VOL. 5, NO. 2, MARCH 2010 ISSN 1990-6145
ARPN Journal of Agricultural and Biological Science


www.arpnjournals.com

SOIL MICROBIAL ENZYMATIC ACTIVITY RELATE TO ROLE OF METHANOTROPHIC BACTERIA IN THE TROPICAL FOREST SOIL OF GUNUNG SALAK NATIONAL PARK

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ABSTRACT

Study on link between soil enzymes activity and net methane emission from the tropical forest soil have not yet been intensively investigated. This observation was intended to elucidate the link between organic substrate hydrolyses, net methane emission, and possible role of soil microbial communities in the soil collected from the forest of Gunung Salak National Park. Microbial community structure of forest soil was reasonably divers, and several important functional microbial groups in soil could become indicator of environmental damage. Higher population of functional microbes of cellulolytic, amylolytic, proteolytic, and phosphate solubilizing bacteria, assumed to be positively maintain the ecological forest in the altitude of 1000 to 900 m above sea level (als), compared to the lower of riparian soil that obtain from tea plantation areas at 800 m asl. Soil enzymatic activities (cellulase, amylase, invertase, urease, and phosphatase) hydrolyze polymer organic substrate to become soluble as importance soil nutrient. Along with continuous bio-processing, some nutrients could take turns to the carbon sources for both of methanogen and methanotrophic organism. Subsequently, the net methane emission likely was governed by polymeric substances hydrolyzing microbes and do to keep methanotrophic and methanogenic activities. In well preserved forest, methanotrophic bacteria were luxurious and controlling net methane emission from forest soil. Accordingly, forest ecosystem is not only important for maintaining ecosystem health but also important to preserve microbial diversity and its genetic resources.

Keywords: soil enzymes, methanotrophic bacteria, forest ecosystem.

INTRODUCTION

Unique and distinctive of microbes in soil compartment of tropical forest is sustaining the role of forest ecology. Microbial mediated processes through enzymatic activities of cellulase, amylase, and invertase are playing major role in the forest floor to decompose plant organic carbon which is typically consists of celluloses and hemicelluloses. Phosphatase is another microbial extracellular enzyme responsible to turn the organic phosphate into inorganic substance which is required for metabolic processes in plant and for the soil microbial activities, as well as protease and urease due to nitrous substance available in soil. Break down of organic compound in the forest floor as caused by soil microbial activities mostly turn the substance to reducing sugar and some organic acid such as acetic acid. Continuously, during the uninterrupted bio-processing, acetoclastic bacteria would turn the acetic acid to improve into methane and carbon dioxide. Methane is the second largest emission contributor to global warming after CO_2.

In deep compartment of soil, some types of anaerobic microorganisms especially methanogen produce methane as caused by oxygen depletion which result in environment that has redox potential in minus 150 mV (Aijin and Mingxing, 1996). In the upper soil layer at which oxygen is not growth limiting factor, methane-oxidizing bacteria (methanotrophs or methanotrophic bacteria) turn to oxidize methane from that environment because their habitats occur mostly in soils, and are especially common near the environment where the methane is produced. Methanotrophs bacteria oxidize the methane through methane monooxygenase (MMO) enzymatic activity (Hanson and Hanson, 1996). Methanotrophs are a unique group of methylotrophy bacteria, have been isolated from a wide variety of terrestrial environments including soils (Whittenbury *et al*, 1970), peat bogs (Dedysh *et al*, 1998; McDonald *et al*, 1996; Ritchie *et al*, 1997), and plant rhizosphere (Gilber *et al*, 1998). Therefore, methanotrophs play a major role in the reduction of the release of methane from forest soil into atmosphere, and it is the only terrestrial sink to reduce methane in soil (Jaatinen *et al*., 2004).

The soils exhibited high uptake rates of atmospheric methane at all measurement times throughout the period measured in an acidic and a neutral forest soil (Kolb *et al.*, 2005). In the laboratory investigation, expression of methanotrophic bacteria fluctuated along with organic composting process, as showed by Halet *et al.* (2006) in-vessel reactor under controlled environment. Methane oxidation in forest soil has been intensively investigated (Roslev *et al*., 1997; Knif and Dunfield, 2005), but study of methanotrophic bacteria in the tropical forest soil is minute.

