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Isolation of potential lignin-degrading bacteria from tropical forest soils in Thailand

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ABSTRACT

Lignin is the second most abundant polymer in plant residues and nature in general. However, lignin recalcitrance poses a challenge to biofuel production from plant biomass. To overcome the problem, this research aims to isolate bacteria strains capable of degrading lignin. Potential ligninolytic bacteria strains were isolated from 16 different soil samples based on their ability to grow on minimal media with alkali lignin as sole carbon source at 40°C under microaerobic conditions. Furthermore, growth on model lignin compounds (guaiacol and veratryl alcohol) and dyes (aniline blue, methylene blue, and Congo red) was also used to isolate four potential ligninolytic strains. Results from 16s rDNA sequence analysis showed that the four isolates belonged to genus *Burkholderia*, *Pseudomonas*, and *Klebsiella*. The gene encoding laccase-like multicopper oxidase was only detected in *Burkholderia*. Thus, the isolates identified in this study are capable of degrading lignin and may possess genes encoding for different ligninolytic enzymes which can be further explored for lignin degradation and optimization of biofuel production from plant biomass.

Keywords: Lignin, Ligninolytic bacteria, Microaerobic bacteria.

INTRODUCTION

In the bid to mitigate the effects of global warming on the environment and develop a sustainable environment, the quest for alternative sources of energy besides fossil fuels is on. This has given rise to interest in biofuels and renewable energy. Plant biomass is being looked upon as a promising source of biofuel (Wang et al., 2014; Bułkowska et al., 2016). This application solves two problems by reusing waste generated by agriculture and also having a greener source of energy. This approach seems promising; nevertheless, there are many challenges besetting it. One of such challenge is the efficient breakdown of the components of the biomass cell wall in order to convert the cellulose and hemicellulose to biofuel. The presence of lignin in the cell wall structure of plants is responsible for this bottleneck in the production of biofuel as lignin is recalcitrant to degradation (Zhu et al., 2017; Welker et al., 2015). Lignin-derived inhibition mechanisms occur in three forms: lignin acts as a physical shield against enzymatic attack of cellulosic surfaces (Kumar et al., 2012), lignin absorbs enzymes in nonproductive binding (Palonen et al., 2004), and soluble lignin fragments inhibit enzyme performance (Jing et al., 2009). Studies have shown that carbohydrate binding module (CBM) in cellulase which improves enzymatic hydrolysis of crystalline cellulose can interact with lignin in non-productive binding. Such binding is promoted through hydrophobic interactions between lignin and CBM. Lignin is more hydrophobic than cellulose, thus, the hydrophobic surface of CBM also interacts with the hydrophobic surface of lignin which leads to non-productive binding (Fritz et al., 2015; Palonen et al., 2004; Rahikainen, 2013).

Conventional methods of removing lignin are alkali and acid hydrolysis which are not environmentally friendly. Alternatively, studies have shown that microorganisms can also breakdown lignin structure in plant cell wall (Huang et al., 2013, Zhu et al., 2017, Yang et al., 2017; Acevedo et al., 2011). Compared to bacteria, extensive studies have been carried out on fungal lignin degradation and the enzymes involved (Zhu et al., 2017; Huang et al., 2013; Xiangqun et al., 2017; Cupul et al., 2014). Nevertheless, the use of bacteria to breakdown lignin holds many advantages over the use of fungi such as faster growth, broader range of habitat tolerance, ease of genetic manipulation and protein expression, and efficient large scale production (Taylor et al., 2012). Thus, the aim of this study is to screen for ligninolytic bacteria from soil samples obtained from a tropical forest in Thailand.

MATERIAL AND METHODS

Collection and preparation of soil samples

Soil samples were collected from 16 different locations in a tropical forest (20-30cm depth) and transported microaerobically for further analysis. The samples were enriched in 1% alkali lignin for 7 days and afterwards transferred to Carboxymethyl cellulose (CMC) agar plates for isolation of potential lignocellulosic degrading bacteria. Microaerobic condition was maintained throughout the process.

