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Screening of ligninase-producing bacteria from south east Pahang peat swamp forest soil

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ABSTRACT

Aims: Research on lignin degradation capability is previously restricted exclusively to fungal enzymes. However, recent studies had successfully revealed several soil bacterial strains that were able to produce ligninolytic enzymes. These bacterial ligninolytic enzymes were claimed to be more specific in catalysing cleavage of certain linkages between phenolic units of lignin polymers as compared to fungal enzymes. The present study focuses on screening for ligninase-producing bacteria isolated from South East Pahang Peat Swamp Forest (SEPPSF) soil using agar-based assay.
Methodology and results: Thirteen isolates used in this study, which were selected based on distinctive colony morphology from our previous isolation work, showed decolourisation zone on Azure B plates screening. The ratio of decolourisation zones were measured to the ratio of the colony size and the biggest ratio was 2.22 by isolate AR1. Only 4 out of the 13 isolates were able to grow on lignin plates. Subsequently, the 4 isolates, AR3, AR8, AR10 and AR13 were tested on M1 agar supplemented with 3 ligninolytic enzyme indicator compounds which were tannic acid (TA), guaiacol and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) respectively. All four isolates showed growth on TA plates while only AR10 showed a clear brown coloration. An Intense reddish-brown colour formation was observed around the colony of isolates AR3 and AR10 on guaiacol plates while none exhibited green coloration around the colonies when tested on ABTS plates.
Conclusions, significance and impacts of study: Isolate AR10 that was identified as *Serratia* sp. was perceived to be a potential ligninase-producer, though in-depth analysis has to be conducted in the future to determine the specific ligninolytic enzyme activities and characteristics. The application of different substrates is essential to investigate the ligninolytic potential and reaction of those bacterial enzymes towards different indicator compounds. This study is a preliminary endeavour concerning potential ligninolytic enzymes from bacteria as biocatalysts in various industrial processes. This is the first report on preliminary study for ligninolytic activities of soil bacteria from SEPPSF soil.

Keywords: ligninolytic enzymes, bacteria, peat swamp forest, tannic acid, guaiacol

INTRODUCTION

Lignin is the most abundant aromatic polymer in the biosphere (Bholay *et al*., 2012). It confers the backbone rigidity of secondary structure of plants cell wall by holding cellulose and hemicellulose fibres together (Bandounas *et al*., 2011). Naturally, saprophytic microorganisms which live on dead decomposing matter are responsible for the continuous bio-degradation of leaf litter in nature (Hammel and Cullen, 2008). In some cases, the decomposition of leaf litter, roots and woods is relatively slow especially in extremely acidic, anoxic and poor nutrient ecosystems such as tropical peat swamp forests in which conditions may hinder microbial and fungal activities (Yule and Gomez, 2009). Lignin is likely to be a recalcitrant compound due to its intense brown colour, hydrophobicity and poor mechanical properties (Bholay *et al*., 2012). Contamination of toxic industrial effluent containing chlorinated lignin which is mostly discharged by pulp and paper industries triggers immense environmental and economic problems. Chemical oxidation or precipitation techniques which cause more environmental problems have led to the search for environmentally-friendly alternative approaches. Biological approaches in removing the dark-brown colour from the effluent seem more engaging and have been explored by researchers using ligninolytic enzymes produced by several groups of test microorganisms (Prasongsuk *et al.*, 2009).

Ligninase-producing microorganisms have been extensively studied especially on fungal species such as white rot basidiomycetes. They are able to secrete an array of lignin-modifying enzymes (LME's) such as laccase (LAC), lignin peroxidase (LiP) and manganese peroxidase (MnP) (Arantes and Milagres, 2007; Shary *et al.*, 2008; Huang *et al.*, 2013;) which are efficient in depolymerising and mineralising plant cell wall components including the most recalcitrant lignin (Narkhede *et al.*, 2013). On the contrary, the ability of

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ligninolytic bacteria is rather limited by the fact that they consume only low molecular weight portion of lignin (Raj *et al*., 2007). Ligninolytic activities of bacteria have been recently reported in various species such as *Pandoraea norimbergensis*, *Pseudomonas* sp, (Bandounas *et al*., 2011), *Bacillus pumilus, Bacillus atrophaeus* (Huang *et al*., 2013) and *Thermus thermophilus* which have been proven to acquire high lignin degrading and laccase-producing potential (Popovitch, 2012). Besides that, the application of both aerobic and anaerobic ligninase-producing bacteria in a bacterial treatment systems has resulted in a significant increase in removal efficiency of the dark colour from paper mill effluent as well as reducing biological oxygen demand (BOD) of the water bodies (Raj *et al*., 2007; Oliveira *et al*., 2009; Bholay *et al*., 2012).