Abundance and diversity of soil microbe related to methanotrophic activity in the tropical forest relatively unclear, and subsequently investigations completed throughout this exertion. Soil samples gathered from three levels of elevation then measured at some stage in cellulase, amylase, invertase, urease, and phosphatase enzymatic activities. Afterward, those enzymes activities thus compared to methanotrophic bacterial population as well as their performance in reducing methane observed in

the laboratory. Analyses among soil enzymatic activities quantified to correlate with the role of methanotrophic bacteria in the soil.

MATERIALS AND METHODS

Soil sample and usage

Soil collected from forest floor of Gunung Salak National Park (West Java) at three different altitudes, located around S $06^0$46'24.3" - $06^0$46'49.8" and E $106^0$42'09.9" - $106^0$42'25.9", in June 2009. Five replicates of samples obtained by using auger in 0-10 cm deep of soil surface (upper soil) below the humus layer, to 15-20 cm deep (lower soil). First sample deprived at 1000 m altitude dominated by Fagaceae vegetation, and covered with shrub and fern (A-soil). Second sample gathered at 900 m altitude under Fagaceae vegetation, and covered with shrub and zingiberaceae plants (B-soil). Third sample acquired in the altitude of 800 m above sea level, at the tea plantation as riparian area (C-soil).

Soil pH was measured with a glass electrode using soil-water of 2 over 5 ratios. Total soil N was determined by Kjeldahl digestion (Bremner, 1965), and the organic-C was resolute by dichromate digestion. Metal concentrations were determined by using atomic absorption spectrometry after digestion (McGrath and Cunliffle, 1985). The clay contents of the soils were determined by pipette method (Tanner and Jackson, 1947). Soil chemical and physical properties had examined to illustrate in Table-1. Total microbial population in soil was determined by serial dilution technique in nutrient agar media. Population of phosphate solubilizing bacteria was determined in Pikovkaya media, and proteolytic microorganism was verified in skim milk medium.

Table-1. Main physicochemical properties of the soil.

Analysis of	**Soil sampling** (above sea level)		
	1000 m	900 m	800 m
C - organic (%)	3.46	4.06	4.04
N - total (%)	0.26	0.15	0.17
C/N ratio	13	25	25
P (ppm)	14.90	11.15	5.40
Exchangeable alkaline ($me.g^{-2}$)			
Ca	4.06	4.96	4.74
Mg	1.02	1.24	1.00
K	0.27	0.27	0.29
Na	0.21	0.17	0.14
Al	0.57	0.47	0.92
CEC ($me.g^{-2}$)	26.41	23.04	21.01
Texture (%)			
sand	11	24	27
silt	46	47	45
clay	43	29	28

Enzymatic activity

Urease activity examines in soil samples as follow; 2 ml of 0.1 M phosphate buffer (pH 7) and 0.5 ml 6.4 % urea added to 0.5 g of soil, which incubated at 30^OC for 90 minutes. The volume made up to 10 ml with distilled water. The ammonium released was measured using phenate methods (Nannipieri *et al*., 1980).

Cellulase activity measured in mix up 2 ml of 0.1 M phosphate buffer (pH 7) and 0.5 ml 1 % carboxymethyl cellulose and added to 0.5 g of soil sample, which was incubated at 30^OC for 90 minutes. The volume was made up to 10 ml with distilled water. Reducing sugar produced during the incubation determined by 3,5-dinitrosalycilic methods measured spectrometrically at 540 nm.

Amylase and invertase activities determined in 0.5 g of soil sample added with 2 ml of 0.1 M phosphate buffer (pH 7) and 0.5 ml 1 % of amylum (for amylase) or 0.5 ml 1 % of sucrose (for invertase), respectively; then it was incubated at 30^OC for 90 minutes. The volume was made up to 10 ml with distilled water. The reducing sugar produced during the incubation was determined by 3,5-dinitrosalycilic acid methods and measured spectrometrically at 540 nm.

