PROTOCOL FOR EXTRACELLULAR ENZYME ASSAYS

Kuzyakov Lab

Rev.	Date	Prep.	Modify.	Check.	Approv.
1	2015	Bahar S. Razavi		E. Blagodatskaya	Yakov Kuzyakov
2	2015	Bahar S. Razavi	M. Sanaullah	E. Blagodatskaya	Yakov Kuzyakov
3	20.09.2016	Bahar S. Razavi	B S. Razavi	E. Blagodatskaya	Yakov Kuzyakov

1. Introduction

Extracellular enzymes (EEs) produced by soil microorganisms and plants (or both) are involved in innumerable biogeochemical processes, and play key role in the processing, stabilization, and destabilization of soil organic matter and nutrient cycling in terrestrial ecosystems.

Extracellular enzyme activity (EEA) is most strongly controlled by the concentration of enzymes and corresponding substrates. The abundance of different nutrient-degrading (C, N, P etc.) enzymes in soils is controlled by numerous factors including microbial biomass, community composition, substrate availability, microclimate, and stoichiometric demands. However, *in situ* EEAs within the soil environment are also affected by temperature, the binding of enzymes to soil clays and humic properties, and diffusion constraints, which ultimately regulate the active enzyme pool, in terms of size, substrate availability, and turnover rates. Therefore, acknowledging *in situ* soil conditions is critical when using laboratory enzyme assays to interpret soil microbial function across different environmental sites.

2. Principle

By using microplate reader (e.g. Victor^3 1420-050 Multi label Counter, PerkinElmer, USA), the simultaneous analysis of multiple soil enzymes is possible. It includes a variety of substrates which are associated with either 4-Methylumbelliferone (MUF) or 7-amino-4-methylcoumarin (AMC) (see figure 1 and figure 2). When the substrates are depleted, fluorescent MUF or AMC material is liberated. The fluorescence is measured by microplate reader and quantification of soil enzyme activities is done by comparing with standard concentrations.

Examples of enzymes (Stemmer, 2002)

- β-glucosidases (IUEIMB enzymes nomenclature EC 3.2.1.21): catalyze the removal of terminal glucose from 1-4-beta-glucosidic linkages (e.g. from cellulose)

- β-xylosidases (EC 3.2.1.37): Part of the xylanase enzyme complexes necessary for the degradation of xylan (vegetable polysaccharide)

- N-acetyl-β-glucosaminidases (EC 3.2.1.52): Crucial role in the degradation of chitin (fungi) and Peptidoglycan (bacterial cell wall component).

- Leucine aminopeptidases (EC 3.4.11.X): split as exopeptidase (protease) terminal amino acids of peptide chains (proteins), necessary for the breakdown of proteins

- Phosphatases (EC 3.1.3.2 Acidic; EC 3.1.3.1 Basic): Represent microbial enzyme activities at alkaline pH and plant and microbial originated enzyme activities at acidic pH.

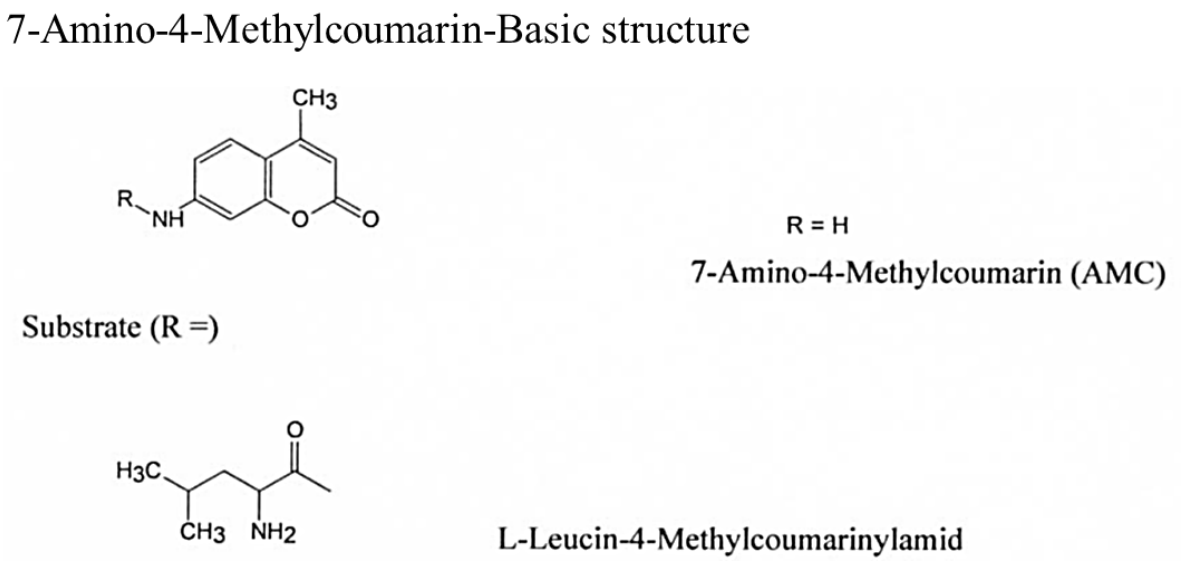
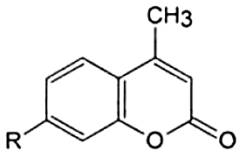


