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Comparison of various culture media effectiveness in the isolation of bacteria from Pekan peat swamp forest soil

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ABSTRACT

Aims: Previously described as non-favorable-microbial habitat, peat swamp forest has its own features, which are extremely acidic, poor in nutrient, water-logged and anoxic environment where rate of decomposition of plant litters is quiet slow. Interestingly, current research has proven that there is diversity of microbial communities in this ecosystem. The main objective of this study is to isolate bacteria from Pekan peat swamp forest soil that play a role in the decomposition of plant litters through cultivation on different agar-based medium. The success of isolation of bacteria from this neglected habitat could open the opportunity in unleashing the specific role of bacteria in peat swamp plant litter degradation as well as potential biotechnological application of these bacteria in lignocellulose-related industry.
Methodology and results: To mimic the peat condition that is low in nutrient and comprised of plant debris, M1 and peat agar supplemented with cellulose, glucose, lignin and xylan were used. Specifically, for the isolation of actinomycetes, dry and wet heat pre-treatments were applied to the soil samples. Then, the samples were cultivated on three different agars which were oatmeal agar as well as M1 and peat agar supplemented with glucose. Enrichment method was applied in the isolation of cellulase-producing bacteria. It was found that higher number of bacteria and actinomycetes were successfully isolated from peat agar, followed by oatmeal agar and M1. In fact, more actinomycetes were isolated from soil that was treated with wet heat pre-treatment compared to dry heat pre-treatment and on peat agar compared to M1 and oatmeal agar. This finding is promising, indicating that the application of peat water in the agar-based medium is useful to mimic the actual environment of peat swamp and increase the possibility to isolate indigenous bacteria. Primary screening of isolates from samples enriched with carboxymethyl cellulose (CMC) showed positive result of decolourisation zone on Azo-CM-Cellulose agar indicating the ability of isolates to degrade cellulose compound.
Conclusions, significance and impacts of study: The study indicates the effectiveness of different culture media in successful isolation of bacteria including actinomycetes. Using the enrichment method, bacteria that are able to degrade cellulose compound was successfully isolated even though it is well known that plant litter degradation in the peat swamp environment happens at very slow rates.

Keywords: peat swamp forest, soil bacteria, peat water, lignocellulose

INTRODUCTION

Peat swamp forests are formed from the accumulation of peat where the organic materials are decayed slowly and the environment is extremely acidic, water-logged, nutrient-poor and anaerobic (Yule and Gomez, 2009; Dedysh, 2011). The rate of organic matter decomposition is slower as compared to its rate of accumulation. In spite of being assumed as a neglected forest, peat swamp forest actually plays an important role to the carbon cycle of the environment and supplies a variety of goods and services like forestry and fisheries products, flood mitigation and groundwater recharge (UNDP, 2006). Although the appearances of peat swamp forest were dull and uninspiring when compared to other tropical forests, novel isolates and role of microorganisms in plant litter degradation and role of bacteria are yet to be discovered. According to Dedysh (2011), the existing knowledge about microorganisms liable for decomposition of plant residues in this ecosystem is very limited. A study done by Jackson *et al.* (2009) found that the peat swamp environment was dominated by *Acidobacteria*, while Dedysh (2011) and Kanokratana *et al.* (2011) recorded in their studies that both phyla *Acidobacteria* and *Proteobacteria* are the most abundant types of microbes found in this harsh environment. Subdivisions 1, 3, 4 and 8 are belonging to *Acidobacteria* where only subdivision 1 is well established with cultured and characterised strains. Whereas,