Bacterial isolates screening on lignin minimal salt media (L-MSM)

To test for ligninolytic potential, the bacterial isolate were cultured on lignin minimal salt media (L-MSM) agar plate with 0.1% alkali lignin as sole carbon source. The minimal salt media is made up of (g/L) Na_2HPO_4 (2.4), K_2HPO_4 (2.0), NH_4NO_3 (0.1), $MgSO_4$ (0.01), $CaCl_2$ (0.01), alkali (kraft) lignin (1.0) pH 7.2. A culture subset of L-MSM plate with 1% glucose (alternative carbon source) was also prepared to check for isolates that showed lignin tolerance properties. Strains that showed growth on both media were selected for identification and further study.

Bacterial isolation based on dye decolourization

To further identify the degradation activity, the selected isolates were cultured on Luria-Bertani (LB) plates supplemented with 0.02% aniline blue and methylene blue to check for decolourization of the dyes. Furthermore, isolates were inoculated on minimal salt agar supplemented with Congo red as sole carbon source.

Utilization of lignin as sole carbon source for bacterial growth

Due to the interference background of lignin media, the growth of bacteria in the media was determined by counting of colony forming unit (cfu). Two *Klebsiella* spp. were pre-cultured in LB media until OD_{600} 1.0 was reached. The bacteria cells were collected by centrifuging at 5,000 rpm for 5 minutes, washed with normal saline (0.9% NaCl) for three times and afterwards resuspended in L-MSM at initial OD_{600} of 0.1. Then proper dilution of the cultures on days 1, 2, 3 and 7 were plated on LB agar, and cfu was calculated to represent bacterial growth.

Detection of bacterial growth on different lignin-associated monomers

Isolates were cultured on MSM agar plates containing 1g/l guaiacol, veratryl alcohol, and 2,6-Dimethoxyphenol (2,6-DMP) as sole carbon source. Bacteria growths on these media were observed by comparing the differences of colony morphology on each media.

Identification of bacterial isolates

Genomic DNA of selected bacteria was extracted and purified using phenol-chloroform technique. 16s rDNA was amplified using universal primers 27F and 1492R (27F 5'-AGAGTTTGATCCTGGCTCAG-3'; 1492R 5'-GGTTACCTTGTTACGACTT-3') followed by DNA sequencing using the corresponding primers.

Identification of potential laccase-like multicopper oxidase gene in isolates

PCR reaction was carried out using degenerate primer Cu1AF and Cu2R (Wang et al., 2016) (Cu1AF 5'-ACMWCBGTYCAYTGGCAYGG-3'; Cu2R 5'-GRCTGTGGTACCAGAANGTNCC-3') to screen for the presence of potential laccase like multicopper oxidase gene in the bacteria.

Bands of interest were purified and used for ligation into pGEM®-T Easy vector following the Promega protocol. Plasmids were transformed into *Escherichia coli* DH5α. Extracted plasmid was sequenced and homology analysis of the result was performed using BLAST against NCBI database.

RESULTS

Culture on lignin minimal salt media (L-MSM)

Thirty three bacteria colonies were selected on Carboxymethyl cellulose (CMC) agar plates after initial enrichment in 1% alkali lignin. When subcultured on L-MSM plates, 16 bacterial isolates were able to grow on the L-MSM media with lignin as sole carbon source. All isolates that could grow on L-MSM were also able to grow on L-MSM supplemented with 1% glucose while the rest were only able to grow on L-MSM supplemented with 1% glucose. Only isolates that grew on L-MSM plates were selected for further study

Figure 1. Bacteria growth on L-MSM plates

Bacterial decolourization of aromatic dyes

To test for lignin degrading ability, bacterial isolates were inoculated on LB medium supplemented with aniline blue, methylene blue, or Congo red. All isolates exhibited growth on aniline blue. Some isolates exhibited deep colour of blue around their colonies (figure 2). As the incubation period extended to day 4, decolourization was observed as the blue colour of the dye disappeared.