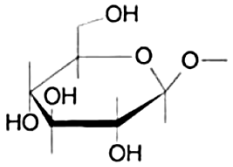
Fig. 1. Example for 7 amino-4-methylcoumarin-associated substrates (Stemmer, 2002)

4-Methylumbelliferone-Basic structure

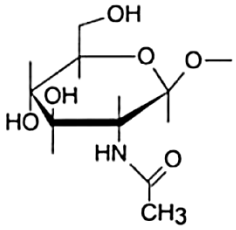


R = OH
4-Methylumbelliferone (MUF)

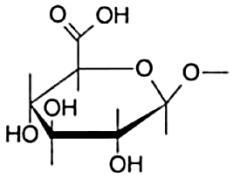
Substrate (R =)



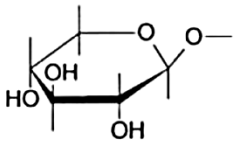
4-Methylumbelliferyl β-D-Glucopyranoside



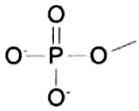
4-Methylumbelliferyl-N Acetyl-β-Glucosaminide



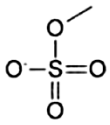
4-Methylumbelliferyl β-D-Glucuronide



4-Methylumbelliferyl β-D-Xylopyranoside



4-Methylumbelliferyl Phosphate



4-Methylumbelliferyl Sulphate

Fig. 2. Example for 4-methylumbelliferone-associated substrates (Stemmer, 2002)

3. Materials and Equipments:

Glassware and plasticware

All the material listed below MUST be autoclaved and sterilized before usage for each experiment.

- 1 storage bottle with polypropylene lid (100 ml) for each sample
- 50 ml Volumetric pipettes
- 1000 ml flasks
- 500 ml flasks
- Petri dishes
- To keep the substrate solutions: 15 ml centrifuge tubes
- 2 and 10 ml pipettes tips
- 200 µl pipettes tips

Equipments

- Electronic Multi-channel pipette (200 µl)
- Pipette (1, 2, 10 ml)
- Microplate Reader (360/460 nm) with PP microplates (black, 96 wells)
- Incubator (30 ° C)
- Ultrasonic probe
- Autoclave
- Magnetic stirrer
- Timer

Chemicals

- MES buffer
- Trizma buffer (Trizma-base + TRIZMA hydrochloride)
- 0.1 M NaOH sol. (for maintaining alkaline phosphatase pH)
- 4-Methylumbelliferon
- 7-Amino-4-Methylcoumarin
- Substrate powder

Some useful notes:

1. Wear gloves
2. Write on all flasks and tubes your name, date and name of material or solution which is inside
3. Use very precise scale to weight substrate
4. Before autoclaving the stuff, remove all marker signed with Ethanol
5. Use cut off edge pipette (tips) for soil suspension pipetting

4. Procedure

4.1. Preparation of soil suspensions

Soil condition:

It is preferred to use fresh soil samples for enzyme assays. If number of samples are high, then soil samples should be stored at 4 °C.

