Removal of phenolic compounds from soil irrigated with palm oil mill effluent using *Acinetobacter* sp. OPB and grass rhizosphere bacteria

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Abstract

Palm oil mill effluent (POME) in stabilization ponds usually contains phenolic compounds and color higher than standard. The wastewater may exceed the pond capacity during raining season. Soil irrigation might be used to reduce the wastewater volume; however the accumulation of phenolic compounds could be toxic to plants. Previously, we examined rhizoremediation of POME irrigated soil and found that Mulato grass and its rhizosphere bacteria could remove phenolic compounds from the soil. To increase rhizoremediation efficiency, the addition of phenol-degrading bacteria *Acinetobacter* sp. strain OPB with and without grass was investigated. In synthetic medium *Acinetobacter* sp. strain OPB shown completed degradation of 100 mg L^{-1} and 500 mg L^{-1} phenol in 6 h and 15 h. respectively. Particularly, the addition of strain OPB increases the number of phenol-degrading bacteria in planted soil. The bacterial augmentation significantly enhanced both bioremediation and rhizoremediation. The highest efficient of removal was inoculated rhizoremediation with up to 82% of phenolic compounds removal. Phytotoxicity test indicated that the leachate from treatment with bacterial inoculation was non-toxic. Thus, strain OPB could be inoculated with grasses to remove phenols from contaminated soil.

Keywords: phenol, phenolic compounds removal, POME, *Acinetobacter* sp. OPB, rhizoremediation

Introduction

Oil palm is one of the world's most rapidly expanding tropical crops. Indonesia, Malaysia and Thailand are the three largest palm oil producing countries in the world (Rupani et al. 2010). Palm oil processing released large quantities of wastewater. They are concerned as environmental problems when it was discharged to natural water and directly toxic to aquatic plants and animals. Several chemical and physical technologies have been utilized for the treatment of this colored effluent such as adsorption, electrocoagulation (Zawawi et al. 2013), membrane technology (Ahmad et al. 2003), electrochemical method, and ultrafiltration (Mohammad et al. 2013). However, some of them have limitations related to its low efficiency, high construction and operation costs, and lead to more toxic by-products. In palm oil mill wastewater treatment process, stabilization ponds were final place where the treated POME from other process was stored. However, the treated POME still has phenolic compounds and color higher than wastewater standard. In addition, the volume of this wastewater can exceed the pond capacity in raining season. Thus, soil irrigation is commonly used to reduce the wastewater volume. The POME amended soil can enhance soil microbiological activities which ultimately increase soil fertility (Nwoko and Ogunyemias, 2010). In contrast, the accumulated of toxic compounds such as phenolic compounds and other organic acids in POME amended soil is reported (Barbera et al. 2013). Recently, grasses have been used in technology such as phytoremediation, rhizoremediation and constructed wetlands for the treatment of phenols polluted environments (Kuiper et al. 2004).

They are considered as low cost, easily to operate, environmental friendly and strong potential for application (Kivaisi, 2001). Rhizoremediation is a new biological treatment technique for treatment of difference pollutants (González et al. 2013) and also known as an environmental friendly technology. Rhizoremediation uses microbial activity in the plant root zone to breakdown contaminants (Gaskin and Bentham. 2010). Specific plants may promote degradation of specific type of pollution; however they have limitation when there are high concentrations of pollutants (Phillips et al. 2012). Thus, bioaugmentation with efficient bacteria could overcome the limitation of plants used (Glick, 2010).
Brachiaria hybrid (*B. ruziziensis* x *B. Decumbens* x *B. brizantha*) or Mulato II is one of common used as forage grass. Previously experiment, this plant and its rhizosphere bacteria has potential to remove phenolic compounds from soil irrigated with palm oil mill effluent (POME); however their efficiency was decreased when many irrigation cycles were conducted. Thus, this extension research aims to study on *Acinetobacter* sp strain OPB to degrade phenol in synthetic medium and in soil after irrigated with POME using bioaugmentation.

