Eppendorf tube. Place tubes in racks on shaker and incubate for 1-6 h at 20°C. Add 75 µl 0.3% H_2O_2 to the peroxidase samples and substrate controls.
NOTE: Be sure homogenates stay well mixed by stirring on the magnetic stirrer. To prevent clogging of the pipet tip, snip off the end to make an opening about 0.5 cm in diameter.

Do 3-4 analytical replicates per sample.

Prepare sample controls by placing 0.750 ml of sample and 0.750 ml of acetate buffer in a tube and incubate it concurrently. Prepare substrate controls by mixing 0.750 ml of buffer with 0.750 ml of substrate solution. Prepare controls in duplicate.

Incubate phosphatase samples for 45 min, PPO for 1-2 h, NAGase for 3 h, CBHase for 4 h, β-Glu for 1 h, and GA/LA for 4-6 h.

6) Spin the reaction mixture for 1-2 min in table top centrifuge at 10,000 rpm and pipet 0.750 ml supernatant into 10 ml culture tube or Eppendorf tube (as much as possible for PPO). Skip to (11) for PPO assay.

7) Add 0.075 ml 1.0 N NaOH to each tube to terminate the reaction and develop the color.

8) Add 3 ml DI water to each tube and vortex.

9) Read absorbance at 410 nm. Zero spectrophotometer with DI water.
NOTE: In general, if sample absorbances exceed 2.000 the assay should be repeated using a shorter incubation time.

10) 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)/(1.5 ml/assay) (incubation, hr) (gDOM/ml sample homogenate) (0.75 ml homogenate/assay)]

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

NOTE: The micromolar extinction coefficient for p-nitrophenol is ~3.4 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. Mix 0.75 ml of standard, 0.075 ml of 1.0 N NaOH, and 3 ml of distilled water. Read absorbances. Do a linear regression of OD vs. concentration. The slope of the line is the extinction coefficient (my slope was about 3.4). Absorbance is linear with concentration up to an OD of about 2.000.

--PPO--

11) Measure the absorbance of the supernatant at 460 nm, using distilled water to zero the spectrophotometer.

12) 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)/(1.5 ml/assay) (incubation, hr) (gDOM/ml sample homogenate) (0.75 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 460 nm maxes out after about 6 hours at room temperature. Measure the absorbance 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 1.8.