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Proteobacteria are most commonly belonging to the *Alpha*-class which have three different physiological groups like methanotrophs, chemoorganotrophs and phototrophs, or *Delta*-class. This finding was in line with recent studies done by Woo *et al.* (2014) and Mohamad Roslan *et al.* (2015) where they found that *Burkholderia* genus, under phylum of *Proteobacteria* was among the most abundant taxa in this forest's soil. Apart from these two phyla, there are also groups of microorganisms present in this environment such as *Verrucomicrobia*, *Actinobacteria*, *Planctomycetes*, *Bacteroidetes*, *Firmicutes*, *Chloroflexi*, *Spirochaetes* and one unclassified group of bacteria (Dedysh, 2011). Furthermore, Rice *et al.* (2006) reported the presence of ascomycetes and basidiomycetes (microfungi) in this environment which contributed in the decomposition of *Sphagnum* up to 50% and 35% respectively. The effort to isolate microorganisms from peat swamp forest by using conventional media such as nutrient agar is quite challenging (Dedysh *et al.*, 2006). This is due to the characteristics of the peat swamp itself which have been mentioned earlier. Due to this reason, there is limited knowledge about microorganisms inhabiting the peat swamp forest and their functional role for the degradation of plant litters (Dedysh, 2011). However, Dedysh (2011) has experimented a dilute (1:10-1:100) and acidic (pH 4.0-5.5) media and has successfully isolated novel acidophilic methane-oxidising bacteria from northern wetlands (Dedysh *et al.*, 1998). As reported by UNDP (2006), peat sediment from peat swamp forest is mostly soil with more than 65% of organic matters that is composed largely of vegetation residue. This residue comprises of cellulose, hemicellulose (xylan) and lignin that are strongly bonded (Pérez *et al.*, 2002) and usually known as lignocelluloses. Even though the rate of decomposition in this environment is slow, there should be microorganisms responsible in the breakdown of all these plant residues through secretion of lignocellulose enzymes. Microorganisms that were known with the ability to utilise lignocelluloses belonged to the dominant phyla which are *Proteobacteria*, *Firmicutes* and *Actinobacteria* (Woo *et al.*, 2014). *A*ctinomycetes which belong to the phylum *Actinobacteria* is another microbial group focused in this research. Actinomycetes (within the order of *Actinomycetales* and family *Actinomycetaceae*) are fungus-like bacteria and commonly found in soil and known with astounding abilities to degrade wide range of more complex polymers such as lignocellulose, protein and polysaccharides (McCarthy and Williams, 1992). Therefore, this study aims to develop a range of suitable culture media for cultivating bacteria from Pekan peat swamp forest's soil that is rich with plant debris.

MATERIALS AND METHODS

Sample collection and processing

Soil samples and peat water from five different locations were collected at Pekan Peat Swamp Forest. The GPS coordinates for these five locations are N03°29´49.5´´ E103°18´33.9´´ for S1, N03°29´32.0´´ E103°18´38.8´´ for S2, N03°29´17.9´´ E103°18´36.2´´ for S3, N03°29´06.7´´ E103°18´35.9´´ for S4 and N03°29´39.1´´ E103°18´36.4´´ for S5. The soil samples were taken from the surface layers (0-10 cm depth) and peat water was taken with water sampler and analysed by using Hydrolab Quanta Water Quality Monitoring System (Brand: Hydrolab, Model: Quanta). Moisture content (MC) and pH of these soil samples were immediately analysed in the lab. Peat water and the remaining soil samples were stored at 4 °C until further use.

Isolation of bacteria from peat soil samples

The isolation of bacteria was performed using two types of medium which were M1 minimal medium (M1) and peat agar. M1 contained (g/L): 0.25 g of potassium nitrate (KNO_3); 0.1 g of monopotassium phosphate (KH_2PO_4); 0.1 g magnesium sulphate ($MgSO_4$); 0.1 g yeast extract; 0.005 g of sodium molybdate (Na_2MoO_4); 0.02 g of calcium chloride ($CaCl_2{\cdot}2H_2O$) and 1.8% agar. Peat agar contained (g/L): 2 g peptone, 2 g yeast extract and 18 g agar in 1 L of peat water. The pH for both media was adjusted to 4.5 with alginic acid (Dedysh, 2011). For selection purposes, three different carbon sources namely glucose, lignin and xylan were incorporated into both M1 and peat agar at 0.1% (w/v). The soil samples were diluted 1:10 v/v with 0.85% sterile saline solution, serially diluted down to 10^{-5} and plated onto a set of selective isolation plates in duplicate. The inoculated plates were incubated for 24 h at 30 °C and monitored for bacterial growth.