Materials and methods

Palm oil mill effluent (POME)
Palm oil mill effluent (POME) samples used in this study were collected from the last stabilization pond of a palm oil mill in Surat Thani province, Thailand. Although this effluent was previously treated by anaerobic process, it still has phenolic compounds and color higher than standard of industrial wastewater parameters. The samples were collected and stored in 20 L bottles and kept at 4°C prior to use to avoid degradation or changes.

Bacterial strain
Acinetobacter sp. OPB was isolated from rhizosphere of Green Pakchoi (*Brassica Chinensis* var. Chinensis Mansf.) that was hydroponically grown in palm oil mill effluent (POME). The bacterium was identified by using 16S rDNA sequence and stored in the laboratory.

Analysis of phenol degradation
Phenol degradation was tested in 250 mL flask containing 100 mL carbon free mineral medium (CFMM); content NH_4NO_3 3 g L^{-1}, Na_2HPO_4 2.2 g L^{-1}, KH_2PO_4 0.8 g L^{-1} with add 1 mL trace metal solution. The stock phenol solution (10,000 mg L^{-1}) was filter-sterilized and individually added to different flasks containing sterilized CFMM medium to provide final concentrations of 100 and 500 mg L^{-1}. Then, 10% of inoculum (10^7 CFU mL^{-1}) was added to the medium and placed on shaker at 200 rpm for 24 h at room temperature. CFMM with only added phenol for each concentration were used as control. Each treatment was set for triplicates. Every 3 h, samples were collected to measure bacterial growth by spectrophotometer at absorbance 600 nm and phenol remaining using Folin-Ciocalteau method. Phenol used in all experiments was purchased from Merck. Solutions were prepared with deionized water and all substances used were of analytical reagent grade.

Rhizoremediation of phenolic compounds in POME irrigated soil
Mulato II or Brachiaria hybrid (*B. ruziziensis* x *B. Decumbens* x *B. brizantha*) seeds were provided by Prof.Dr. Michael Hare, Ubon Ratchathani University. In this experiment, grass seeds were grown under greenhouse condition which temperature control around 30°C and soil humidity around 60% of water fill pore capacity for one month. The healthy grass seedlings were selected to use in rhizoremediation experiment. Soil samples for planting experiment were excavated from a planting location in the palm oil mill. Soil was screened

through a 4 mm sieve and then homogenized. Samples of the homogenized soil were analyzed. The soil was loamy sand, which contained sand (83.8%), silt (12.3%) and clay (3.9%) and has initial pH 7.8. The soil moisture was adjusted at 60% of field capacity before put in the pots. Each plastic pot contained 300 g of soil and one plantlet of grass.
There were four treatments viz. soil without plant and bacteria (S), soil with inoculated bacteria but without plant (SB), soil with plant but without inoculated bacteria (M) and soil with both plant and inoculated bacteria (MB). Triplicates were set for each treatment and pots were randomized in greenhouse.
For soil bioaugmentation, bacteria were cultured in 250 mL flasks containing 100 mL of 25% LB medium on rotary shaker at 200 rpm at room temperature (30°C) overnight to reach the mid-log growth phase. Then, cultures were centrifuged (8000 rpm), and pellets were washed twice with 0.85% NaCl. After this, the pellets were re-suspended in sterile DI. Next 50 mL of this suspension was poured into pots (SB and MB) resulting in the initial bacterial number of 10^8 CFU g $soil^{-1}$ and settling for one day before start the experiment. POME was irrigated at 50mL per pot every 3 day interval as one cycle. At the end of each irrigation cycle, soil was collect to measure bacterial number and each pot were flushed with about 100 mL of tap water to collect approximately 20 mL of leachate. The leachate was examined for residual phenolic compounds and phytotoxicity.

Analytical methods

Analysis of total phenolic compounds
Analysis of total phenolic compounds is modified from Barlocher and Graca (2005) using Folin-Ciocalteau method. Briefly, 1 mL of water leachate sample was centrifuged at 10,000 rpm for 10 min to separate supernatant and sediment. For analysis, 100 µL of supernatant was adding to 150 µL DI water followed by 1 mL of 2% Na_2CO_3. After 5 min of incubation, 50 µL of Folin-Ciocalteau reagent was added and mixed. The sample was incubated for 1 h at room temperature (30°C), measure absorbance 760 nm. The remaining phenolic compounds is calculated by comparing to the standard curve and reported as gallic acid equivalents.

