Enzyme Assays for Fresh Litter and Soil  
Adapted from Bob Sinsabaugh Lab, 1994  
Steven Allison, 2005

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

Substrates:
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.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Substrate</th>
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<tr>
<td>acid phosphatase (AP)</td>
<td>5 mM pNP-phosphate</td>
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<tr>
<td></td>
<td>185.6 mg/100 ml</td>
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<tr>
<td>cellobiohydrolase (CBH)</td>
<td>2 mM pNP-cellobioside</td>
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<td>92.7 mg/100 ml</td>
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<td>β-glucosidase (BG)</td>
<td>5 mM pNP-β-glucopyranoside</td>
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<td>150.7 mg/100 ml</td>
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<tr>
<td>β-N-acetylglucosaminidase (NAG)</td>
<td>2 mM pNP-β-N-acetylglucosaminide</td>
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<td></td>
<td>68.5 mg/100 ml</td>
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<tr>
<td>glycine aminopeptidase (GAP)</td>
<td>5 mM glycine p-nitroanilide</td>
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<td></td>
<td>97.6 mg/100 ml</td>
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<tr>
<td>leucine aminopeptidase (LAP)</td>
<td>5 mM leucine p-nitroanilide</td>
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<td></td>
<td>125.7 mg/100 ml</td>
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<tr>
<td>polyphenol oxidase (PPO)</td>
<td>5 mM L-DOPA</td>
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<td></td>
<td>98.6 mg/100 ml</td>
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<tr>
<td>OR (in soils)</td>
<td>50 mM pyrogallol, 50 mM EDTA (for soil)</td>
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<td></td>
<td>631 mg/100 ml</td>
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<tr>
<td>peroxidase (POD)</td>
<td>5 mM L-DOPA</td>
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<td>98.6 mg/100 ml</td>
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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% H2O2 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 (level 5 on the Treseder Titer Plate Shaker). 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:

\[ \text{OD} = \text{Sample Abs} - (\text{Substrate control Abs} + \text{Sample control Abs}) \]

\[ \text{Activity (µmol h}^{-1} \text{gDOM}^{-1}) = \frac{\text{OD}}{[(\text{EC}/\text{µmol/ml})/(0.200 \text{ mL/assay}) \text{ (incubation, hr)} \text{ (gDOM/ml sample homogenate)} \text{ (0.050 mL homogenate/assay})]} \]

\[ \text{gDOM} = (\text{g wet litter mass}) \text{ (oven dry mass/wet litter mass)} \]

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.

12) Measure the absorbance of the supernatant at 450 nm.

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

\[ \text{OD} = \text{Sample Abs} - (\text{Substrate control Abs} + \text{Sample control Abs}) \]

\[ \text{Activity (µmol h}^{-1} \text{gDOM}^{-1}) = \frac{\text{OD}}{[(\text{EC}/\text{µmol/ml})/(0.200 \text{ mL/assay}) \text{ (incubation, hr)} \text{ (gDOM/ml sample homogenate)} \text{ (0.050 mL homogenate/assay})]} \]

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 maxes out after about 6 hours at room temperature. Measure the absorbance of 100 µL
aliquote 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:

\[
\text{OD} = \text{Sample Abs} - (\text{Substrate control Abs} + \text{Sample control Abs})
\]

\[
\text{Activity} (\mu\text{mol h}^{-1} \text{ gDOM}^{-1}) = \frac{\text{OD}}{[(\text{EC}/\mu\text{mol/ml})/(0.200 \text{ ml/assay}) \times (\text{incubation, hr}) \times (\text{gDOM/ml sample homogenate}) \times (0.050 \text{ ml homogenate/assay})]}
\]

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:

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<tr>
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<th>Bl</th>
<th>Sub</th>
<th>Ho 1</th>
<th>As1</th>
<th>Ho 2</th>
<th>As2</th>
<th>Ho 3</th>
<th>As3</th>
<th>Ho 4</th>
<th>As4</th>
<th>Ho 5</th>
<th>As5</th>
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</table>
7 samples

Bl
Sub
Ho1
As1
Ho2
As2
Ho3
As3
Ho4
As4
Ho5
As5
Ho6
As6
Ho7
As7