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RESEARCH PAPER

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## Methodology for Measurement of Enzyme Activity in Soil

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### ABSTRACT

*Enzymes are proteins that have catalytic functions indispensable to maintenance and activity of life. All chemical reactions occurring in a living organism are dependent on the catalytic actions of enzymes, and this is why enzymes are called Biotransformation. At present, there are about 4,000 kinds of enzymes whose actions are well known. Enzymes function in a mild environment similar to the body environment of a living organism, and they support life by synthesizing and degrading materials that constitute the building blocks of the organism and by creating energy. Enzymes function as highly selective catalysis in such a way that they selectively catalyze specific reactions (reaction specificity) and specific materials (substrate specificity).*

*Technology for utilizing enzymes for the improvement of our life is a key feature of biotechnology. Mankind has been concerned with enzymes from ancient times when enzymes were unknown; mankind unknowingly used microbial fermentation techniques in food processing. In ancient Egypt, they produced breads or beer by fermentation. In Japan too, we have a long history of producing sake by fermentation even from the Jomon period. It is only in the nineteenth century that fermentation was unmasked. Now we know that enzymes are the true entity of fermentation. The word "Enzyme" is derived from the Greek, en (in) +zyme (ferment).*

**Key words:** Soil, Enzyme, Catalytic Functions,

## INTRODUCTION

The soil microbial community contains a wide range of species and an immense number of microbial cells, and they carry out several types of reactions. One gram of soil may contain as many as  $10 \times 10^9$  microbial cells; in comparison, the number of humans on Earth is  $7 \times 10^9$ . Since it is impossible to determine the number, richness, and structure of species, an alternative method for measuring the microbial diversity is to assess the type and rate of reactions mediated by soil enzymes. The objective of the dissertation was to develop a rapid and sensitive small-scale method for the simultaneous measurement of the activity of several soil enzymes. The method involves analyzing homogenized soil suspensions using fluorescent substrate analogues freeze-dried onto multi well plates. The feasibility of the enzyme activity assay was assessed by measuring soil samples obtained from experimental sites, treated in different ways, and supporting different cover plants. In addition, the effect of the season on the enzyme activities was studied. Bulk samples were obtained from experimental sites established in agricultural and forest soils.

### **Crop plants had the largest effect on soil enzyme activity**

Eight different crop plants and added peat caused statistically significant changes in enzyme activity levels in two consecutive sampling years. The effect of crop plants was the most pronounced, and it was observed in eight of the ten enzymes measured in both years. The sampling year affected the activities of six enzymes. The effect of peat addition was detectable only after two years. In another experiment, green or composted plant residues were added to soil. This enhanced enzyme activities slightly compared to chemical

fertilizers, but the effect was not consistent. When calculated on the basis of organic matter, forest soils showed higher enzyme activities than agricultural soils, and enzymes showed higher potential activity around alders than around pine trees. Cluster analysis was used for data analysis in order to combine the data for all measured parameters. Clustering allows for the grouping of samples, revealing differences in the entire pattern, even though the differences in individual enzymes were not statistically significant and even though the results showed correlation. The sophisticated cluster analysis software was developed at the Finnish Environment Institute in connection with this research project. Due to the diversity of species and the wide taxonomic diversity in soils, methodological development in soil microbiology is still a major challenge. A reference sample for comparison purposes is generally required for the interpretation of results. The method developed in this study may be used as a sensitive indicator of soil diversity. The experimental part of the study was conducted at the Laboratory of the Finnish Environment Institute with funding from Tekes, and the author wrote the analysis part as an external researcher at the Natural Environment Centre's Ecosystem Change Unit.

The dissertation defense will be held on Friday, 9 November 2012, at 12 noon at the Building of Forest Sciences on the Viikki Campus (Latokartanonkaari 7, lecture room B2, Helsinki).

