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To cite this article: A Kanti and I M Sudiana 2019 *IOP Conf. Ser.: Earth Environ. Sci.* **308** 012073

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IOP Conf. Series: Earth and Environmental Science **308** (2019) 012073 doi:10.1088/1755-1315/308/1/012073

Fungal diversity and its functions in tropical peatlands as plant growth promoting microorganism or associated with green house emission

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Abstract. Peat land deterioration is global issue. Exploitation of peatland ecosystem, especially extreme drainage has caused severe environmental problem. Among other destruction of fresh water stock, emission of greenhouse gas in the form of carbon dioxide, methane, and nitrous oxide. The objective study was to assess the influence of land use type on fungal diversity and its activity which indirectly contribute plant growth promotion or associated with greenhouse emission from peatland in Central Kalimantan. Four land use types: Nearly-pristine peat swamp forest (n-PSF), Un-drained deforested peatlands (UDP), Drained deforested peatlands (DDP), and Degraded peatlands under agriculture (DPA) were evaluated representing the disturb and less disturb peatland ecosystem. The hydrolytic enzymes assessment, particularly those linking to polymeric substances mineralization, was conducted to estimate microbial activities which contribute to greenhouse gas emission. The result indicates that fungal diversity and its activities of less disturb peatland was quite distinctive from exploited areas, which implies that higher greenhouse gas emission in exploited areas. It is recommended that exploitation of peatland ecosystem should consider water regimes through managing oxic and anaerobic environment to limit greenhouse gas emission. The presence of IAA, phosphatase producing fungi could be important for genetic resources for the growth promotion in peatland ecosystem.

1. Introduction

The important of peatland as Carbon stock is well documented [1] . Although the tropical peatland only covering about 0.25 % of terrestrial land surface, and estimated containing about 88.599 Gt representing about 3 % of terrestrial carbon stock [2], but much concerns on its future owing to most of tropical peat land is under rapid change [3]. The role of peatland as C-stock is sustained due to acidic, anaerobic environment and poor nutrient status result in slow bioconversion of organic carbon. Conversion of peatland into agricultural land is initiated with construction of large drainage which ultimately changes the physico-chemical and biological properties of peat soil [4]. The most critical phenomenon is anaerobic surface peat becomes aerobic, resulting in an increase of redox potential from reducing to oxidizing state leading to enhancing aerobic decomposition and stimulating oxygenated-respiration [5].

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The fungi are an immensely diverse group of organisms, encompassing a huge range of forms from microscopic single celled yeasts to large macrofungi [6]. Of which Ascomycetes fungi is important soil fungi which greatly contribute to biodegradation of organic material (wood), and partly also on soil enzymes in peat land [7]. Fungal diversity in the soil remains unclear. A figure of 1.5 million fungal species has been widely quoted as an indication of the true number of fungi that exist [8]. Soil enzymes play significant role in governing microbial mediated processes in peatland ecosystem [9]. Aerobic microorganism releases extracellular and cell membrane bound hydrolytic enzymes among others; those involved in carbon metabolism: cellulase, xylanase, invertase, β-glucosidase, chitinase, laccase, lipase [10]. The one acting on phosphorus metabolism: phosphomonoesterase, phosphodiesterase, phosphotriesterase, phospholipase [11]. The one is responsible for sulfur metabolism: arylsulfatase, dimethyl sulfoxide reduction. Dynamic profile of these enzymes include synthesis, releases, acting on specific substrate are playing an important role in the transformation of polymeric and recalcitrant substances (humic acids), and producing soluble and less toxic substances [12]. Hence peat are rapidly oxidized generating a large amount of dissolved organic carbon and CO_2 [13]. Those substances are easily taken up by peat microbes for intracellular metabolism. Not only creating aerobic environment, peatland drainage also exposing the upper horizon of peat with higher temperature, and may lead to more rapid hydrolyses rate [14].Peatland ecosystem contains quite complex microorganisms owing to availability of a wide range of electron donors and acceptors [15]. When high water table is maintained then NO_3 and NO_2 could be important electron donor for heterothrophic denitrifying microorganism (Keller & Bridgham, 2007). But when those potential electron acceptors are absent then the sulfate reducing microbes will prevail provided by the presence of sulfur oxidizing microbes active in upper layer of oxic environment [16]. The activity of microbes under anoxic and anaerobic condition is primary influenced by the aerobic microorganisms. The interlink metabolism in peatland ecosystem affect the level of greenhouse gas emission in peat land ecosystem [17]. Fungi, and bacteria are prominent group of microbes in the peat land ecosystem [18]. Population of those microbes and soil enzymes activities could be important criterion to assess the contribution of land use change on green gas emission. The objective of this study was to evaluate fungal diversity of several land use type in Central Kalimantan. The activities of hydrolytic enzymes, especially those linking to C-metabolism, were used to assess land use change affects microbial activities.