Soil suspension:

Weigh 0.5 gram soil (dry weight equivalent) in 100 ml storage bottle with polypropylene lid. Then add 50 mL sterile water and make soil suspension by using low-energy sonication (40 J s^{-1} output energy) for 2 min. Due to the very small amount of sample weighed must absolutely be observed carefully as possible homogenization of the soil sample

Note: *After ultra-sonication for each sample the rod should be washed and dried. Rod should be in the soil suspension up to 1-1.5 cm bottom of the bottle.*

4.2. Preparation of buffers

0.1 M MES-buffer

Weigh 20.673 g of MES ($C_6H_{13}NO_4SNa_{0.5}$, *molecular weight 206.73 g/mol*) salt in one 1000 ml volumetric flask and make the volume by adding sterile water.

0.05 M Trizma-buffer

Weigh 0.985 g of TRIZMA-Base (α- α- α –Tris-(hydroxymethyl)-methylamin A.C.S. 0.016M) and 2.66 g of TRIZMA-HCl (Tris (hydroxymethyl)aminomethan-hydrochlorid buffer, 0.036M) salts together in 500 ml volumetric flask and make the volume by adding sterile water**.**

4.3. Standard preparation:

10 mM MUF stock solution

In a 100 ml volumetric flask, 0.1762 g of 4-methylumbelliferone and 50 ml of methanol are added. After the 4-methylumbelliferone has completely dissolved in methanol, final volume of 100 ml is made with sterile water.

10 µM MUF working solution

Take 100 µl of MUF stock solution and add it to 900 µl of MES buffer in a PP tube. From this solution (1 mM) take an aliquot of 500 µl in 50 ml volumetric flask and make the volume with MES buffer.

5 mM AMC stock solution

Weigh 0.0875 g of 7-amino-4-methylcoumarin into a 100 ml volumetric flask and add 50 ml of methanol. After complete dissolution of 7-amino-4-methylcoumarin in methanol, make the final volume of 100 ml with sterile water.

10 µM AMC working solution

Add 200 µl of the AMC stock solution in 800 µl TREZMA buffer in a PP tube. Take 500 µl aliquot from this solution (1 mM) in 50 ml volumetric flask and add 49.5 ml TREZMA buffer to make the volume.

Note: *The MUF and AMC-working solutions can be stored in a refrigerator for ~2 months. However, for AMC standard solution, the salt is poorly soluble during the preparation of the stock solution and precipitates again very easily in the fridge. Therefore, the AMC stock solution should always be made fresh as needed or should be the same depending on the number of processed samples obtained from freshly prepared stock solution of AMC. For the analysis of a few samples, you should reduce the amount of MUF and AMC stock solution accordingly.*

4.4. Standards execution

For an analysis set of 2 soil types with four replicates, one 96-well microplate is needed for both MUF and AMC standards. First, 6 columns of the plate will be used for MUF (1-6) and next 6 columns (7-12) are located for AMC. The soil solution is stirred during pipetting on a magnetic stirrer. Avoid cross contamination from one sample to another on stir bars and stir bar capsules, so rinse and dry in between with deionized water. The scheme and sequences of pipetting will be as followed (Fig. 3):

- 50 µl soil suspension (multi-channel electronic pipette with 12 tips)
- MES and TRIZMA buffers in the following order: 150, 140, 130, 100, 70, 30 µl (Multi-channel electronic pipette with 8 tips)
- MUF or AMC standard in order: 0, 10, 20, 50, 80, 120 µl
  (Multi-channel electronic pipette with 8 tips)

		Standard: MUF Buffer: MES						Standard: AMC Buffer: Trizma					
		1	2	3	4	5	6	7	8	9	10	11	12
Soil 1	A												
Soil 1	B												
Soil 1	C												
Soil 1	D												
Soil 2	E												
Soil 2	F												
Soil 2	G												
Soil 2	H												
Soil suspension in µl		50	50	50	50	50	50	50	50	50	50	50	50
Standard in µl		0	10	20	50	80	120	0	10	20	50	80	120
Buffer in µl		150	140	130	100	70	30	150	140	130	100	70	30
Conc. picomoles/ well		0	120	200	500	800	1200	0	100	200	500	800	1200

Figure 3: Pipetting scheme of the standard plates

The measurement of the plates in the microplate reader is carried out at 360/460 nm after a time of 30, 60 and 120 min. The files are stored in the form of an Excel spreadsheet. Between the measurements, the soil and standard plates remain at room temperature. Record initial time as Time 0 so you know when to start next measurement e.g. after 30 min and before each reading, shake the plates for 1-2 min.