### **Description of the methods**

Soil Enzyme Activity (Soil Microcosms) Part 1

#### **Materials**

Equipment  
- Balance

- Scissors

**Supplies**

- Plastic flowerpots
- Dry, sieved soil

- Yeast extract
- Fertilizer (15-30-15)
- Markers
- Filter paper

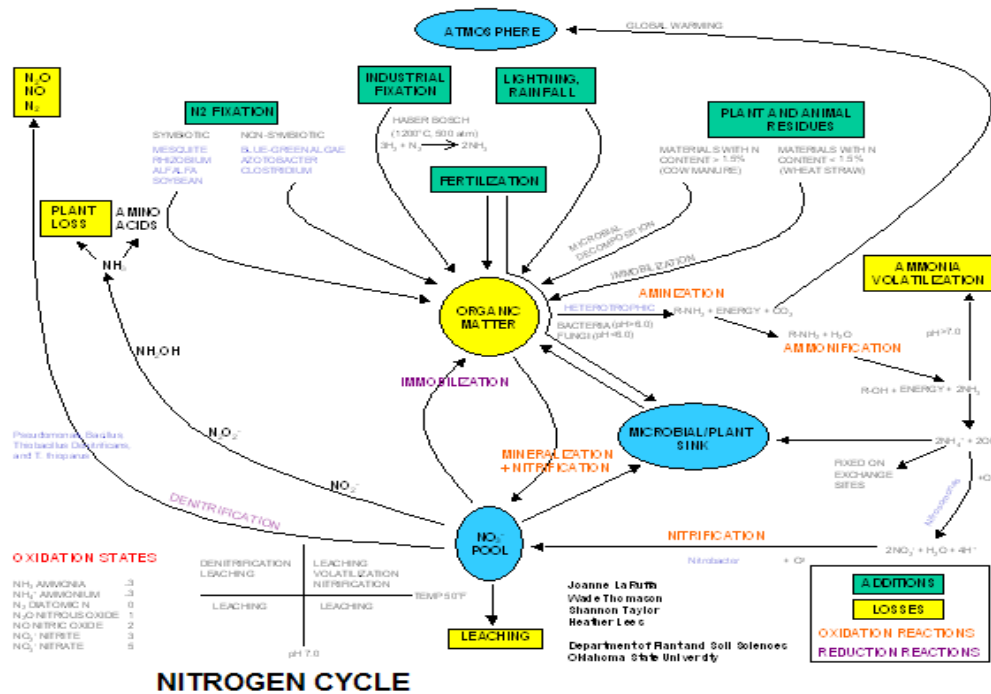


Fig 1. Show nitrogen cycle.

**Activities**

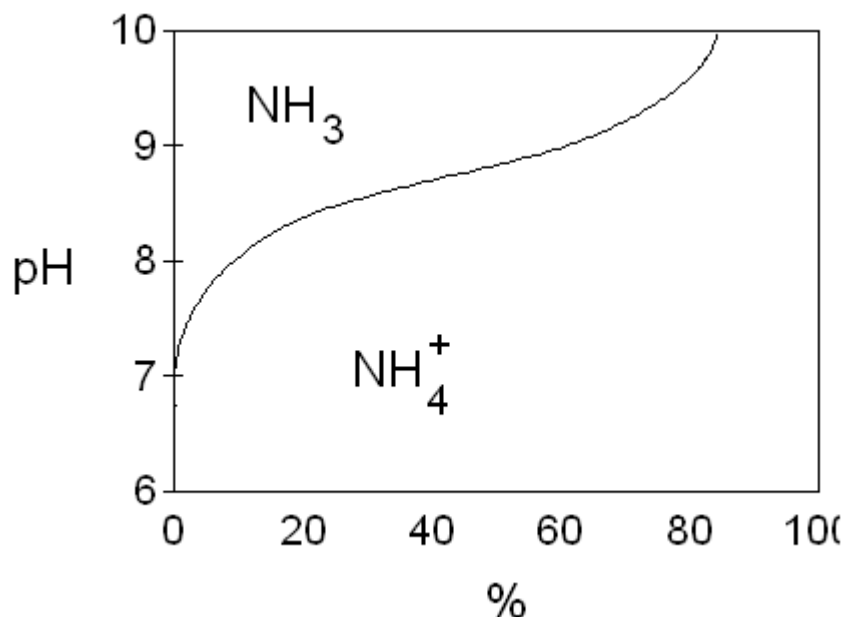
- Set up five soil microcosms with different fertilizer treatments

Treatment	Fertilizer	Yeast Extract
Organic	0.0 g	1.6 g
Inorganic	0.2 g	0.0 g
Combined Low	0.1 g	1.6 g
Combined High	0.4 g	1.6 g
Unamended	0.0 g	0.0 g

**Procedure**

1. Cut out a circle of filter paper to fit the bottom of the flower pot.
2. Use a glass beaker to measure out ~300ml of soil.
3. Weigh out the appropriate amount of fertilizer and mix it into the soil.

4. Put the filter paper circle into the bottom of the flowerpot
5. Pour in the soil and fertilizer mix until it is ~1/2 inch from the top.
6. Water the soil until it is all moist.
7. Keep moist and incubate at room temperature for 2-3 weeks



**Fig 2. Equilibrium relationship for ammoniacal N and resultant amount of  $\text{NH}_3$  and  $\text{NH}_4^+$  as affected by pH for a dilute solution.**

### Soil Enzyme Activity (Alkaline Phosphatase Assay)

Bacteria and fungi that break down insoluble nutrient sources in the soil produce extracellular enzymes. These are proteins that are produced inside the cell and exported out into the soil solution. The enzymes are active outside the cell where they catalyze reactions to break down the structure of the nutrient source to make it more accessible. The amount of an extracellular enzyme in the soil depends on the metabolic abilities of the soil organisms, the number of organisms present, the presence of substrate and the environment of the soil (pH, temp., ionic strength etc.). Because enzymes are costly for the cells to make, they are tightly regulated. Enzymes will only be made when they are needed.