Bacteria producing ligninolytic enzymes are still mostly unexplored and many novel ligninolytic enzymes may lie in wait for discovery. In fact, ligninolytic enzymes from bacteria are more specific as it was observed that particular strains are able to grow only on either *β-O-4* model or *β-1* model to catalyse the rupture of certain linkages between phenolic units of lignin polymers as compared to fungal enzymes (Vicuña, 1988). The ligninolytic bacterial isolation was done as described in our previous work (Mohamad Roslan *et al*., 2015) using soil sample collected at Compartment 74 in the Peat Swamp Forest, Pekan Forest Reserve, Pahang, Malaysia. The present study screened the isolated bacteria for ligninolytic enzyme activities by using a lignin-mimicking dye and three different ligninolytic enzyme indicator compounds.

MATERIALS AND METHODS

Bacteria collection and media preparation

All bacterial isolates were retrieved from the glycerol stocks collection (- 80°C) by streaking on peat-water (PW) agar plates (Mohamad Roslan *et al*., 2015) and routinely cultured two to three times prior to screening procedure. Two types of agar media were used for this agar-based screening which were modified M1 minimal medium (Dedysh *et al*., 1998) and also peat-water medium (PW). The final pH of both media was adjusted in range of 4-5 by using 20% alginic acid to mimic the natural acidic condition of peat swamp. The plates were incubated at 30 °C and aseptic techniques were applied throughout the procedure to avoid microbial contamination.

Dye decolourisation test

The primary screening process involved lignin-mimicking dye assay whereby isolates were grown on PW medium containing 0.01% w/v Azure B (Sigma Aldrich) and the ratio of decolourisation zone to colony size was measured after 24 h incubation at 30 °C. *Escherichia coli* ATCC 25922 was grown overnight on LB agar supplemented with 0.01% w/v Azure B as a negative control.

Screening for ligninolytic bacteria

The selected isolates were tested separately on M1 agar supplemented with three ligninolytic indicator compounds as in Pointing (1999), which were 1 mL of 1% w/v tannic acid (TA) (Sigma Aldrich), 0.01% w/v guaiacol and 0.1% w/v 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid (ABTS) (Amresco). The growth and reaction of isolates towards each of the three compounds were compared to ligninolytic fungi, *Fusarium verticillioides* IMB 11778 as a positive control and *E. coli* ATCC 25922 as a negative control on PDA and LB plates respectively. All plates were incubated at 30 °C and observed for colonies growth and change of media colour up to 10 days.

RESULTS AND DISCUSSION

In this study, we sought to explore the ligninolytic activities of bacteria isolated from South East Pahang Peat Swamp Forest (SEPPSF) soil using solid media containing three ligninolytic indicator compounds. At least 13 isolates from 29 previously isolated bacteria (Mohamad Roslan *et al*., 2015) exhibited clear decolourisation zone when grown on Azure B plates. Azure B is among the most common lignin-mimicking dye used in measuring activities of ligninase-producing microorganisms. A clear decolourisation zone around bacterial colonies indicates positive ligninase activities. As shown in Table 1, the diameters and ratios of the decolourisation zone to colony size recorded for all 13 isolates were varied. Isolate AR1 produced the most notable zone ratio which was 2.22 while the smallest ratio was 1.75 by isolate AR5. More than half of the isolates showed double the size of decolourisation zone over the colony size. It was also observed that the decolourisation of the dye by all isolates was associated with cell-dye-adsorption (Figure 1).

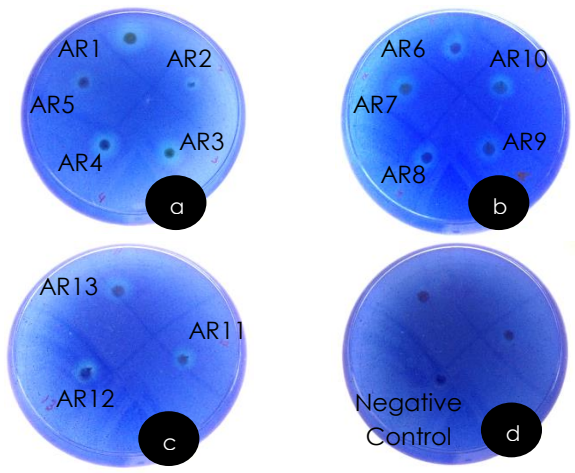

Figure 1: Decolourisation zones of 13 isolates on AB dye-containing plates after 24 h of incubation at 30 °C. Plate a, b and c are PW media inoculated with the isolates while plate d is *E. coli* as negative control on LB agar.