Phosphatase activity analyzed in 0.5 g soil sample which is added with 2 ml of 0.1 M maleate buffer (pH 11) and 0.5 ml of 0.115 M p-nitrophenyl phosphate, and incubated at 37^OC for 90 minutes. The reaction was stopped by cooling to 2^OC for 15 minutes, then 0.5 ml of 0.5 M $CaCl_2$ and 2 ml of 0.5 M NaOH were added; and the mixture put in centrifuges at 4000 rpm for 5 minutes. The released p-nitrophenol was established spectrometrically at 398 nm (Tabatai and Bremner, 1969). Controls were made in the same means, but the substrate was added before the $CaCl_2$ and NaOH solution.

Substrate induced respiration

Biomass-C was also measured by substrate induced respiration. Two sub-samples of each moist soil, equivalent to 30 g oven-dry soil were weighed into 300-ml flasks, amended with a mixture of glucose and talcum powder in a proportion of 1:4 to give a concentration of 6 mg glucose per g soil and held at room temperature for 30 minutes. The flasks were then sealed and incubated for 2 hours at 25^OC. The flask head-space was sampled and analyzed for CO_2 by GC Shimadzu 14 B. Carbon dioxide was determined on FID (flame ion detector). Biomass-C was calculated and equal to 16.18Y+5.1; where Y is ml CO_2 evolved $g.soil.h^{-1}$ (Kalembasa and Dunfield, 1973).

Soil aerobic respiration

The sieved soils from each sampling transect were adjusted to 40% of water holding capacity (WHC). Sub-samples, each equivalent to 50 g oven-dry soil (105ºC, 24 h) were placed in 60-ml glass bottles which were then put into one liter jars and each jar containing 10

ml water, to avoid desiccation. A vial containing 20 ml 1 M NaOH placed inside the jar to trap the CO_2 produce. The jars were sealed and incubated at 25ºC in the dark for 10 days. The NaOH vials were replaced at intervals follow of observation. The trapped CO_2 was determined by auto-titration with standardized 1 M HCl.

Methane oxidation rates

Ten soil slurries were prepared in 100-ml bottles by mixing 20 g of air-dried forest soil, a representative with 20 ml of water. The bottles were closed with butyl stoppers and incubated in an initially 20 % O_2 and 80 % CH_4 at 25^OC, the concentration of CH_4 in the head space was adjusted to 100 mg/L. The samples were then incubated for 24 h, on rotary shaker at 125 rpm. Every 24 hours, methane in the headspace was replaced, and maintained for 1 week. Negative controls (without soil) were treated similar to soil samples. Head space pressure was maintained at 140 kpa. After 1 week, the methane concentration in head space was measured every 2 hours. Methane was analyzed using GC 14 B SHIMADZU with FID.

Methanotrophic population

After finishing methane absorption test, soil slurry of dilution series were prepared in triplicate down to 10^{-8}/g dry weight of soil with a step size of a factor of 10, and then each dilution series was placed on NMS-agar for plate count. Other slurries mixed with broth of NMS-medium to evaluate of population through most probable number (MPN), after incubated with 20 % O_2 and 80 % CH_4 at 25ºC. Culture without addition of methane also was used as negative control. Grown colonies after 3 days, and the change of culture turbidity (MPN) were recorded to determine the population of methanotrophic in soil sample.

RESULTS AND DISCUSSIONS

Soil of Gunung Salak National Park is very rich with functional microbial population as indicated by quite high population of proteolytic, cellulolytic, and phosphate solubilizing bacteria (Table-2). This is clearly indication that the soil microbes in tropical forest not only important for genetic resources but also play central role on mediating biotransformation of complex carbon sources of plant, animal, and soil microbes origin. There is a clear differentiation on the population of Ca-P solubilizer, proteolytic, amylolytic, and cellulolytic bacteria in soil gathered from the well conserved forest A-soil when it was compared to the converted forest into tea plantation ones (C-soil) as riparian area. When forest has been converted into tea estate, the soil have been intensively exploited and exposed to pesticide and herbicide. Those certainly have suppressed the soil indigenous microbes and thus also affect ecosystem function. In the forest buffer zone location, situated at 900 m asl at which some vegetation was cut and the original vegetation might be changed, also has an effect to the microbial communities. Respiration and soil biomass also less in this area, it might due to organic substances leaching owing to surface run off.