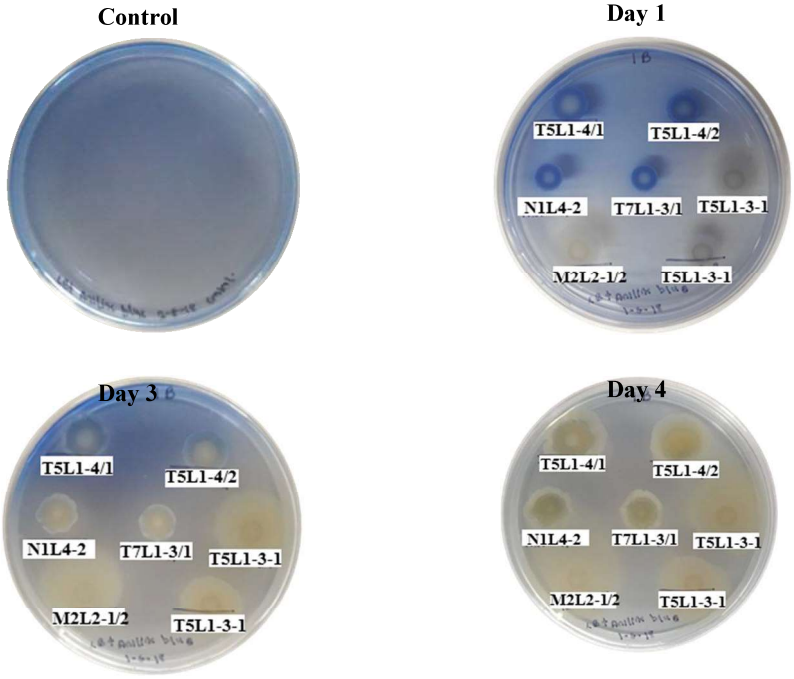


Figure 2: Aniline blue dye decolourization assay.

When bacterial isolates were cultured on methylene blue, adsorption of dye rather than decolourization was noticed after 3 to 4 days of incubation (figure 3). Similar phenomenon was observed when bacterial isolates were grown on agar plate supplemented with Congo red as sole carbon source as the adsorption of dyes rather than decolourizaton was noticed (figure 4).

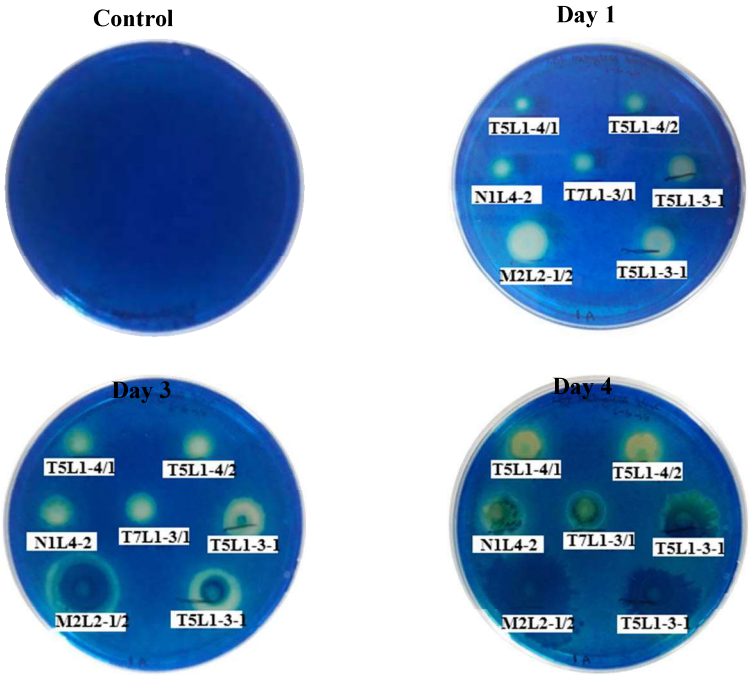
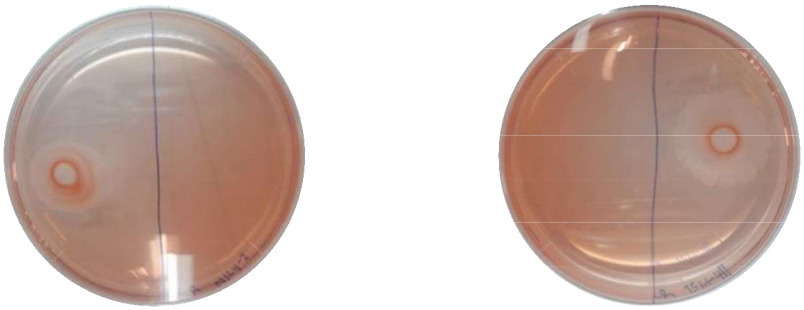


Figure 3: Methylene blue decolourization plate assay



Klebsiella spp. A1 *Klebsiella* spp. A2

Figure 4: *Klebsiella* spp. grown on minimal salt media supplemented with Congo red for 7 days.