4.5. Substrate preparation

10 mM Substrate stock-solution

Weigh 1/10000 of the molecular weight of each substrate in 15ml sterile centrifuge tubes and dissolve it in 300 µl of dimethyl sulfoxide (DMSO) (C_2H_6SO). Then add 9.7 ml of sterile water and shake it properly to make it homogeneous solution.

1mM Substrate working-solution

Take 1 ml aliquot of an enzyme substrate of the respective 10 mM substrate stock solution and add 9 ml of sterile buffer (MES buffer for MUF substrates and TRIZMA buffer for AMC substrates, see Table 1) in 15ml sterile centrifuge tubes.

Table 1: List of the substrates along with their molar mass and associated buffers

Substrate	Abbreviations	Formula	Molar Mass [g/mol]	Buffer
4-Methylumbelliferyl-ß-D-Glucoside	ß-Glu	$C_{16}H_{18}O_8$	338.31	MES
4-Methylumbelliferyl-• -D-Glucoside	• -Glu	$C_{16}H_{18}O_8$	338.31	MES
4-Methylumbelliferyl-N-Acetyl-ß-D-Glucosaminide	N-Ac	$C_{18}H_{21}NO_8$	379.36	MES
4-Methylumbelliferyl-ß-D-Xylopyranoside	Xyl	$C_{15}H_{16}O_7$	308.28	MES
4-Methylumbelliferyl-ß-D-Cellobioside	Cello	$C_{22}H_{28}O_{13}$	500.45	MES
4-Methylumbelliferyl-Phosphate	Phos	$C_{10}H_7O_6PNa_2$	300.11	MES
L-Leucine-7-amido-4-methylcoumarin hydrochloride	L-Leu	$C_{16}H_{20}N_2O_3$*HCl	324.81	TRIZMA
L-Tyrosine-7-amido-4-methylcoumarin	L-Tyr	$C_{19}H_{18}N_2O_4$	338.36	TRIZMA

Note: *Few substrates, such as L-tyrosinde-7-AMC etc. are poorly soluble, so it is recommended to prepare their working solutions directly from the substrates and avoiding concentrated stock-solutions. It would be better to use warmer water for dissolution of salts. The stock solutions of the substrates can be stored frozen at -20 °C for approx. 6 months. While, the substrate working solutions in a refrigerator at 4 °C can be used maximum for 3 weeks.*

Substrate concentrations

In order to determine enzyme kinetics, different substrate concentrations are required (Table 2). This concentration range depends on substrate saturation points of soil samples under analysis. It is recommended to use 1-8 substrate concentrations but if required 9th concentration can also be prepared. Buffers (MES or Trizma) must be used for dilutions.

Table 2. Substrate concentrations

Sr. #	Substrate (ml)	Buffer (ml)	Substrate conc. (µmol/g soil)
1	0.0	10.0	0
2	0.1	9.9	10
3	0.2	9.8	20
4	0.3	9.7	30
5	0.4	9.6	40
6	0.5	9.5	50
7	1.0	9.0	100
8	2.0	8.0	200
9	4.0	6.0	400

4.6. Substrate pipetting and execution

For enzyme kinetics, four samples can be analyzed for one substrate in one 96-microwell plate. For each sample, 3 analytical replicates are required. All precautionary measures for pipetting must be taken for precise results as mentioned in "standards execution" (section 4.4). The scheme and sequences of pipetting will be as followed (Fig. 4):

- 50 µl soil suspension (multi-channel electronic pipette with 8 tips)
- 50 µl MES or TRIZMA buffer depending on the substrate (multi-channel electronic pipette with 12 tips)
- 100 µl substrate (multi-channel electronic pipette with 12 tips). Always start pipetting of substrate from lower concentration to higher concentrations so that same pipette tips can be used.

Substrate conc. (µmol/g soil)		Soil 1			Soil 2			Soil 3			Soil 4		
		1	2	3	4	5	6	7	8	9	10	11	12
0	A												
10	B												
20	C												
30	D												
40	E												
50	F												
100	G												
200	H												
Soil suspension (µl)		50	50	50	50	50	50	50	50	50	50	50	50
Buffer (µl)		50	50	50	50	50	50	50	50	50	50	50	50
Substrate (µl)		100	100	100	100	100	100	100	100	100	100	100	100

Figure 4: Pipetting scheme of the substrate plates for enzyme kinetics.