The percent removal of phenol can be calculated from the equation below:

$$\text{Percent removal (\%)} = \frac{\text{Initial phenolic compounds (mg)} - \text{Final phenolic compounds (mg)}}{\text{Initial phenolic compounds (mg)}} \times 100$$

Where the initial phenolic compounds were the total mass of phenol in irrigated POME and the final phenolic compounds were the total mass of phenol in water leachate from soil. Mass of phenolic compounds (mg) was calculated from the concentration in the sample (mg L^{-1}) time the total volume of the sample (mL).

Total number of phenol-degrading bacteria in soil
Five gram of soil (from the top 5-10 cm of each pot) was collected using a 6 mm sterilized cork borer. The soil was sampled three times for each pot. The sampling protocol is adapted from Lamichhane et al. (2012). The samples from all replicates of each treatment were combined and mixed. One gram of the mixed soil from each treatment was used to estimate the phenol degrading bacteria by drop plate technique using carbon free mineral medium (CFMM) supplement with 50 mg L^{-1} phenol as carbon source.

Phytotoxicity test
In biological degradation, ecotoxicity of treated pollutant was recommended to assess due to some degradation product was found to be more toxic than untreated pollutant (Anastasi et al.

2011). Mungbean (*Vigna radiata* (L) Wilczek) and cucumber *(Cucumis sativus* L.var) were used to determine whether the leachate from soil was toxic or not compare to control (untreated POME) and DI (positive control). All of these plant seeds represent common vegetable seeds, which are sensitive to organic and inorganic pollutants (Lin and Xing, 2007; Nisha and Sreedevi, 2008). The experiments were conducted in triplicates by placing ten seeds in separate Petri dishes and adding 5 mL sample daily. Percent of germination index (GI %) and the length of radicle (root) were recorded after 4 days.

Percent of germination index (GI %) was calculated according to the following formula:

$$GI\% = (Gt \times Lt)/(Gc \times Lc) \times 100$$

Where Gt is the mean number of germinated seeds in the treatment sample, Lt is the mean root length of the treatment sample, Gc is the mean number of germinated seeds in the control (DI water), and Lc is the mean root length of the control.

Results and discussion

Phenol degradation

The results of batch studies for phenol degradation in carbon free mineral medium (CFMM) by *Acinetobacter* sp. strain OPB are shown in **Fig. 1A**. The bacterium could remove phenol completely after 6 and 15 h incubation for 100 and 500 mg L^{-1} phenol, respectively. The increasing of bacterial growth was correlated with decreasing of phenol as in **Fig. 1B.** This result indicated that the bacterium could grow with phenol as sole carbon and energy source. Similarly, Ahmad et al. (2012) and Wang et al. (2007) reported that, *Acinetobacter* sp. strains could rapidly degrade phenol at concentrations between 100-500 mg L^{-1} within 3-16 h. Thus, *Acinetobacter* sp. strain could be considered as a great bioaugmentation to enhance phenol removal in contaminated site.