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Table 1: Ratios of decolourisation zone to colony size of isolates on PW agar containing AB dye after 24 h incubation at 30 °C.

Isolates	Results	Colony Size, (C) (cm)			Zone Size, (Z) (cm)			Ratio Z:C
		1st	2nd	Mean	1st	2nd	Mean	
AR1	+	0.5	0.4	0.45	1.0	1.0	1.00	2.22
AR7	+	0.4	0.5	0.45	1.0	0.9	0.95	2.11
AR3	+	0.3	0.5	0.40	0.7	0.9	0.80	2.00
AR6	+	0.3	0.3	0.30	0.5	0.7	0.60	2.00
AR8	+	0.3	0.2	0.25	0.5	0.5	0.50	2.00
AR10	+	0.5	0.4	0.45	0.9	0.9	0.90	2.00
AR12	+	0.3	0.2	0.25	0.4	0.6	0.50	2.00
AR13	+	0.4	0.3	0.35	0.6	0.8	0.70	2.00
AR4	+	0.5	0.3	0.40	0.6	0.9	0.75	1.88
AR9	+	0.3	0.4	0.35	0.6	0.7	0.65	1.85
AR11	+	0.4	0.3	0.35	0.6	0.7	0.65	1.85
AR2	+	0.2	0.3	0.25	0.4	0.5	0.45	1.80
AR5	+	0.3	0.5	0.40	0.6	0.8	0.70	1.75

Studies have reported that adsorption is the most typical mechanisms taken by bacteria to decolourize dye substrates in both solid and liquid phase assays (Kumar *et al*., 2008; Kulandaivel *et al*., 2014). Bandounas *et al*., (2011) also reported visible decolourisation zone on solid phase assay of Azure B by *Pseudomonas* sp. LD002 and *Bacillus* sp. LD003 and the dye appeared to be adsorbed to the cells instead of being degraded. The ability of isolates to decolourize the recalcitrant Azure B dye demonstrates their lignin peroxidase activities (Bandounas *et al*., 2011) which are the high redox potential agents required in the dye decolourisation mechanisms (Archibald, 1992). Since among all 13 isolates only AR3, AR8, AR10 and AR13 were able to grow on minimal M1 medium containing 0.1% w/v alkali lignin (Mohamad Roslan *et al*., 2015), these four isolates were further screened for their ability to exhibit ligninase activity by using ligninolytic indicator compounds. Previously, 16s rRNA gene sequencing analysis identified isolates AR3, AR8 and AR13 as *Burkholderia* sp. while isolate AR10 as *Serratia* sp (Mohamad Roslan *et al*., 2015). *Burkholderia* sp. are common bacteria species in the lignocellulasic activities studies and their ability to break down plant cell wall was widely studied (Vargas-Asensio *et al*., 2014; Woo *et al*., 2014). Ligninase of *Serratia* sp. has been reported to exhibit efficient decolourisation of effluent from textile industry (Sahadevan *et al*., 2013) and significant percentage in paper-pulp mill effluent bio-bleaching (Bholay *et al*., 2012).

Agar-based assay containing three ligninolytic indicator compounds which were TA, guaiacol and ABTS were conducted. All isolates showed growth on all indicator agar except for AR8 and AR13 which did not grow on ABTS agar (Table 2). The modified TA agar-based assay that was also called Bavendamm test was incorporated to show the effect of overall polyphenoloxidase and lignolytic activity of the isolates (Pointing, 1999; Alfarra *et al*., 2013). Even though all four isolates were able to grow on TA plates after 10 days incubation at 30 °C, only AR10 exhibited a clear brown coloration around the colonies (Figure 2). Since TA oxidation alone is not a precise indicator for any specific lignin-modifying enzymes (LME'S), additional experiments with other model compounds are necessary to support the TA assay results (Alfarra *et al*., 2013).