One example of a common extracellular enzyme in soil is alkaline phosphatase. This enzyme is produced by many organisms in the soil. Its purpose is to remove the phosphate molecule from organic compounds such as phospholipids and

nucleic acids. Once the phosphate is cleaved it becomes soluble and can be taken up by the cell. This is a very important activity because phosphate is often the limiting nutrient for microbial growth in soil.

In this lab you will be measuring the amount of active enzyme in soil samples by using a chromogenic substrate assay. In the presence of alkaline phosphatase, the colorless chemical para-nitrophenol phosphate is converted to para-nitrophenol, which is bright yellow. The amount of product formed can be measured using a spectrophotometer and the amount of enzyme activity can be calculated. You will also calculate the dry weight of the soil in order to standardize the results. The soils that you will be analyzing have been kept moist and incubated for ~2 weeks with the following amendments: 1.6g of yeast extract, 0.2g of inorganic fertilizer, 1.6g of yeast extract and 0.1g of inorganic fertilizer, 1.6g of yeast

extract and 0.4g of inorganic fertilizer, or no addition.

### Materials

#### Equipment

- incubator (37 C)
- clinical centrifuge
- 5 ml pipettes and pumps
- screw-top tubes (wide-mouth)
- 16 X 100 mm test tubes
- balance
- spectrophotometer (440nm)
- drying oven (100 C)
- aluminum weighing dishes

#### Samples

- soils from microcosms

#### Media and Reagents

- buffer (pH 10)
- 2 mM p-nitrophenol
- 0.5 M CaCl<sub>2</sub>
- PNPP test solution  
(para-nitrophenol phosphate in buffer)

#### Procedures

##### Phosphatase Assay

1. Weigh out two 2-gram portions of your group's soil sample and pour them into screw-cap tubes labeled "test" and "soil blank".
2. Label one other screw-cap tube as "reagent blank"
3. Pipette 5ml of 0.5 M CaCl<sub>2</sub> solution into each of the three tubes and shake well.
4. Pipette 1ml of PNPP solution into the tubes labeled "test" and "reagent blank".

##### Calculations

Water Content of Soil and Dry Weight

Water content = (wet weight of soil – dry weight of soil) / dry weight of soil

Dry weight of sample = (wet weight of sample / water content + 1)

5. Pipette 1ml of phosphate buffer into the "soil blank" tube to serve as a control.
6. Incubate all three tubes at 37C for 1 hour.
7. Transfer 4ml of the liquid from each tube into labeled 16 X 100mm test tubes. (Be careful to avoid transferring sediment.)
8. Centrifuge the test tubes for 5 min. at 2500 rpm.
9. Transfer 3ml of the supernatant into clean test tubes (re-centrifuge if liquid is at all cloudy).
10. Set the wavelength on the spectrophotometer to 440nm.
11. Set the absorbance to zero with the "soil blank" tube.
12. Read and record the absorbance for the "test" and "reagent blank" tubes.
13. Set the absorbance to zero with a blank tube containing 3 ml of CaCl<sub>2</sub>.
14. Read and record the absorbance of the prepared standards.
15. Plot the absorbance vs. concentration to make a standard curve.

##### Water Content Analysis

1. Weigh an aluminum dish and record the weight.
2. Weigh out ~10g of your soil sample in the aluminum dish. Record the exact weight.
3. Put the samples in a 100 C oven overnight and let them cool in a desiccator.
4. Weigh the dried sample and record the weight.

##### Lab Report

##### Alkaline Phosphatase Assay

##### Enzyme Activity

One unit of enzyme activity (U) is defined as the amount of enzyme that is able to convert 1 mole of substrate to product in one minute. For soil assays, activity is reported as U per gram of dry soil.