Identification of bacterial isolates

Bacteria that exhibited growth in L-MSM plates were identified using 16s rDNA. Results showed that the 16 isolates belong to 3 different genera namely *Klebsiella*, *Burkholderia*, and *Pseudomonas*. Four isolates that exhibited the best growth amongst others were chosen for further study.

Bacterial growth on different lignin-associated aromatic monomers

Bacterial isolates were cultured in minimal salt agar supplemented with 0.1% veratryl alcohol, guaiacol, 2, 6-dimethoxyphenol (2,6-DMP), and Congo red as sole carbon source at 40°C for 7 days. Each isolate reacted differently to the different carbon sources used. The isolates were rated based on their colony size on each media as shown in table 1.

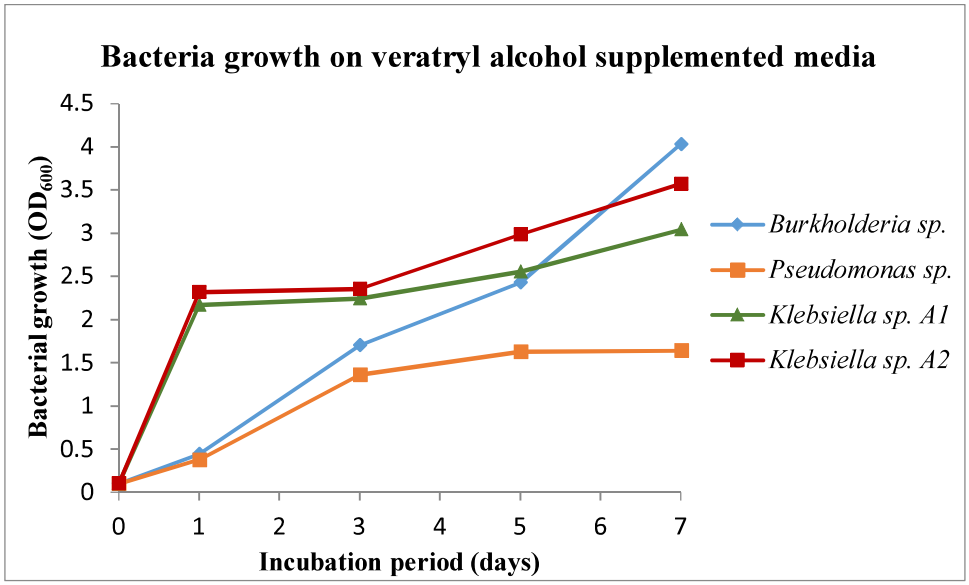
Table 1. Growth intensity of bacteria on different carbon sources

Bacteria	Growth intensity			
	Guaiacol	Veratryl Alcohol	2,6 DMP	Congo Red
***Burkholderia* sp.**	+	++	++	++
***Pseudomonas* sp.**	++	+	+	++
***Klebsiella* sp.A1**	++	++	+	+++
***Klebsiella* sp.A2**	++	++	++	+++

Note: + little growth, ++ more visible growth, +++ profound growth

The four bacteria were cultured in LB media containing 0.1% veratryl alcohol and guaiacol and growth kinetics were followed over a period of 7 days (figure 5). The result obtained in figure 5 showed that *Burkholderia* sp. as well as *Klebsiella* sp. A2 preferred veratryl alcohol rather than guaiacol, while *Pseudomonas* sp. exhibited better growth in guaiacol. Variation of growth pattern in different media implied that different isolates have a capability to degrade different types of aromatic compounds. No difference in growth pattern was observed for *Klebsiella* sp A1 and A2. It should be noted that *Burkholderia* sp. was unable to grow on medium supplemented with guaiacol.