2. Materials and methods

2.1. Site description and sampling

Our study site was located in Central Kalimantan, Indonesia with geographic location at $2^0$16’74”S and $114^0$2’23”E, and altitude of 53 m above sea level. The samples were taken using an auger that was pushed till 30-40 cm depth at four land-use types, namely, a) nearly-pristine peat swamp forest (n-PSF), b) un-drained deforested peatlands (UDP), c) drained deforested peatlands (DDP) and d) degraded peatlands under agriculture (DPA). *Palaquiumleiocarpus Boerl* and *Syzygiumcreaghii* (Ridl.) Merr.& Perry were dominant vegetation at the n-PSF). According to the Von Post Scale for classification of peat surface soils (0-5 cm) in DPA were designated almost completely decomposed with indistinct plant structure, and soils from the n-PSF, UDP and DDP were designated weakly decomposed with distinct plant structure. Similar auger samples were collected from three locations within the same land-use pattern. Samples were collected from 15 and 30 cm from the top and bottom of the auger, respectively, representing surface and sub-surface samples of the peat. Samples were stored in 4°C until they were transferred to laboratory in Research Center for Biology, LIPI.

2.2. Isolation of fungi

In order to understand the fungal diversity from different land-use patterns in peatlands that can be cultured in lab conditions, isolation was done following the methods previously described [19]. Fungi were aseptically isolated from soil using potato dextrose agar. Plates were incubated at ambient temperature (30°C) for two to five days. One colony of particular morphology on each plate was selected, assigned a unique identification number, and purified by streaking for single colonies at least twice.

2.3. Identification of fungi

2.3.1 DNA extraction

DNA extraction was done by using Microbial Colony PCR amplification methods of 26S rDNA for fungi [20]. The cultured were cultivated in R2A, potato dextrose agar, HV for bacteria, fungi and actinomycetes respectively for 24 hours or more. Pipet 50 ul of MilliQ-purified, autoclave water into PCR tube. Using a sterile pipet tip or inoculating needle, place about ¼ of a colony into PCR tube containing water. Place in the thermocycler and run in 94°C for 10 minutes. This lyses enough of the cells to release the DNA. Spin down the tubes or plate to bring the liquid down to the bottom of the well.

2.3.2. *rDNA sequence determination*

Five uL of suspension was used for PCR amplification with primers ITS1 and ITS4, using GoTaq master mix (Promega, M7122) rDNA sequence were amplified using ITS1 (5′TCC GTA GGT GAA CCT GCG G 3′), and ITS4 (5′TCC TCC GCT TAT TGA TAT GC 3), primers [20] and amplified in a TAKARA thermocycler with the following PCR program: 95°C for 2 min; 36 cycles consisting of 1 min at 94°C; 1 min at 52°C and 2 min at 72°C, with a final extention of 8 min at 72°C. PCR products were visualized on 2% agarose (1st BASE, BIO-1000) stained with ethidium bromide (Amersham Biosciences, 17-1328-01).Sequencing of 16s rDNA and D1/D2 of LSU rDNA were determined with Big Dye terminator v3.1. Cycle Sequencing Ready Reaction Kit (Applied Biosystems) following the manufacturer's instructions. The LSU gene sequences determined in this study were manually aligned with published sequences of reference strain available from the EMBL/GenBank/DDBJ databases [21].