Selective isolation of actinomycetes using dry and wet heat pre-treatments

Soil samples were air dried at room temperature and ground by using mortar and pestle. The samples were sieved using 125 µm siever at 60 A for 10 min to remove any large particles. Samples were stored at 4 °C before application of dry and wet heat pre-treatment. For both heat pre-treatment approximately 1 g of dried soil was incubated in water bath at 60 °C for 20 min. 9 mL of sterilised peat water was added into samples before incubation in water bath for wet heat pre-treatment. The pre-treated soil samples were diluted 1:10 v/v with sterile saline and serially diluted down to 10^{-5} and plated on M1, peat agar and oatmeal agar in duplicate. M1 and peat agar were prepared as described above and supplemented with glucose only while oatmeal agar preparation (in g/L) included 20 g oatmeal; 1.0 mL trace salts solution (0.1 g ferrous sulphate heptahydrate ($FeSO_4{\cdot}7H_2O$); 0.1 g manganese chloride tetrahydrate ($MnCl_2{\cdot}4H_2O$) and 0.1 g zinc sulphate heptahydrate ($ZnSO_4{\cdot}7H_2O$) diluted in 100 mL distilled water) and 21 g agar. The pH of the oatmeal media was also adjusted to 4.5 with alginic acid. The inoculated petri dishes were incubated at 30 °C for 1-3 weeks. Based on morphological differences, selected actinomycetes were picked and subcultured to obtain pure culture on respective media.

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Selective isolation of cellulase-producing bacteria using enrichment method

Approximately 10 g of soil samples, 1 g of carboxymethyl cellulose and 10 mL of peat water were put in conical flasks. The mixtures were then stirred and kept in a shaker incubator at 30 °C and 200 rpm for 2 weeks. The enriched soil samples were serially diluted with 10% sterile phosphate buffer saline 1:10 v/v and plated on M1 and peat agar supplemented with cellulose, in duplicate. M1 and peat agar were prepared as the above recipe. The inoculated plates were incubated for 24 h at 30 °C and then were selected and streaked on the original media. For the screening of cellulolytic activity, M1 minimal media and peat agar containing Azo-CM-Cellulose were prepared. Selected bacteria were patched on these dyed-agars in duplicate, incubated and observed daily until halo-formation was seen. Negative control was prepared by using *Escherichia coli* patched on nutrient agar containing Azo-CM-Cellulose.

Statistical analysis

A paired-samples t-test was performed to compare the number of isolated bacteria in M1 and peat agar as well as dry and wet heat pre-treatments. IBM SPSS Statistics 20 was used to conduct this test.

RESULTS AND DISCUSSION

Physicochemical analysis of soil and peat water

Both water and soil samples were in acidic condition whereby the pH of peat water samples was around 3.47-3.67 while pH of the soil samples ranged from 4.16-4.29 (data not shown). Moisture content of the peat soil samples was in the range of 69.8-83.3%. The high percentage of moisture content was in accordance with the well-known fact of water-logged condition of peat swamp forest (Page *et al.*, 1999; UNDP, 2006; Yule and Gomez, 2009; Dedysh, 2011).

Selective isolation of bacteria

The bacterial colonies were characterised based on their morphological differences in shape, elevation, margin, texture, appearance, optical property and pigmentation. Based on these morphological observations, a selection of 141 bacteria was isolated from M1 and peat agar supplemented with three carbon sources while from dry heat and wet heat pre-treatment, a selection of 66 and 103 actinomycetes was successfully isolated respectively.

The number of isolated bacteria was significantly higher in peat agar compared to M1. As illustrated in Figure 1, there was a significant difference in the scores between M1 and peat agar in terms of mean of number of isolated bacteria where, M= 23.5, SD= 9.5, t(5)= -5.894 and p= 0.002. These results suggest that in terms of the media used for isolation of bacteria from peat swamp forest soil, peat agar is better than M1.