Table-2. Population of functional microbial group in soil collected from different altitude of Gunung Salak National Park.

Soil sampling of upper and lower layer (mean value of 3 replicates)	**Ca-P solubilizer microbes** (soil.g-^{1}) (x 10^7)	**Cellulolytic microbes** (soil.g-^{1}) (x 10^7)	**Amylolytic microbes** (soil.g-^{1}) (x 10^7)	**Proteolytic microbes** (soil.g-^{1}) (x 10^5)	**Total microbial population** (x 10^8)	**Soil respiration** (µg CO_2-C.g.soil^{-1}. hour^{-1})	**C-microbial biomass** (µg.g.soil^{-1})
A-soil of 1000 m asl (0-10 cm)	8.2±1.3	8.7 ±1.4	9.2±1.7	7.9±1.6	4.2±1.9	3.41	8.53
(15-20 cm)	4.2±1.3	7.2 ±1.4	7.8 ±1.5	6.8±1.3	2.5±1.5	3.10	7.75
B-soil of 900 m asl (0-10 cm)	6.8±1.2	8.0±1.3	7.6±1.6	7.1±1.8	2.9±1.6	2.61	6.73
(15-20 cm)	5.4±1.4	4.2 ±1.5	6.7±1.5	6.2±1.5	2.3±1.6	2.51	6.41
C-soil of 800 asl (0-10 cm)	0.6±0.1	0.5±0.1	4.6±1.5	0.4±0.2	2.1±1.5	2.20	5.50
(15-20 cm)	0.2±0.1	0.3 ±0.2	2.2±1.3	0.2±0.2	1.1±1.8	1.40	3.50

Enzymatic activities

Performance of soil enzymatic activities for all samples is expressed in Figure-1. Phosphate solubilizing bacteria that having phosphatase activities (data not shown), is very important mediator for biodegradation of organic phosphorous of plant and animal origin. Their populations were quite high in well conserved forest. Meaning that it has large amount demand of phosphate for plant growth, and also might indicate that forest ecosystem is stable. But when forest was converted into tea domain areas (C-soil), their population was clearly much less. Populations of phosphate solubilizing bacteria could be good indicator for ecosystem deterioration. Other group of microorganisms such as cellulolytic and amylolytic bacteria, which play key important role in biodegradation of less biodegradable substances such as cellulose and starch of plant were also less in converted areas (C-soil),

evenly proteolytic bacteria were significantly less in the riparian as tea plantation areas.

Microorganisms with their enzymatic activities involved in methane oxidation, and a better understanding of the environmental parameters that regulate this biological process. The mechanism by which soil enzymatic activities correlated to enhance methane oxidation efficiency has been investigated with particular emphasis on the bacterial methanotroph community. Methanotroph present in extreme environment such as in very low nutrition (Kussmaul *et al.*, 1998). However, fungi are being able to grow producing organic acids, like formate and acetate, which support growth of methanotrophic bacteria (Bock and Sand, 1993; Whittenbury *et al.*, 1970). Soil enzyme activity is variable in time and limited by available substrate supply (Degens, 1998; Tateno, 1988), and may provide useful linkage between microbial community composition and carbon processing (Waldrop *et al*., 2000). Enzymatic activities as caused by soil microbial activities were sensitive indicators to detect changes occurring in soils (Gonzalez *et al*., 2007). Forest soil is clearly defined to have ability for reducing methane emission. Methane is clearly produced in forest ecosystem (Roslev *et al*., 1997). The mechanism by which methane produced and generated were not fully understood, this work is trying to see that soil enzymes activities would be end up with the precursor of acetic acids formation. It is well known that acetic acid is the gate through which methane is synthesized by acetotrophic bacteria.