2.4. Validation of isolated fungi producing Indole acetic acid (IAA) and phosphate dissolution

2.4.1. Determination of IAA production by isolated fungi

Isolated fungi were grown overnight in 5 ml MD which contain (g/L) peptone 5.0, yeast extract 2.5, glucose 10, KH_2PO_4 0.1, $MgCl_2$ 0.1, NH_4SO_4 0.1.20µl aliquots were transferred into 5 ml of fresh MD media supplemented with 500 µg/ml of L-tryptophan. After incubation for 42 h, the density of each culture was measured spectro-photometrically at 600 nm. The solution was centrifuged at 5500X g for 10 min. A 1-ml aliquot of thesupernatant was mixed vigorously with 4 ml of Salkowski's reagent [22], and allowed to stand at room temperature for 20 min before the absorbance taken at 535 nm. The concentration of IAA in each culture medium wascalculated against a standard curve.The IAA produced by each strain was measured in triplicate.

2.4.2. Phosphate solubilizing fungi

To study the physiological properties of Fungi Solubilizing bacteria (FSB). The isolate was picked up from the plate after enumeration of total fungi in PDA. Each morphologically distinct colony was taken and transferred into Pikovskaya medium, and incubated at 30°C for 4 days. FSB colonies were indicated by the formation clear zone surrounding the growing colonies. To study the amount of phosphate released by PSB, then the isolate which has the highest the ration clear zone formation per colony ratio was selected. They were grown in Pikovskaya media, and amount of orthophosphate released was measured

using ascorbic acid methods [23]. The orthosphosphosphate released was then plotted against the number of sell in the growing cultures.

2.5. Soil enzyme activities

The activities of 4 hydrolases (cellulase, amylase, phosphatase), and FDA (fluorescein diacetate) representing (protease, esterase, and lipase) were measured. The FDA was measured following [24]. One gram peat soil sample added to 9 ml sterilized aquadest in 50-ml test tube, then homogenized with vortex. 200 µl of each suspension were transferred into new tube, added with1500 µl of phosphate buffer pH 7.6 and mixed with vortex. All ofthe mixtureaddedwith60 µl ofFDAsolution, incubated and shaken at 150 rpm for 3 hours at 37°C. Afterward, 500 µl chloroform-methanol (2:1) was added to stop the reaction, and finally the tubes were centrifuged at 5000 rpm for5minutes. Positive results were noticeable by the formation of yellow-green fluorescent; finally, supernatant were measured by spectrophotometer (490nm).

For hydrolytic enzyme activities, two grams of peat soil was suspended in 10 ml of acetate butter (50 mM, pH 5.0), and shaken for 15 minutes at 125 rpm, and afterward centrifuged for 20 minutes at 3000 g for 20 minutes at 4ºCTo determine cellulose activities, 1 ml of soil suspension was added with 200 µL 0.70% (w/v) carboxymethyl cellulose sodium salt low viscosity, (SIGMA**),** and incubated at 50ºC for 24 hours. After incubation 500 µL of DNS (dinitrosalicylic solutions, SIGMA-Aldrich) were added, and the enzymes reaction finished by dipping the tube into boiling water for 7 minutes, and stabilize to reach room temperature, and measured at the at 540 nm, against the reagent. For xylanase similar protocol was done except the substrate was 1.2 % w/v xylan from oat (SIGMA Aldrich). The enzyme activity is expressed as µMoll glucose equivalent released per 24 hours per g. soil. Phosphatase was determined according to [25] using *p*-nitrophenylphosphate (*p*-Nitrophenyl Phosphate Disodium Salt, Hexahydrate, Fisher BioReagents) as substrate incubated at 37ºC for 1 hour, and the enzyme activity is expressed as µMol nitrophenol released per g soil per hour.

3. Result and discussion

3.1. Total population of Fungi

Fungi is important microorganisms to reduce erosion by helping bind the particles of soil together. When the filaments of fungi become wet, they absorb water and swell up to ten times their original size which helps to store moisture within the upper layer of soil where many plants root systems. The population of fungi were varied (Figure 1), the highest was in Degraded peat lands under agriculture (DPA).