The measurement of the plates in the microplate reader is carried out at 360/460 nm after a time of 30, 60 and 120 min. The files are stored in the form of an Excel spreadsheet. Between the measurements, the soil and standard plates remain at room temperature. Record initial time as Time 0 so you know when to start next measurement e.g. after 30 min and before each reading, shake the plates for 1-2 min.

5. Fluorescent measurements on a microplate reader

Select the correct protocol. An excitation wavelength of 355 nm and an emission wavelength of 460 nm, slit width of 25 nm, in Victor^3 1420-050 Multi label Counter.

Representative Results

Enzyme assays can be used to quantify potential EEAs and to compare activities among similar samples. Michaelis–Menten equation can be used to determined enzyme activity (V), $V = V_{\max}[S]/K_m + [S]$, where V_{max} is the maximal rate of enzymatic activity; K_m is the half

saturation constant, or the substrate concentration at $½V_{max}$ and S is the concentration of the substrate.

Exercises for practice

Exercise # 1

Mr. X prepared 25mM stock solution of β-Glucosidase in 100 ml volumetric flask. He took 0.5, 1.0, 2.0 and 4.0 ml from this stock solution and made their volume to 10 ml with MES buffer, separately. What will be the final concentration of these four solutions?

Exercise # 2

In order to determine enzyme kinetics in soil, different concentration of substrate were prepared for pipetting them in 96-well microplate along with soil suspension and buffer. To calculate different concentrations of a substrate in µmol/g of soil, please fill the missing table by considering following data:

Working sol. Concentraion (mM) = 1

Volume of substrate pipetted / well (µl) = 100

volume of soil suspension / well (µl) = 50

volume of buffer / well (µl) = 50

Final volume / well (µl) = 200

Total volume of soil suspension (µl) = 50000

Soil weight used for soil suspension (g) = 0.5

Sr. #	Substrate (ml)	Buffer (ml)	Substrate conc. (µmol/g soil)
1	0.0	10.0	
2	0.1	9.9	
3	0.2	9.8	
4	0.3	9.7	
5	0.4	9.6	
6	0.5	9.5	
7	1.0	9.0	
8	2.0	8.0	
9	4.0	6.0	

Exercise # 3

What will be the enzyme activity (nmol/g soil/h) of following data:

MUF value after 60 min =	20282.0
MUF value after 120 min =	42615.0
Standard scale (pmol) =	0.00258
Soil suspension aliquote (µl) =	50
Total vol. of soil suspension (µl) =	50000
Soil weight for suspension (g) =	0.53

Exercise # 4

Calculate V_{max} and K_m from the following data:

Substrate Conc. (µmol/g)	Activity (nmol/g/h)
0	6
5	140
10	222
15	283
20	337
25	398
50	462
100	484

Answers to the questions

Exercise # 1: 1.25 mM

2.5 mM

5.0 mM

10.0 mM

Exercise # 2:

Sr. #	Substrate (ml)	Buffer (ml)	Substrate conc. (µmol/g soil)
1	0.0	10.0	0
2	0.1	9.9	10
3	0.2	9.8	20
4	0.3	9.7	30
5	0.4	9.6	40
6	0.5	9.5	50
7	1.0	9.0	100
8	2.0	8.0	200
9	4.0	6.0	400

Exercise # 3: 108.2 nM/g/h

Exercise # 4: V_{max} = 578±25 nmol/g/h

K_m = 14±2 µmol/g

References:

German DP, Weintraub MN, Grandy AS, Lauber CL, Rinkes ZL, Allison SD. 2011, Optimization of hydrolytic and oxidative enzyme methods for ecosystem studies. *Soil Biology and Biochemistry*, 43, 1387–1397.

Koch O, Tscherko D, Kandeler E. 2007, Temperature sensitivity of microbial respiration, nitrogen mineralization, and potential soil enzyme activities in organic alpine soils: Temperature sensitivity in alpine soils. *Global Biogeochemical Cycles*, 21.

Razavi BS., Blagodatskaya E., Kzyakov, Y. 2015. Nonlinear temperature sensitivity of enzyme kinetics explains canceling effect - a case study on loamy haplic Luvisol. *Frontiers in Microbiology* 6:1126.