All four isolates showed growth on agar-based assay containing guaiacol but an intense reddish-brown colour surrounding the colony due to guaicol oxidation was only exhibited by isolates AR3 and AR10. Guaiacol oxidation was always correlated with decolourisation of polymeric dye such as Remazol Brilliant Blue R (RBBR) which make it suitable for detection of laccase activities (Kiiskinen *et al*., 2004). However, in ABTS assay, only isolates AR3 and AR10 showed growth on ABTS plates and none exhibited green coloration around the colonies. ABTS is a reagent commonly used as laccase-mediated testing in

Table 2: Growth of isolates and colour changes of agar containing indicator compounds.

Isolate	Tannic Acid (TA)		Guaiacol		ABTS	
	G	R	G	R	G	R
AR3	+	-	+	**	+	-
AR8	+	-	+	*	-	-
AR10	+	*	+	**	+	-
AR13	+	-	+	-	-	-
F. verticillioides IMB 11778 (positive control)	+	**	+	**	+	**
E. coli ATCC 25922 (negative control)	+	-	+	-	+	-

Notes: G, Growth; R, Reaction; -, no growth/no colour change; +, colony growth; *, slight colour change; **, intense colour change.

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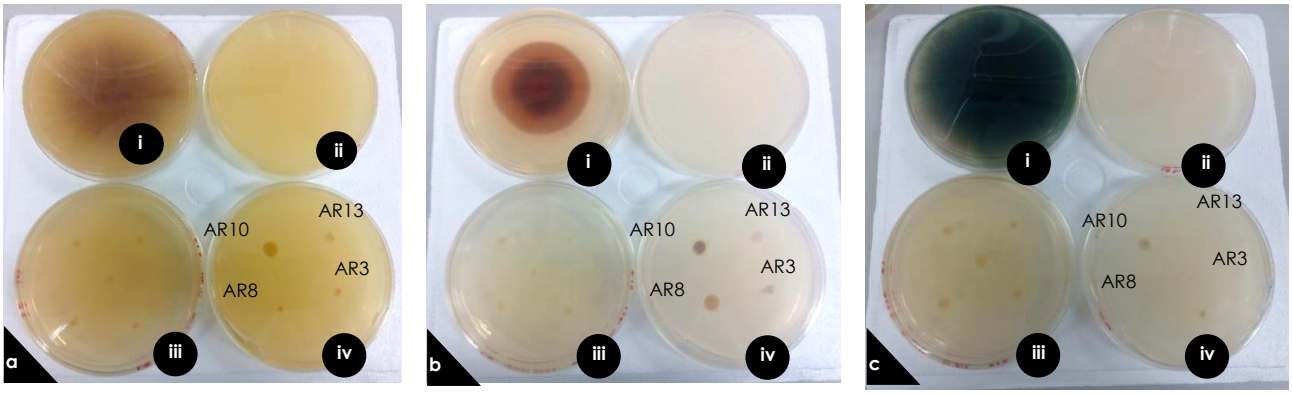

Figure 2: Colour changes of agar by isolates towards media containing: a, 1 mL of 1% w/v tannic acid; b, 0.01% w/v guaiacol; c, 0.1% w/v ABTS respectively after incubation at 30 °C up to 10 days. i) *F. verticillioides* IMB 11778 (positive control) on PDA; ii) control plate (M1 medium); iii) *E. coli* ATCC 25922 (negative control) on LB; iv) Isolate AR3, AR8, AR10 and AR13 on M1

ligninolytic enzyme screening and was a reliable indicator for the presence of syringaldazine (Pointing, 1999; Popovitch, 2012; Huang *et al.*, 2013; Rehmann *et al.*, 2014). The negative results indicated that all isolates were unable to exhibit laccase activity under those parameters used in the procedure.

CONCLUSION

The ability of the isolates to decolourize lignin-mimicking dye Azure B is a positive indicator of ligninolytic enzyme activities. Tannic acid, guaiacol and ABTS are reliable indicators of laccase through the appropriate media and the optimisation of chemical and physical parameters must be set to achieve the expected results. Based on the overall agar-based screening results, *Serratia* sp. AR10 was identified as a potential ligninase-producing bacterium even though specific and further experiments should be conducted in the future to isolate and characterize ligninolytic enzymes produced by *Serratia* sp. AR10. These ligninolytic enzymes may hold a great potential in bio-bleaching and bioremediation prospects.

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