A



B

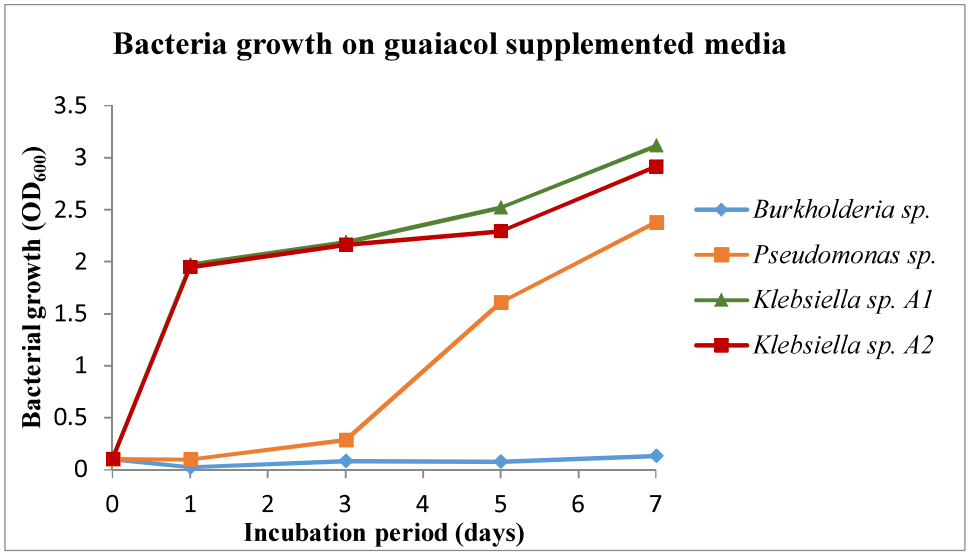


Figure 5: Bacterial growth (OD_{600}) on veratryl alcohol supplemented media (A) and guaiacol supplemented media (B).
Note: Values plotted in graph represent average of 2 replicates.

Identification of potential laccase-like multicopper oxidase (LMCO) gene

Degenerate primers designed for the conserved copper binding regions I and II of Laccase-like multicopper oxidase gene were used to detect a presence of laccase gene. Of all the isolates, only *Burkholderia* sp. showed expected band on agarose gel at the size of 140 bp. The PCR fragment was cloned into pGEM®-T Easy vector. Six transformants were selected with 4 positive clones (figure 6).

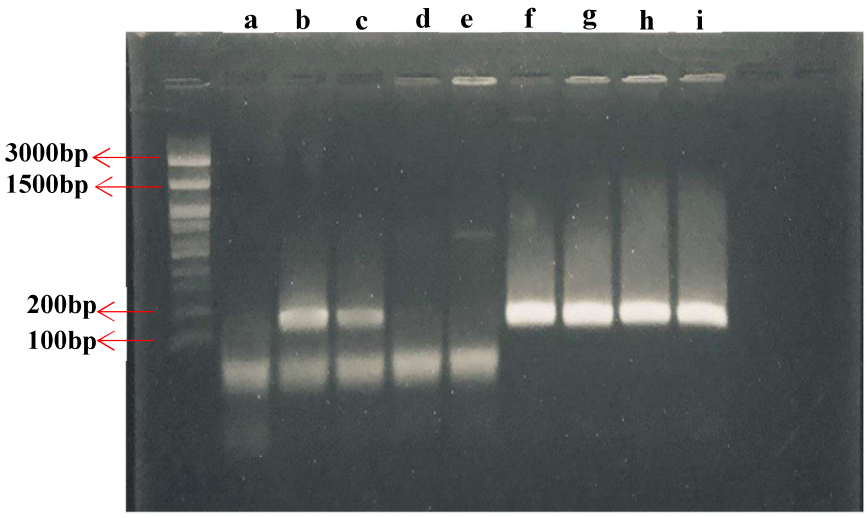


Figure 6. Gel electrophoresis of transformed cells containing insert of conserved copper binding regions from *Burkholderia* **a-** negative control **b,c-** positive control **d-i–** selected transformants