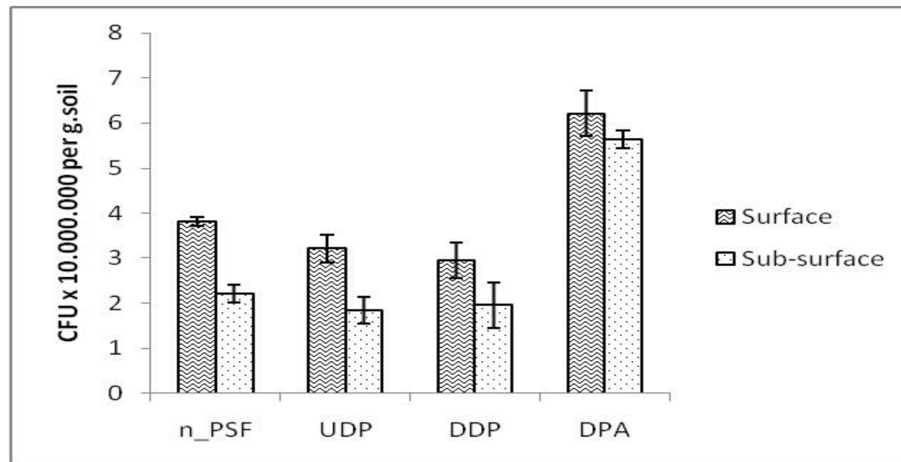


Figure 1. Profile of fungi population of peatland soil.

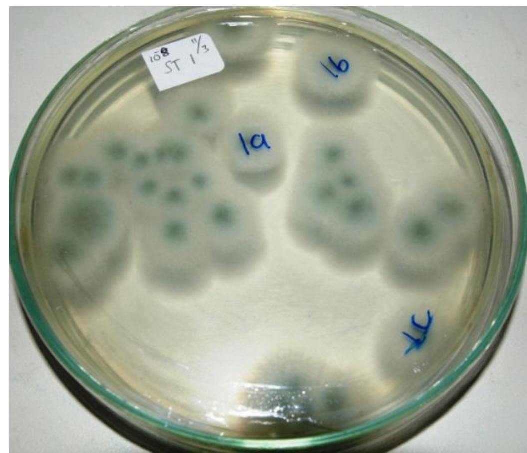
Table 1. Population of fungi in peat soil.

Land use pattern	Genera	Species	CMC-ase	C1 Avicelase	Xylanase	IAA production	Phosphatase
n-PSF	*Aspergillus,*	*A. flavus*	+	+	+	-	-
		A.niger	+	+	+	+	+
	Trichoderma	*T.harzianum*	+	+	+	-	+
		T. viride	+	+	+	+	-
	Penicellium	*P. hirsutum*	+	+	+	-	-
		P. lanosoum					
		P. roseopurpureum					
	Pythium	*P. graminicola*	+	+	+	-	+
		P. spinosum					
	Cladosporium	*C.cladosporioides*					
	Fusarium	*Fusarium oxysporum*	+	+	-	+	-
	Acremonium		+	+	-	-	-
	Paecilomyces		+	+	+	-	-
	Verticillium		-	-	+	+	-
	Chaetumium	*Chaetumium globosum*	+	+	-	+	+
	Verticellum		+	+	-	+	+

Contribution of fungi on biodegradation of plant material in peatland is well documented [18]. We observed 12 genera *Aspergillus, Trichoderma, Penicellium, Pythium, Cladosporium, Fusarium,*

Acremonium, Paecilomyces, Verticillium, Chaetumium, and *Verticellum* dominant genera isolated from peat soil (Figure 2, Table 1), and other unidentified Basidiomycetes fungi which could be belonging to Ectomychorhizal group [26]. Most of the isolated fungi were cellulolytic (Table 1). We observed morphologically divers fungi in the study site. Fungi rapidly oxidized polymeric substances of plant materials into complex of humic acids producing brown black color of peat soil (Figure 3). Macroscopically, surface horizon of peat containing greatest fraction of oxidized material having black color with less of wood fragments and therefore could be classed as a sapric or sapric-hemic peat. We also found some fungi were able to hydrolyze cellulose (Figure 4).

The population of fungi was affected by water table regimes. Highest population was observed in DPA (both in surface and subsurface soil horizon), while the population of aerobic fungi was almost similar in water submerged peat soil. Implying that aeration of peat soil stimulates the growth of aerobic fungus, under aerobic condition fungus- mycelia is able penetrate deeply (about 1-1.5 m) into peat soil in Palangkaraya. Here we observed quite morphologically diverse of fungi grow rapidly on surface soil horizon. We also observed the isolated fungi having cellulolytic capacity, verifying that they are important contributor for rapid degradation of plant material.