**Table 1. Spectrophotometer readings.**

Spectrophotometer Readings

	Absorbance	Net Absorbance (test – soil blank)	Concentration of <i>p</i> -Nitrophenol	Enzyme Activity
Organic fertilizer (test)				
control (reagent blank)		XXXX	XXXX	XXXX
Inorganic fertilizer				
control (reagent blank)		XXXX	XXXX	XXXX
Combined Low				
control (reagent blank)		XXXX	XXXX	XXXX
Combined High				
control (reagent blank)		XXXX	XXXX	XXXX
Unamended				
control (reagent blank)		XXXX	XXXX	XXXX

**Table 2. Standard Curve.**

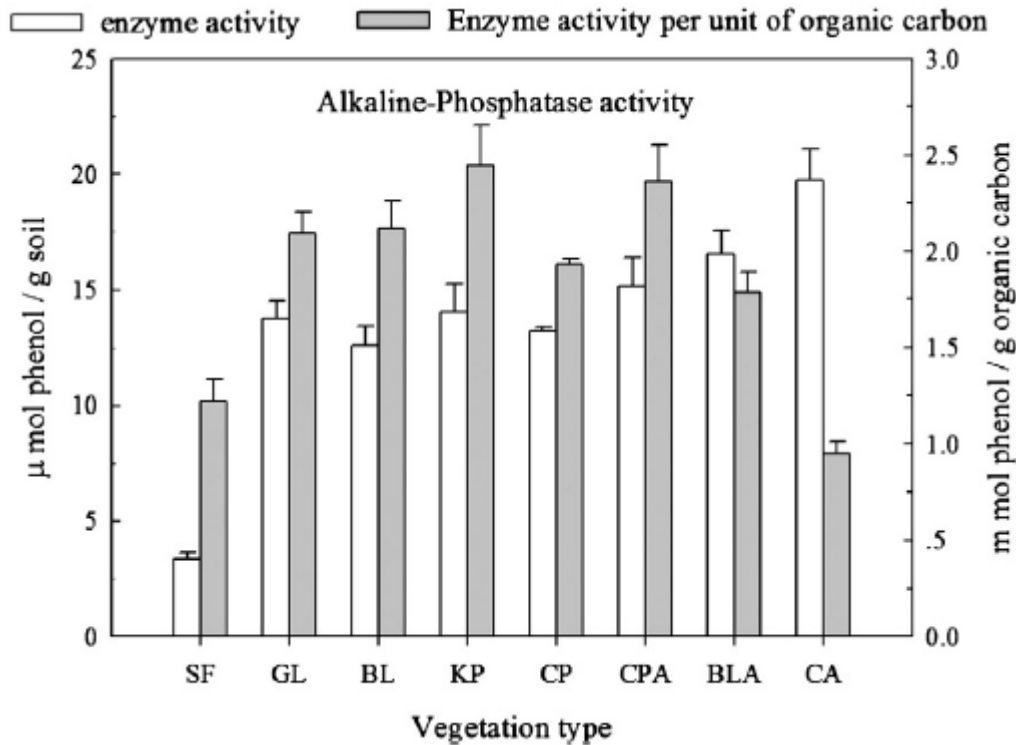
Concentration	2.0 mM	1.0 mM	0.5 mM	0.25 mM	0.125 mM	0.063 mM
absorbance						

**Table 3. Water Content Analysis.**

Sample	Organic	Inorganic	Combined low	Combined high	unamended
Dish weight					
Wet weight with dish					
Wet weight of soil					
Dry weight with dish					
Dry weight of soil					
Water content					

1. Calculate the amount of *p*-nitrophenol that was produced using the standard curve (remember that the total volume of liquid was 6ml even though you only measured the concentration in 3ml) \_\_\_\_\_  $\mu$ moles in 6 ml.
2. Divide the amount of product by the number of minutes that the samples were

- incubated to find the value of U \_\_\_\_\_  $\mu$ moles / minute.
3. Calculate the dry weight of the soil sample that was used in the incubation \_\_\_\_\_ grams.
4. Calculate the activity per gram of dry soil \_\_\_\_\_ U / gram of dry soil.



**Fig. 3. Vegetation influence on alkaline phosphatase activity and alkaline phosphatase activity per unit of organic carbon.** SF is sloped farmland, CA is Chinese arborvitae, GL is grassland, KP represents korshinsk peashrub, CP is Chinese pine, CPA is mixed forest.

## CONCLUSION

For nitrogen cycle enzymes, urease and catalase activities varied significantly under different vegetations. Urease plays an important role in soil nitrogen cycle and utilization because it can hydrolyze urea to ammoniacal nitrogen. In this study, urease activity was found to be high in the soils of restored forest lands and low in grassland soil. Enzymes are classified into six categories based on the types of reaction catalyzed that is, oxidoreductase, transferase, hydrolase, lyase, ligase, and isomerase, according to the Enzyme Commission, International Union of Biochemistry and Molecular Biology. Individual enzymes are further classified systematically based on the chemical name of the substance modified by the enzyme

(substrate) and its reaction mechanism. Enzymes are named with the use of letters and numbers: EC plus four numbers representing four elements. For example, 1,4-β-D-Glucan glucanohydrolase (common name: α-amylase) is named as EC 3.2.1.1.

## ACKNOWLEDGMENTS

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