Utilization of lignin as sole carbon source for bacterial growth

Due to fast growth on lignin associated monomers (figure 5), *Klebsiella* spp. A1 and A2 were inoculated into L-MSM and growth of the two isolates was measured by plate counting. The initial inoculums were 1.98x 10^6 cfu/ml and 1.53x10^6 cfu/ml for *Klebsiella* sp. A1 and *Klebsiella* sp. A2, respectively. After the first two days, *Klebsiella* spp. A1 and A2 showed significantly increased growth up to 2 order of magnitude of the original number (figure7). Both strains showed a steady decline in growth afterwards. The results of cfu/ml were calculated from triplicate samples.

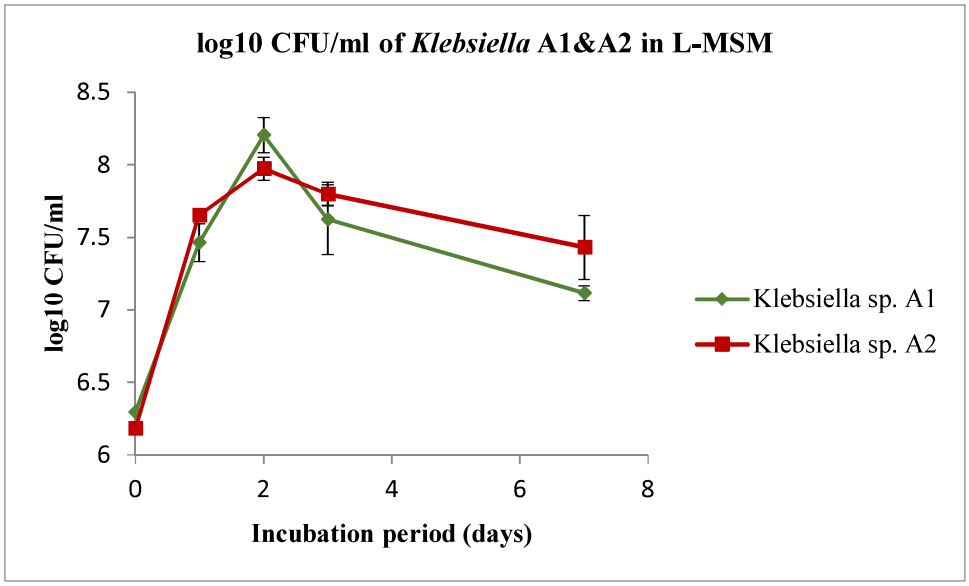


Figure 7: Growth of *Klebsiella* A1 and A2 in L-MSM.

DISCUSSION

Tropical forest soils have a very wide range of microbial diversity and they also have the highest rate of plant litter decomposition compared to other biomes (Parton et al., 2007). Fast decomposition in tropical forest soils indicates the presence of active lignocellulolytic microorganisms (Woo et al., 2014). Several studies had successfully isolated lignin degrading bacteria from tropical forest soils include *Bacillus pumilus*, *Bacillus atrophaeus, Gordonia,* and *Enterobacter ligninolyticus* SCF1 (Huang et al., 2013; Woo et al., 2014; DeAngelis et al., 2013).

Here in this study, we could isolate bacteria from tropical forest soils in Thailand that have the potential to degrade lignin given their ability to grow on the different media supplemented with different model lignin compounds. As stated previously, the growth observed indicates lignin tolerance, depolymerization and utilization of degraded compounds as carbon source. Bacteria without any of these abilities will not be able to grow on the medium because aromatic compounds obtained from lignin degradation are believed to disrupt membranes, enzyme functions and damage DNA (Xu et al., 2018; Zeng et al. 2014).

The results obtained in this study shows that isolated *Burkholderia* sp., *Pseudomonas* sp., and *Klebsiella* sp. have ligninolytic potentials. Previous studies have also shown that some bacteria in the genus *Klebsiella, Pseudomonas,* and *Burkholderia* have ligninolytic abilities. In accordance with the previous finding, ligninolytic *Klebsiella* strains also have been isolated and identified in some studies (Gaur et al., 2018; Xu et al., 2018: Woo et al., 2014; Too et al., 2018). *Pseudomonas* and *Burkholderia* strains with lignin degrading abilities have also been isolated in other researches (Xu et al., 2018; Yang et al., 2018; Prabhakaran et al., 2015 Akita et al., 2016, Yang et al., 2017; Woo et al., 2014).