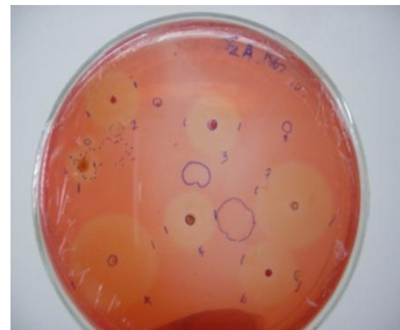


(A) (B)

Figure 2. Photograph representative of morphologically diverse fungi isolated from study site (A), some plates also observed to be dominated by *Aspergillus* (B).

Figure 3. Photograph of oxidized surface horizon of DPA showing the black color due to oxidation of decomposed plant materials.

(A)



(B)

Figure 4. The cellulolytic test to evaluate the ability of isolate utilizing cellulose.

3.2. The profile of cellulase

Cellulase is a group of hydrolytic enzymes that catalyze the breakdown of glycosidic bonds in cellulose. Complete degradation of cellulose requires at least three enzymes: endo-β-1,4-glucanase, exo-β-1,4-glucanase, and β-glucosidase. Endoglucanase disrupts crystalline structure of cellulose via random cleavage of internal bonds; exoglucanase removes oligosaccharides, such as cellobiose and tetrasaccharides from the non-reducing ends of carbohydrate chains generated by endoglucanase; and glucosidase releases glucose from oligosaccharides. Several analytical methods have been developed for estimating the potential activity of soil endoglucanase, exoglucanase, glucosidase or their combination. We used CMC-ase as substrate for endo-β-1,4-glucanase to assess the contribution of this enzyme on polymeric substances hydrolyses. This enzymes was more active at the surface layer of peatsoil (Figure 5).

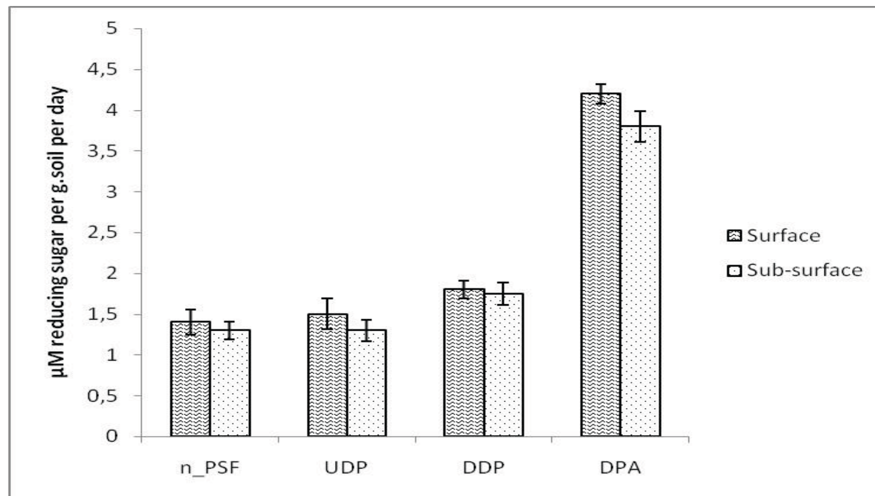


Figure 5. CMC-ase profile of several land use type in surface soil horizon.

3.3. Profile of xylanase

Since peat soil composed of complex of organic substances mainly originated plant material we determined xylanase activity to estimate the contribution of this enzyme of carbon emission from peatland ecosystem. We observed the activity of xylanase was quite variable (Figure 6).

