Extracellular enzyme assays (NAG, BG, and CBH) Version 2

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Abstract

We conducted fluorimetric assays for each of three hydrolytic enzymes: b-glucosidase and cellobiohydrolase, and *N*-acetyl-glucosaminidase in black microplates. We measured fluorescence at 365 nm excitation and 450 nm emission. From each sample, we recorded fluorescence values for MUB substrate (substrate control), homogenate (homogenate control), MUB standards in the presence of maleate buffer (standard), and MUB in the presence of homogenate. We calculated potential extracellular enzyme activity of NAG, BG, and CBH from each litter sample.

Citation: Mia Maltz,Allison SD,German D,Looby C. Extracellular enzyme assays (NAG, BG, and CBH). **protocols.io** dx.doi.org/10.17504/protocols.io.jg3cjyn
Published: 19 Aug 2017

Before start

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; pH can vary with soil)

4.374 g sodium acetate trihydrate

1.1 ml glacial acetic acid (add more to make pH = 5)

1 L sterile DI water (miliQ water)

Substrate solutions (1000 µM unless otherwise noted):

Enzyme	Substrate
Cellobiohydrolase (CBH)	4-MUB-β-D-cellobioside (in refrigerator) 12.5 mg/50 mL **500 µM**

β-Glucosidase (BG)	4-Methylumbelliferyl β-D-glucopyranoside 16.9 mg/50 mL **1000 µM**
α-Glucosidase (AG)	4-Methylumbelliferyl α-D-glucopyranoside 16.9 mg/50 mL **1000 µM**
β-xylosidase (BX)	4-Methylumbelliferyl β-D-xylopyranoside 15.4 mg/50 mL **1000 µM**
MUB standard	1000 µM 4-Methylumbelliferone 0.89 mg/50 ml **100 µM**

Make substrate and fluorescent standard solutions in 125 mL red glass bottles using milliQ water. Store solutions in the 4°C refrigerator. Substrates are in the freezer unless noted. Remake solutions every week, and make them one day BEFORE you want to start working, as this affects the measured activity levels. MUB standard should be diluted to 25 µM before use, depending on expected sample fluorescence. MUB is made by heating the solution to 90°C for a few minutes, with constant stirring (don't let it boil).

Materials

- NaOH by Contributed by users
- Sodium Acetate Buffer by Contributed by users
- MilliQ Water by Contributed by users
- 4-MUB-β-D-cellobioside (in refrigerator) 12.5 mg/50 mL 500 µM by Contributed by users
- 4-Methylumbelliferyl β-D-glucopyranoside 16.9 mg/50 mL 1000 µM by Contributed by users
- 1000 µM 4-Methylumbelliferone 0.89 mg/50 ml 100 µM by Contributed by users
- 4-MUB-N-acetyl--D-glucosaminide by Contributed by users

Protocol

Sample Preparation: Soils

Step 1.

Weigh other part (1-2 g wet weight), record mass, and place in labeled 500 ml container.

Step 2.

Add 125 ml acetate buffer and blend on highest speed for 1 minute to make a homogenate slurry.

AMOUNT

125 ml Additional info: acetate buffer

Step 3.

Rinse blender with DI water between samples.

Assay Set-up

Step 4.

1	2	3	4	5	6	7	8	9	10	11	12
CBHbl	BGbl	AGbl	BXbl	CBH	BG	AG	BX			Hombl	Buf
CBHbl	BGbl	AGbl	BXbl	CBH	BG	AG	BX			Hombl	Buf
CBHbl	BGbl	AGbl	BXbl	CBH	BG	AG	BX			Hombl	Buf
CBHbl	BGbl	AGbl	BXbl	CBH	BG	AG	BX			Hombl	Buf
CBHbl	BGbl	AGbl	BXbl	CBH	BG	AG	BX			Hombl	Buf
CBHbl	BGbl	AGbl	BXbl	CBH	BG	AG	BX			Hombl	Buf
CBHbl	BGbl	AGbl	BXbl	CBH	BG	AG	BX			Hombl	Buf
CBHbl	BGbl	AGbl	BXbl	CBH	BG	AG	BX			Hombl	Buf

Step 5.