Besides lignin, various xenobiotic compounds and dyes could be degraded by ligninolytic enzymes such as laccase, manganese peroxidase, and lignin peroxidase. The dyes used in this study were selected based on their aromatic structure given that lignin has a large aromatic structure. Thus, breakdown of these aromatic rings in dyes leads to decolourization as bacteria grow on the media (Bandounas et al., 2011). Aniline blue is a triphenylmethane dye which can be degraded by laccase, manganese peroxidase, and lignin peroxidase. The decolourization of aniline blue suggests that the isolates possess one or more of ligninolytic enzymes (Li et al., 2018). *Klebsiella, Pseudomonas,* and *Burkholderia* have been reported to degrade triphenylmethane dyes including aniline blue in previous studies (Yang et al., 2017; Zabłocka-Godlewska et al., 2014). *Pseudomonas* isolates exhibited the fastest decolourization of aniline blue having the most visible decolourization within 24 hours of incubation (figure 2). Decolourization of thiazyne dyes such as methylene blue can be achieved by high redox potential agents such as lignin peroxidases (LiPs) as it cannot be degraded by low redox potential oxidases such as manganese peroxidases (MnPs) and laccases (Ferreira-Leitao et al., 2007; Tian et al., 2016). Although all isolates showed initial methylene blue decolourization, absorption of the dye to the colony of the isolates was observed after two days of incubation. Congo red dye was also absorbed to the colonies of *Klebsiella* isolates in this study. Although the reason for the latter absorption is not yet fully understood, Melo-Nascimento et al. (2018) also reported absorption of methylene blue and Congo red dye to the colonies of *Klebsiella* isolates. *Pseudomonas* has also been reported to degrade methylene blue (Eslami et al., 2017).

Screening for the presence of laccase enzyme showed that only *Burkholderia* sp. had the laccase like multicopper oxidase gene amongst the isolates. Laccase-like gene in *Burkholderia* spp. has been identified and expressed in *Escherichia coli* by Ma et al. (2018) which supports the presence

of LMCO in the *Burkholderia* isolates in this study. Genome sequencing and functional characterization studies on lignin degrading *Klebsiella* isolates show that *Klebsiella* genome has no laccase genes present in it (Woo et al., 2014; Melo-Nascimento et al., 2018). There are reports on *Pseudomonas* possessing laccase-like genes; however, no laccase-like gene was detected with PCR amplification in the *Pseudomonas* isolate in this study (Prabhakaran et al., 2015; Wang et al., 2016). The primers used for LMCO amplification are degenerate primers designed for the conserved copper binding regions I and II of laccase-like multicopper oxidase gene across different genus of bacteria, therefore, the PCR method may need to be optimized to suit specific bacteria of interest in order to successfully amplify LMCO conserved regions in different bacteria. Factors like annealing temperature and DNA template concentration may be adjusted depending on the bacteria of interest. Although only *Burkholderia* sp. showed the presence of LMCO gene, the reported genome of *Klebsiella* and *Pseudomonas* from the NCBI database revealed the presence of other genes associated with lignin degradation besides lignin e.g. peroxidases, gluthathione, gentisate pathway and protocatechuate pathway. (Woo et al., 2014; Melo-Nascimento et al., 2018; Prabhakaran et al., 2015).

CONCLUSION

Burkholderia sp., *Pseudomonas* sp., and *Klebsiella* sp. isolated in this study have shown ligninolytic potentials based on growth on L-MSM, lignin monomers, and aromatic dyes. Amongst them, *Burkholderia* sp. has the laccase like multicopper oxidase gene while *Pseudomonas* sp. and *Klebsiella* sp. may possess other genes that could be associated with lignin degradation such as peroxidases, gluthathione, gentisate pathway and protocatechuate pathway. Thus these isolates can be explored further in studies related to lignin degradation for optimization of biofuel production from plant biomass.

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