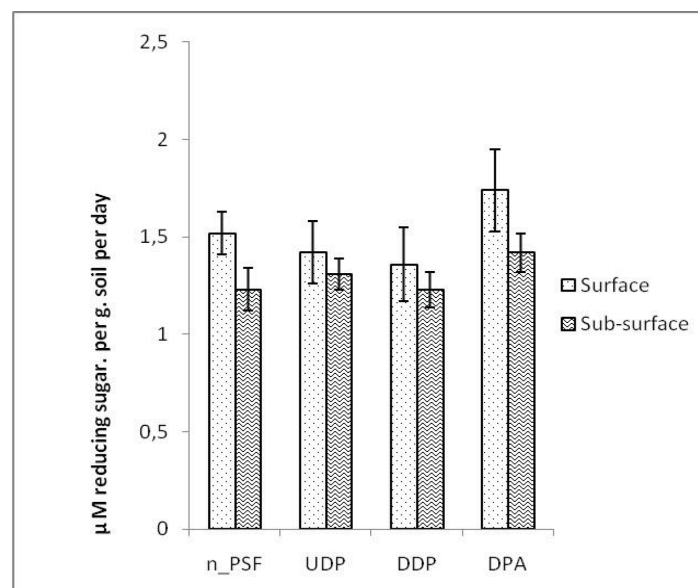


Figure 6. Xylanase activity in sub- surface soil horizon of several land use type.

Soil organic matter is largely heterogeneous in terms of carbon and nutrient chemistry. Provided that substrate positive induction is an important mechanism for soil enzyme production, organic matter

chemistry may be manifested in the relative abundance of various carbon and nutrient mineralization enzymes. There are some endeavors for investigating the relationship between organic matter chemistry and the activity of soil enzymes. For example, positive correlations between peptidase and protein and between β-1,4-N-acetyl glucosaminidase and polysaccharides were found in both light and heavy fraction of soil organic carbon. In a long-term rye monoculture system, xylanase activity was correlated with the relative abundance of xylan and xylose and examined the relationship of soil enzyme activities with the chemical composition of soil organic matter [27]. They found that integrated soil enzyme activity was unrelated to chemical compositions of organic matter characterized by Fourier transform infrared spectroscopy. No correlation between enzymes and organic matter is perhaps because soil organic matter reflects longterm decomposition processes whereas enzyme activity fluctuates with current conditions [28].

3.4. Profile of phosphatase
We used activity of phosphomonoesterase to asses the contribution of this enzyme on mineralization of organic phosphate containing substances originated from plant material. We observed that the activity of this enzyme was higher in surface horizon of peat soil, particularly in DPA land use type (Figure 7). Implying that the demand of phosphorous for microbial metabolism is higher in DPA land use type.

Phosphatase characterizes a broad group of enzymes that hydrolyze phosphate ester and subsequently release phosphate. In general, phosphatase has low substrate specificity and thus can act upon a number of different structurally-related substrates. Extensively studied soil phosphatase includes acid phosphatase and alkaline phosphatase that are mainly found in acid and alkaline soils, respectively [29].

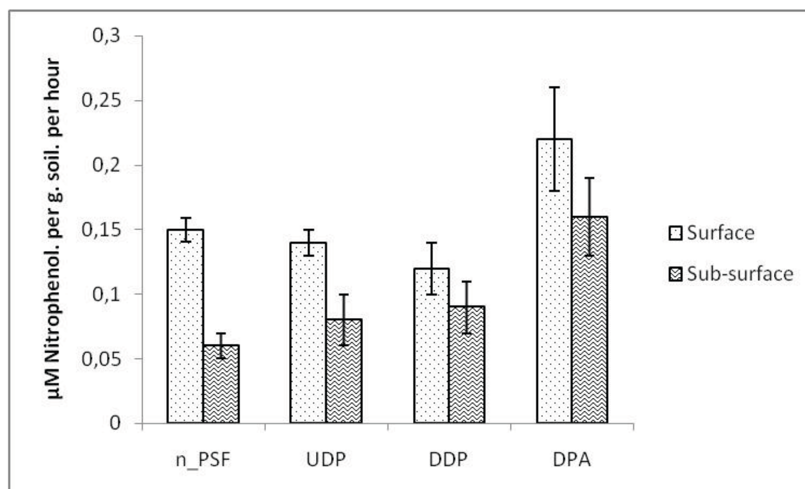


Figure 7. Profile of PME-ase (phosphomonoesterase) activity in sub-surface horizon of peat soil.

3.5. Profile of FDA
FDA analyses are used widely to estimate total soil microbial activities since it is sensitive and rapid. The analyses cover the ubiquitous lipase, protease, and esterase enzymes involved in the hydrolysis of FDA. The FDA activities was varied, the highest FDA activities was in DPA (Figure 8). FDA hydrolysis was correlated with soil respiration augmented with pure culture (data not shown).