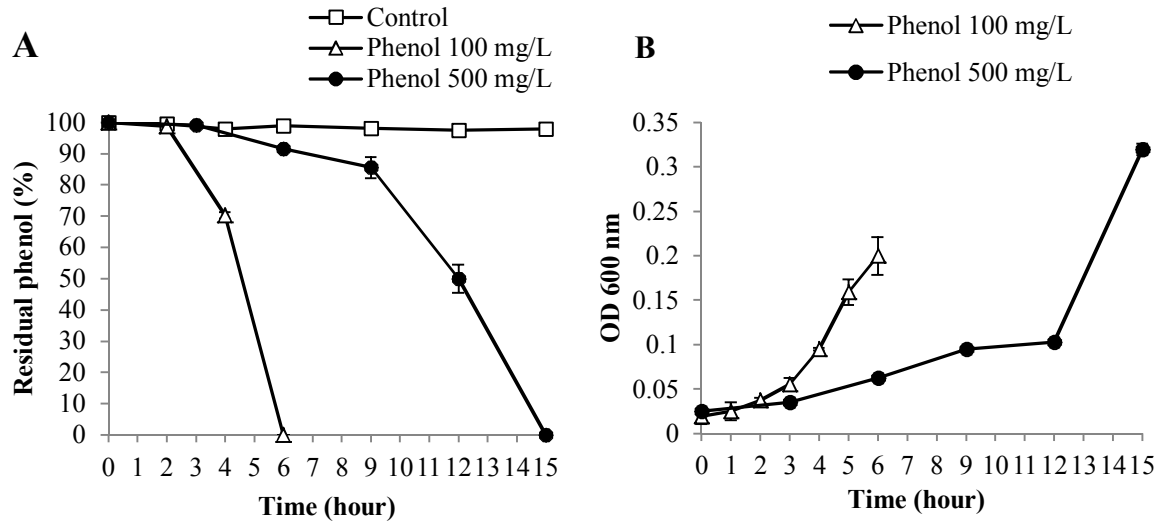

Figure 1: A. degradation of phenol and **B.** growth curves of *Acinetobacter* sp. OPB in concentrations of phenol (100 and 500 mg L^{-1}) after cultured in CFMM media at 30°C for 15 hour.

Rhizoremediation of phenolic compounds in POME irrigated soil

To examine the enhancing ability for rhizoremediation of phenolic compounds, *Acinetobacter* strain OPB was inoculated in both planted and unplanted soil. Phenolic

compounds removal (%) from water leachate at the end of each irrigation cycle is shown in Fig. 2A. Initial phenolic compounds in POME were 25 mg, 26 mg and 24 mg in first, second and third irrigation respectively. Phenolic compounds removal (%) significantly increase in both planted and unplanted soil with inoculated bacteria, but more noted in soil that plant roots were presented. The most effective of phenolic compounds removal (%) were show in treatment that plant and inoculated bacteria interaction (MB) with 78-82% of phenolic compounds removal, which significantly higher than control soil (S) (55-65%). These results showed that bioaugmentation of this bacterial strain during rhizoremediation could be more effective tool. Glick (2010) also suggested that Bioaugmentation with biodegradative bacteria, plant growth-promoting bacteria and bacteria that facilitated phytoremediation could overcome some limitation such as changing pollutant form to increase bioavailability for plant and increasing plant tolerate to various environmental stress.

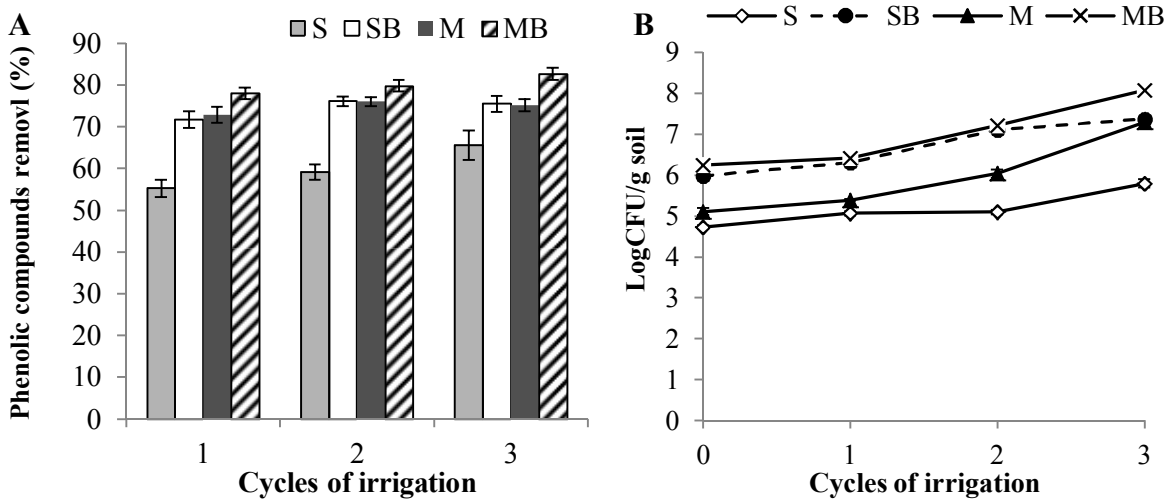

Figure 2: A. Phenolic compounds removal (%) and B. phenol degrading bacteria in Soil (S), Soil with bacteria inoculation (SB), Mulato grass (M) and Mulato inoculated with bacteria (MB).