Cellulose material of plant and animal origin are hydrolyzed by cellulose produce reducing sugar. The activity this enzyme fluctuated in soil surface and bellow layer, implying that organic substrate and other element was evenly distributed soil. In general, surface soil has greater cellulase activities then bellow layer. Well conserved forest clearly has higher cellulose activities then converted forest. The organic material in preserved forest were plant and animal origin were much higher than in converted forest and this might be the main reason for higher cellulase activities in conserved forest. Activities of cellulose complex produce reducing sugar, which is easily, be converted into other substances. Aerobic oxidations of reducing sugar produce organic acids such as acetic acid. The last is good carbon sources for methanogenic organism. Wang and Bettany (1997) reported methane emission from forest soil was about 13-184 mg kg^{-}.soil-^{1} after 8 weeks.That value is quite reasonable since cellulose activities in forest soil is about 600 µg-glucose.g-soil. h^{-1}. The estimation of potential methane emission from one-meter-square should take into account the forest soil density, which soil organic material content dependent. The potential methane emission from soil in Gunung Salak area was quite high, owing to soil total organic content were about 3-4 % (Table-1).

Amylase is complex enzymes that hydrolyze starch to reducing sugar. Similarly to amylolytic activities, the activities of this enzyme were quite high in well conserved forest, but slightly decreased in the below forest, and much lower in degraded land. Activities of both cellulase and amylase will end up with reducing sugar. The later is important carbon sources for oxidative fermentative organism. Oxidative fermentative organisms can syntheses organic acid such as acetic acid for methanogenesis. Proteinase include urease are responsible for hydrolyzing N-containing substances, which will produce ammonium. The last substances are nutrient for plant, and soil microbes' growth as well as to become sources for methanogen and methanotrophic bacteria. Urease activities also show similar phenomenon as other enzymes, which also indicates the important of preserved ecosystem in maintaining ecosystem health.

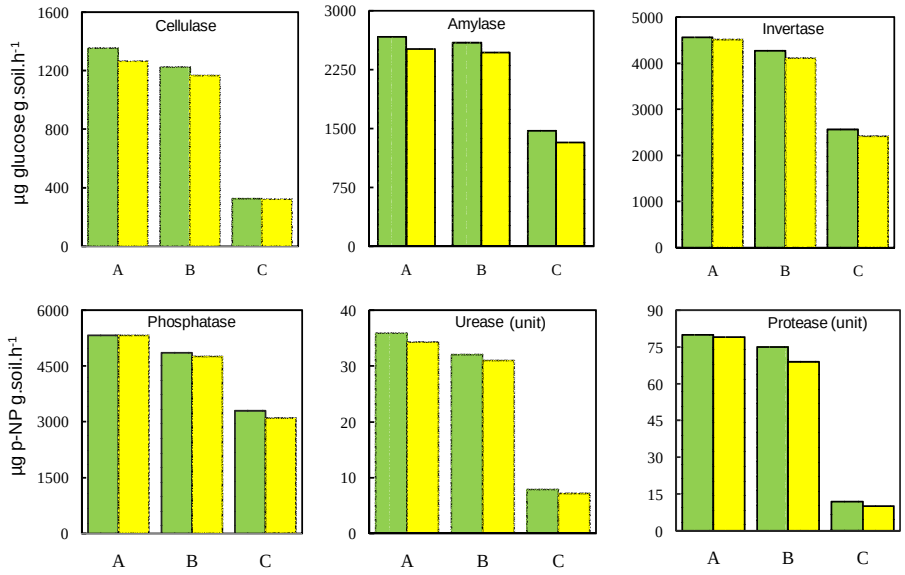

Figure-1. Soil microbial enzymatic activities in soil forest collected from the altitudes of 1000 (A) and 900 m asl (B), evaluated to riparian soil (C) of tea plantation area at 800 m asl. Green bars signify the activities of top layer soil (0-10 cm), and the yellow bars indicate lower layer ones (15-20 cm).