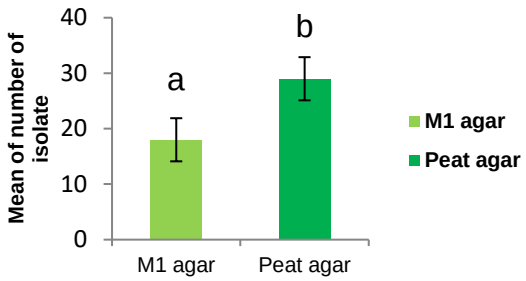

Figure 1: Comparison on mean of number of isolates obtained using M1 agar and peat agar. (Note: 'a' and 'b' represent two different values which were significantly different). * $p < 0.05$.

In comparison to M1, which has greater diversity of colony morphology, domination of bacterial colonies with similar morphology was observed in peat agar supplemented with lignin. Furthermore, the ability of bacteria to grow on all plates supplemented with various carbon sources, even with the most complex structure of lignin, showed the presence of lignocellulase-producing bacteria. Some of the successfully isolated bacteria from different agar medium are shown in Figure 2 (a), (b) and (c).

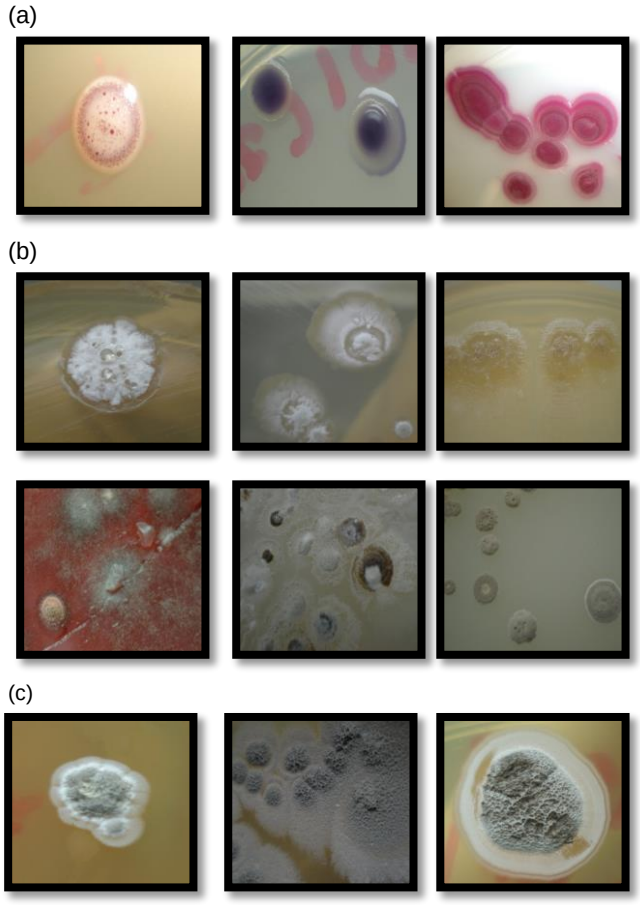

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Figure 2: a, Isolates from peat agar; b, Isolates from dry heat pre-treated soil; c, Isolates from wet heat pre-treated soil.

The incorporation of peat water in the growth medium improved the success rate of bacterial isolation from peat swamp soil samples (Mohamad Roslan *et al.*, 2015) since most of peat-inhabiting bacteria do not grow on conventional media such as nutrient agar, R2A and others (Dedysh, 2011). Peat water contained low concentration of nutrients (Na, Mg, Ca and K < 3 mg/L, N and P < 0.1 mg/L), low level of chlorophyll-a (< 5 mg/L), high in total phenols and a very high dissolved organic carbon content (70-84 mg/L DOC) (Yule and Gomez 2009) that can possibly be the preferable substrate for microbial growth. In this study, M1 was used as an attempt to mimic peat water's low nutrient condition, however it lacked other components such as natural dissolved organic carbon, phenols and chlorophyll-a, which could be the reason why more isolates were obtained on peat agar instead of M1. While synthetic carbon sources such as glucose, lignin and xylan were supplemented in M1 to promote bacterial growth, other carbon sources could be used to develop more defined medium in the future.