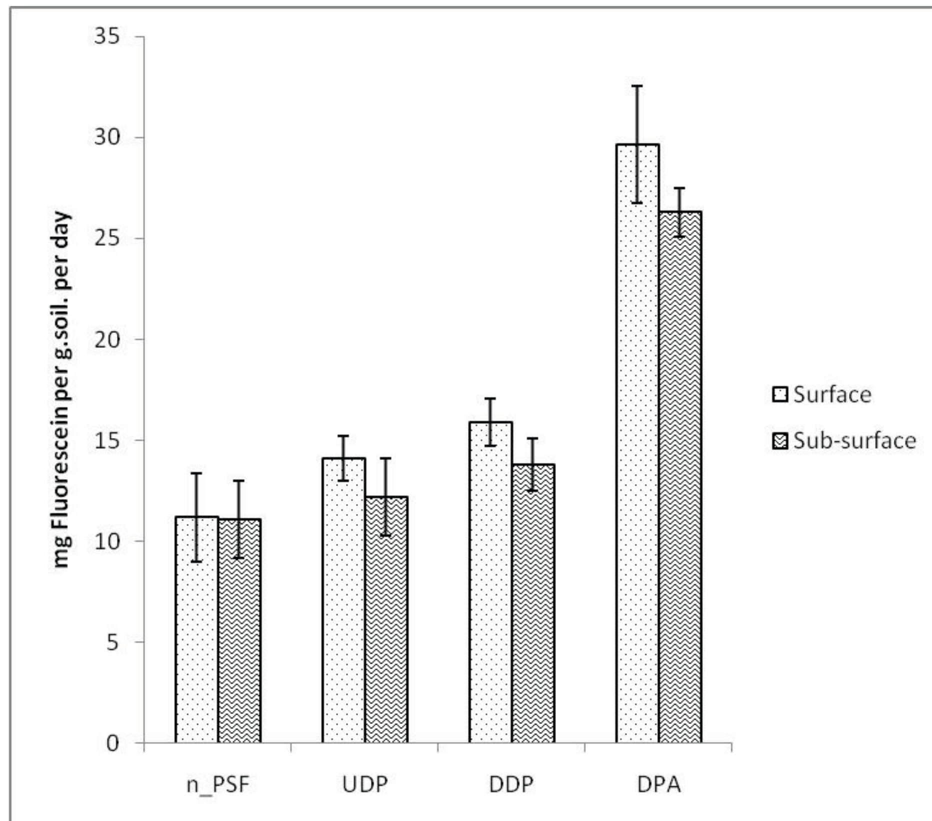


Figure 8. Profile of FDA in sub-surface horizon of peat soil.

Soil enzymes activities in peat soil affected by many factors. For instance the end product of soil organic hydrolyses suppress over all enzymes hydrolytic activities, nutrient-mineralization enzyme is inversely related to the nutrient availability. [30] demonstrated that negative feedback was indeed the cause for the reduction of phosphatase and chitinase activities after long-term respective phosphorus and nitrogen fertilization. They argued that there were no direct and adverse effects of fertilization on enzyme activity because phosphatase and chitinase did not respond to short-term fertilization. Inverse relationship between phosphorus availability and phosphatase activity seems to be more common than the one between nitrogen availability and nitrogen mineralization enzymes.

This is because organic phosphorus largely exists in the form of phosphate esters and is mineralized mainly through phosphatase catalysis. In contrast, organic nitrogen appears in various forms and can also be tightly associated with organic carbon. Not only do diverse nitrogen-mineralization enzymes catalyze mineral nitrogen release from complex organics, but also mineral nitrogen, can be produced as the byproduct of organic carbon decomposition. Therefore, a particular nitrogen-mineralization enzyme activity may not always have a negative relation with the available nitrogen [31]. Above and below a threshold of nutrient availability, negative feedback may be irrelevant to microbial enzyme production. In the case of high nutrient availability, nutrient-mineralization enzyme activity can be low and thus fertilization will not generate additional suppressive effects [30].