Methanotrophic Communities

As already reported elsewhere (Odegaard *et al.*, 1984), due to soil enzymes activities represented by cellulose, amylase, invertase, and urease hydrolyze organic materials into soluble substances. Those soluble substances will be finally undergoes methanogenesis that produce methane. The net methane emission into atmosphere is determined by methane production and consumption rate. Methane is consumed by quite heterogeneous methanotrophic groups including the type I, type II, and Type X. The different of that group are in the specific enzyme used for methane biotransformation. Type I has particulate methane monooxygenase enzymes, Type II has soluble methane monooxygenase and type X has both soluble and particulate methane monooxygenase. The fact that population of methanotrophic bacteria was higher in preserved forest than that of tea plantation areas (Figure-2), then over all microbial community structures, and its role in governing methane emission budget from forest ecosystems are proposed:

a) Microbial community structures which performs organic substrate hydrolyses through enzymes activities cover amylase, cellulase, invertase, phosphatase and urease are higher in preserved forest. The activities of those enzymes provide good substrate for methanogenic communities.
b) Hydrolyses product of those enzymes may penetrate or translocated into deeper soil horizon. At which oxygen is growth limiting factor for aerobic organism. At certain level redox potential value may reach -150 mV, under which methanogenesis could occur simultaneously with other microbial processes.
c) Methanogenesis generate methane, which is known as methane emission potential from soil. This means, when organic substrate concentration is high reflecting high methane emission potential.
d) The presence of methanotrophic bacteria able to consume more than 70% of the methane potential emission. Then net methane emission is account for about zero of potential methane emission. This is shown by population of methanotrophic bacteria is higher in preserved forest than that of converted forest. Ability of methane absorption by methanotrophic bacteria also higher as do to A-soil than that of C-soil.
e) Community of methanotrophic bacteria exist at various soil elevations. Methanotrophic bacteria clearly exist and ecologically important to reduce methane gas emission.
f) Soil microbial enzymatic activity has verified as part of role of methanotrophic bacteria in soil of tropical forest floor of Gunung Salak National Park. Assessment on more variety of elevation is required to make clear variation potential activities.

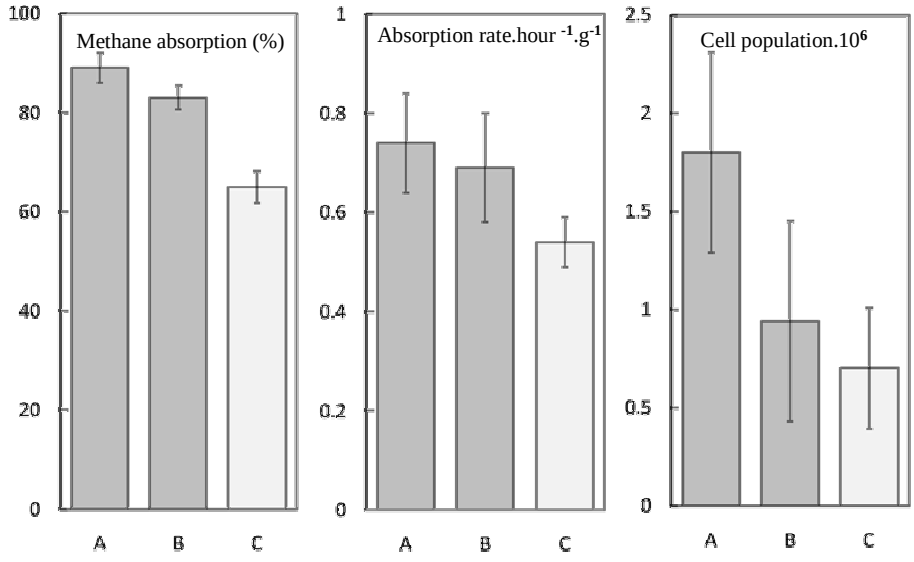

Figure-2. Functioning of methanotrophic bacteria in soil forest collected from the altitudes of 1000 (A) and 900 m asl (B), evaluate to riparian soil (C) of tea plantation area at 800 m asl.

CONCLUSSIONS

Understanding microbial community structure and its physiological behavior in forest soil is important for verifying the significant contribution of soil microorganism in maintaining global ecosystem health especially on the performance of methane evolution in forest ecosystem.

ACKNOWLEDGEMENTS

The authors are thankful to the Directorate General for Higher Education, Ministry of National Education, Republic of Indonesia, for the financial assistance provided for this study in the year 2009.

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