Selective isolation media and effect of pre-treatment for isolation of actinomycetes

Selective isolation of actinomycetes on M1, peat and oatmeal agar resulted in the highest number of isolates from peat agar and wet heat pre-treated samples. The isolates were classified based on aerial spore, colour, substrate mycelia, diffusible pigment, shape, surface, texture as dull, chalky, tough, leathery in appearance and earthly smell. In comparison to dry heat pre-treated soil, high numbers of isolates with different morphological characteristics were isolated from wet heat pre-treated soil. As illustrated in Figure 3, there was a significant difference in the scores between dry and wet heat pre-treatments in terms of mean of number of isolated actinomycetes where, M= 28.2, SD= 9.0, t(5)= -7.030 and p= 0.001. These results proposed that wet heat is a better pre-treatment method for successful isolation of actinomycetes from peat swamp forest soil. The method of wet heat pre-treatment used in this study was similar as stated in Hong *et al.* (2009) but used sterilised peat water instead of sterilised sea water. Wet heat pre-treatment was also found as the best pre-treatment method in isolating varieties of actinomycetes from mangrove (Abdul Malek *et al.*, 2014).

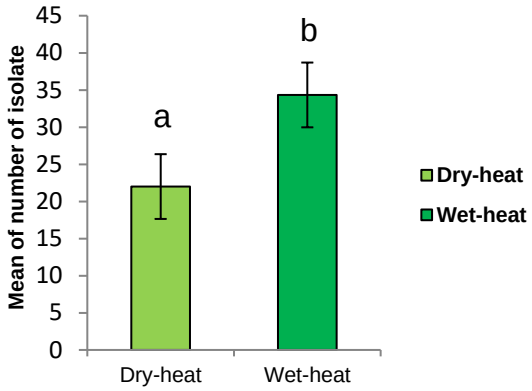

Figure 3: Comparison on mean of number of actinomycetes obtained on all growth medium via application of dry-heat and wet-heat pre-treatment of the soil samples. (Note: 'a' and 'b' represent two different values which are significantly different) * p < 0.05.

Pekan peat swamp forest as a new resource of lignocellulase-producing bacteria

Based on the success of bacterial isolation described above, this study demonstrates the potential of Pekan peat swamp forest as the resource of lignocellulase-producing bacteria. Primary screening of at least 7 and 20 bacteria (not including actinomycetes), patched on M1 and peat agar supplemented with Azo-CM-cellulose respectively, resulted in decolourisation zones around all the colonies. Among the 27 bacteria, isolate 1, patched on peat agar, showed the highest measurement of zone to colony size ratio, which is 1.71 cm (data not shown). The decolourisation zone and the colony size expand as time of incubation increases (data not shown). This observation further supports that the cellulose-degrading ability is present among bacteria of peat swamp soil. In the meantime, none of the successfully isolated actinomycetes have been subjected to the enzymatic screening test. It is expected that some of the isolates are able to exhibit lignocellulolytic activities as previous study indicated that species from the genus streptomycetes, under the same class and order as actinomycetes which are actinobacteria and actinomycetales, have the ability to degrade lignocelluloses materials effectively (McCarthy and Williams, 1992; Pérez *et al.*, 2002).

CONCLUSION

The use of peat agar supplemented with specific carbon source showed promising result in the isolation of bacteria from Pekan peat swamp forest soil compared to oatmeal agar and M1. This indicates the significant of peat water in mimicking the real peat swamp forest soil's condition in the laboratory. The ability of isolated bacteria from the Pekan peat swamp forest to show decolourisation zone on Azo-CM-Cellulose agar plates suggested that bacteria play a specific role in cellulose degradation in peat swamp environment.

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