When nutrient availability is low, the nutrient may greatly limit microbial growth and metabolisms. Thus, fertilization may stimulate microbial biomass and in turn microbial enzyme production. [32]

examined N-acetyl-glucosaminidase and protease activities in response to nitrogen fertilization rates. Soil microbial biomass increased with fertilization rates, and enzyme activities were, therefore, greater in fertilized soils than in the unfertilized controls. Substrate induction on enzymes has been attested through substrate addition studies. For example, chitinase activity can be induced by chitin, phosphatase by organic phosphorus, β-glucosidase by cellobiose, and cellulase by cellulose [33].

However, positive induction may not always take place. [31] reported that cellulose addition had no any effect on β-glucosidase activity and also that collagen and cellulose phosphate addition did not increase the activities of respective glycine aminopeptidase and acid phosphatase. Lack of substrate induction on enzymes is thought to occur under some conditions, such as lack of a small amount of available nutrient that is required to activate inducible enzyme synthesis or enzyme.

Peatlands hold a vast amount of partially decayed vegetation matter due perhaps to hydrolytic and oxidative enzymes mostly produced by soil microorganism [34]. The total fungi population was higher in the surface soil horizon, and enzymes activities especially cellulase, xylanase, protease, esterase, and lipase activities were also higher in the surface horizon reflecting decomposition was higher when peat soil was aerated. Limiting oxidation of C-pools through maintaining redox state in the peatland ecosystem could be one strategy for CO_2 emission management. This finding is in agreement with previous studies [1]. But the decomposition rate could be slower when all easily degraded material used up by microorganism, and reserving the recalcitrant material in the upper layer with lower nutrient content [1].

Oxygen that is required for microbial activities is often absent or limited in peat lands, thereby the decomposition of organic material due to activity soil enzymes produced by microorganism is inhibited. Lowering water table in peatlands has been found to significantly increase the activities of hydrolytic enzymes such as β-glucosidase, phosphatase, and sulphatase. From the CO_2 emission perspective, maintaining the water level at upper horizon could be beneficial to limit the CO_2-emission. Exploiting peat land could be better if the microbial indicator could be included for the assessment. Approximately, 20% of terrestrial dissolved organic carbon entering the ocean comes from peatlands. The soil enzymes produced by microorganism contribute to the release of carbon.

Soil enzymes have been acknowledged as important soil property to capture soil carbon and nutrient dynamics. Of numerous hydrolytic and oxidative soil enzymes, phenol oxidase and cellulase appears to be the proximate control dictating the decomposition of soil organic matter. Environment that is conducive to the activity of soil phenol oxidase has been found to favor decomposition of soil organic matter and plant litters and, therefore, likely to impede soil carbon sequestration.

There are a number of robust cases that soil enzyme activities can be predicted from microbial strategies for extracellular enzyme production and that changes in soil enzyme activities are able to manifest the direction and magnitude of organic carbon decomposition and nutrient mineralization. In forest ecosystems, for example, increase in cellulase activity and decrease in phenol oxidase activity under projected atmospheric nitrogen deposition seem to be in agreement with predictions based on microbial resource allocation and microbial nitrogen mining. Modifications in lignocellulolytic enzyme activities are, therefore, accountable for changes in decomposition rates and quantity of soil carbon retention. Mechanisms that underline microbial enzyme production and the predictability of soil enzymes for soil carbon and nutrient dynamics have great implications in terms of global environmental change as well as management practices. To restore soil organic matter and optimize nutrient cycling in agricultural systems, we need to develop management practice.

4. Conclusion

Peat land ecosystem is composed of high fungal diversity, which has significant contribution on degradation of plant materials leading to emission of greenhouse gas. Fungal population in less disturb peat land was quite distinctive from exploited areas, which implies that higher greenhouse gas emission in exploited areas. Exploitation of peat land ecosystem should consider environmental factor that affect organic material degradation for instance water regimes through managing oxic and anaerobic environment to manage greenhouse gas emission. The presence of IAA, phosphatase producing fungi could be important for genetic resources for the growth promotion in peat land ecosystem.

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Acknowledgement
This research was supported the Indonesian Government Research Project through Thematic Research Grant Project 2015-2019. We would like to express sincere gratitude to all students who assist laboratory works.