Phenol degrading bacteria were counted every cycle of soil irrigation and numbers of bacterial are showed in Fig. 2B. After 3 cycles of soil irrigation, the results show that phenol-degrading bacteria of the soil planted with Mulato were increased from 4.9 log CFU g^{-1} soil to 5.1 log CFU g^{-1}, 6.0 log CFU g^{-1} and 7.4 log CFU g^{-1} soil in planted soil (M), while soil that inoculated with bacteria and planted (MB) were 6.3 log CFU g^{-1}, 7.2 log CFU g^{-1} and 8.1 log CFU g^{-1} soil in the first, second and third irrigation cycles, respectively. The values were significantly higher than control soil in the third irrigation cycle (5.79 log CFU g^{-1} soil). This result concluded that phenol-degrading bacteria increased with the decreasing of phenolic compounds. Thus both of inoculated and indigenous soil bacteria could be stimulated by plant roots. This consists to report from Gaskin et al. (2010) suggested that Australian native grasses can stimulate the rhizosphere microbial community capable of degrading aliphatic hydrocarbons in soil. The plant roots are known to release specific carbon sources such as sugars and amino acids that can stimulate rhizosphere microorganism to degrade organic contaminants in the soil (Khan et al. 2013).

Phytotoxicity study

Toxicity of the leachate from each treatment was examined. Sample with GI % value more than 50 % were consider as non-toxic (Anastasi et al. 2011). The water leachate from three

cycles of irrigation were tested with mungbean and cucumber seeds germination and compared to untreated POME (negative control) and deionize water (positive control). Results from Table 1. showed the percentage of germination index (GI %) was increased from 15-18% (untreated POME) to 66-89% after treat with plant and inoculated bacteria, whereas water leachate from uninoculated soil (S) were only 33-42 % and 16-21% for mungbean and cucumber seeds respectively.

Table 1: Phytotoxicity test using mungbean and cucumber seed germination index (%)

Sample	Germination index (%)					
	First irrigation		Second irrigation		Third irrigation	
	Mungbean	Cucumber	Mungbean	Cucumber	Mungbean	Cucumber
DI	100	100	100	100	100	100
POME	15.9±0.6	11.4±0.3	18.3±0.3	11.9±0.3	17.6±0.3	12.5±0.2
S	39.5±0.7	16.9±1.3	42.9±0.6	21.4±0.2	33.6±0.6	16.7±1.3
SB	75.0±0.8	41.1±0.7	76.5±0.4	41.5±0.6	66.4±0.9	50.2±0.5
M	85.2±0.8	65.0±0.6	82.6±0.5	65.2±0.4	83.2±1.0	65.6±0.4
MB	86.1±0.9	67.3±0.4	87.6±0.5	69.4±0.5	89.2±0.4	68.8±0.5

POME = palm oil mill effluent before treat, DI = deionize water, S = Control uninoculated soil, SB = soil inoculated with bacteria, M = Mulato, and MB = Mulato inoculated with bacteria. Values represent mean ± standard deviation

This result confirmed that water leachate from control soil (unplanted) was toxic, while soil with planted grasses and added phenol-degrading bacteria showed the most effective detoxification of POME.

Conclusion

Acinetobacter sp. strains OPB rapidly and completely degraded phenol in synthetic medium. Bioaugmentation of this bacterium in soil irrigated with POME not only increased the efficiency of phenolic compounds removal but also decreased toxicity of degradation product when compared to control soil. The biological activities were enhanced in soil with grasses and the number of phenol-degrading bacteria was significantly higher than that in unplanted soil. This result suggested that plant roots supported conditions that stimulated rhizosphere microorganisms and maintained the added bacteria population. Further research will study the optimum conditions related to synergistic between Mulato grass and their rhizosphere microorganisms and the pollutant remediation efficiency.

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