1	2	3	4	5	6	7	8	9	10	11	12
NaOAc 200 uL	200	200	200							50	250
200	200	200	200							50	250
200	200	200	200							50	250
200	200	200	200							50	250
200	200	200	200							50	250
200	200	200	200							50	250
200	200	200	200							50	250
200	200	200	200							50	250

Step 6.

1	2	3	4	5	6	7	8	9	10	11	12
milliQ								0	0		
								25	25		
								37.5	37.5		
								43.8	43.8		
								46.9	46.9		
								48.4	48.4		
								49.2	49.2		
								49.5	49.5		

Step 7.

1	2	3	4	5	6	7	8	9	10	11	12
25mM MUB								50	50		
								25	25		
								12.5	12.5		
								6.2	6.2		
								3.1	3.1		
								1.6	1.6		
								0.8	0.8		
								0.5	0.5		

Step 8.

1	2	3	4	5	6	7	8	9	10	11	12
Subs 50	BG 50	AG 50	BX 50	CBH 50	BG 50	AG 50	BX 50				
CBH 50	50	50	50	50	50	50	50				
50	50	50	50	50	50	50	50				
50	50	50	50	50	50	50	50				
50	50	50	50	50	50	50	50				
50	50	50	50	50	50	50	50				
50	50	50	50	50	50	50	50				
50	50	50	50	50	50	50	50				

Using the multi-channel pipette with wide-mouth tips (i.e., trimmed with scissors), add the ***homogenate*** to all wells receiving homogenate

Step 9.

1	2	3	4	5	6	7	8	9	10	11	12
				200	200	200	200	200	200	200	
				200	200	200	200	200	200	200	
				200	200	200	200	200	200	200	
				200	200	200	200	200	200	200	
				200	200	200	200	200	200	200	
				200	200	200	200	200	200	200	
				200	200	200	200	200	200	200	
				200	200	200	200	200	200	200	

All wells should now contain 250 µl. Place plates in the incubator at a proper temperature for soils (e.g., 10°C for Alaskan soils). Incubate for one **hour.**

Assay Termination

Step 10.

After one hour of incubation, add **10 µL** of 1 M NaOH to each well, noting time.

AMOUNT

10 µl Additional info: 1 M NaOH

NOTES
Mia Maltz 19 Aug 2017

This is not done in advance. Add the NaOH to the plates and wait 10 min, then read the plates. This is important. Give the NaOH time to interact with the MUB. MUB will only fluoresce in alkaline conditions.

Reading Plates

Step 11.

Read fluorescence plates at 365 nm excitation and 450 nm emission.

Standard Curve Construction

Step 12.

It is important to calculate the standard curve for the MUB based on the MUB standard wells (wells 9-10).

First, obtain the “homogenate blank” value by averaging the values in column 11.

Step 13.

Then, take the MUB standard values, average them for each MUB concentration, and subtract the homogenate blank from the

fluorescence values for each concentration.

Step 14.

Construct a standard curve using linear regression (MUB concentration on the x-axis, and Fluorescence on the y-axis) to obtain the slope. Make sure this is calculated with the MUB concentrations in µ mols/L (which is synonymous with nmols/mL).

Step 15.

The slope is your Extinction Coefficient (ε).

Step 16.

To calculate the “quench”, simply construct a second standard curve (in a separate plate) that receives buffer and no

homogenate. This “unquenched” curve gives the true fluorescence of MUB.

Step 17.

The ratio of the slope of the standard curve containing soils to the standard curve containing buffer tells you how much the soil

quenches the fluorescence of the MUB.

Fluorescence Activity

Step 18.

Activity (nmol $h^{-1}g^{-1}$) = {NFU x Buffer vol(mL)}/{ε x Homogenate vol(mL) x Time(h) x Soil mass(g)}
NFU = net fluorescence units = {(Assay – Homogenate Control)/Quench Coeff} – Substrate control
Quench coefficient = (Quench control – Homogenate control)/Standard Fluorescence

ε = extinction coefficient Assay volume = 0.250 mL Homogenate volume = 0.200 mL Buffer vol = volume of buffer used to dilute the soil when making homogenate (likely 125 mL) Soil mass = mass of soil included when making homogenate (g) For soils, the mass of the soil can be wet weight, dry weight, organic matter, or Cmic.