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RESEARCH ARTICLE

EFFECTIVENESS OF CRUDE OIL DEGRADING FUNGI ISOLATED FROM PETROLEUM HYDROCARBON CONTAMINATED SOIL IN SIAK, RIAU

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ABSTRACT

Background: Biodegradation of petroleum hydrocarbon needs a specific technique called bioremediation to remove the environmental pollutants. Several indigenous microorganisms including fungi, bacteria, and actinomycetes are effective agents in degrading petroleum derivatives, aliphatic and polyaromatic hydrocarbons (PAHs).

Objective: This research aimed to investigate indigenous fungi isolates from petroleum hydrocarbon contaminated soil in Siak which are capable to degrade hydrocarbon.

Methods: The competence of indigenous fungi was isolated from a crude oil-contaminated soil which collected from one of oil-field in Siak, Riau. The effectiveness of isolates on the degradation crude oil was tested by culturing the isolates in Bushnell-Haas broth containing crude oil (5% v/v) for 16 days. A decrease in pH, change in optical density and amount of CO_2 released were recorded to indirectly indicate the crude oil degradation by the fungi. To measure the percentage of crude oil biodegradation, gravimetric analysis was utilized.

Results: The two colonies were selected and identified as *Aspergillus sp* LBKURCC151 and *Penicillium sp* LBKURCC153. The results showed that *Aspergillus sp* LBKURCC151 reached a higher level (61%) of biodegradation after 16 days under the optimum conditions in degrading total petroleum hydrocarbon than *Penicillium sp* LBKURCC153 (46%).

Conclusion: These results indicated that *Aspergillus sp* LBKURCC151 and *Penicillium sp* LBKURCC153 are potential degraders for bioremediation in crude oil-contaminated area.

Keywords : Biodegradation, Bioremediation, Crude oil-contaminated soil, Indigenous fungi

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INTRODUCTION

Petroleum is one of the most important resources in today's industrial economy. However, oil sludge contaminant is an issue that every petroleum industry must face because mainly its quantity. Sludge oil contains different hydrocarbon as well as non hydrocarbon molecules. The toxicity of oil sludge contaminant can cause health effects.[1]

Nowadays hydrocarbon removal is possible using different techniques. Although physical and chemical technique is mainly used, Bio-based systems offer interesting alternatives being an economic and environmentally friendly method. The specific technique used to degrade the environmental pollutants is called bioremediation.[2]

Riau is one of Indonesia's province with large biodiversity can provide a huge amount of microorganisms isolate source. Indigenous fungi are suitable candidates to clean up the crude oil-contaminated soil. They are known to perform chemical modification and their apical growth mode enables them to reach inaccessible soil regions. These favorable properties are often associated with their ability to grow in environments with low nutrients concentrations, low humidity, and acidic pH.[3]

There are several genus of *Aspergillus* sp, *Fusarium* sp and *Penicillium* sp that have good prospect for bioremediation application in Iraq.[4] Result of isolation and identification for indigenous potential microorganisms from bacterial and fungal group from oil contaminated soil located at Cepu East Java showed fungal isolates identified as *A. fumigatus* (FMC2) *and A. niger* (FMC6) have the potential to degrade hydrocarbon.[4,5] It would be interesting to find the potential indigenous fungi from Siak which are capable to degrade hydrocarbon.

MATERIAL AND METHODS

Soil and Crude Oil Samples

Petroleum hydrocarbon-contaminated samples were collected from one of Oil and Natural Gas Corporation oil-fields, Siak, Riau. Soil sample was collected at a depth of < 1 m from the surface of soil used a hand auger and placed into a sterile container.

Crude oil, which was used to test capability of fungi isolates on biodegradation, was obtained from oil fields in Zamrud, Siak that produce Sumatra crude oil.

Isolation of Strains from Contaminated Soils

Fungi capable of degrading crude oil were isolated by using minimal medium of the following composition (in g L-1 distilled water) : KNO_3, 1; K_2HPO_4, 1; NaCl, 0.1; MgSO4, 0.2; trace of $CaCl_2$ 0.1; $FeCl_3$ 0.02 g. The Minimal Medium (MM) chemicals were from Sigma Aldrich. Medium contained 250 mg L-1 chloramphenicol. A quantity of crude oil-contaminated soil was added into 50 mL sterilized MM containing 1 % (v/v) crude oil in an Erlenmeyer flask. Flask was shaken for 2 weeks at 150 rpm to homogenize the medium for microbial enrichment.[6]

Crude oil-degrading fungi were isolated using dilution plate method. Sodium chloride 0.85 % was used as diluents for inoculums preparation. An aliquot of 1 mL enriched culture was inoculated into a sterile test tube containing 9.0 mL of the diluents. This

gave 10^{-1} dilution. Subsequently, seven-fold (10^{-7}) serial solutions were prepared from the 10^{-1} dilution. 1 mL of dilution was poured on Potato Dextrose Agar (PDA) medium. Plates and media were incubated at 25^0C in the dark. Selected colonies are transferred to appropriate media to allow fungal development. Stock cultures were maintained on the PDA slant, subcultured periodically and store at 4^0C.

Fungal Growth on Crude Oil

All isolated fungi were tested for their capabilities to grow on crude Sumatera light oil as sole carbon source. Fungi were pre-grown in 9 cm diameter Petri dishes containing PDA Medium and incubated at room temperature in the dark for three days. Mycelium disks (5 mm diameter) were taken from the margin of the actively growing colonies, and inoculated in 9 cm plates containing Bushnell-Haas medium[7] added with 15 g L-1 agar, 0.1%(v/v) tween 80 and 5% (v/v) crude oil and without crude oil (control). Plates were inoculated in triplicates and incubated in the dark at room temperature and the mycelium growth were evaluated after 7 days. Two of the highest diameter grown fungal colonies were selected for further experiments.

Identification of The Fungal Isolate

Lacto Phenol Cotton Blue (LPCB) was employed for microscopic observation. The characteristics were compared with the standard description of "A Manual of Soil Fungi"[8].

Crude Oil Degradation Assay

Two strains were selected from the previous experiment and tested for their capability to degrade crude oil in liquid culture by determining the following parameters. Fungi were inoculated as two agar plugs (5 mm diameter) to 98 mL Bushnell Haas Broth medium. This medium was then supplemented with 2 mL crude oil in 250 mL flask. The control flask had no organisms. Incubation was at room temperature (28-30°C) with constant shaking at 180 rev/min for 16 days and all cultures were performed in triplicate.

To measure pH, pH of the fermented broth collected aseptically from each fungal isolated was determined using pH meter after 0, 4, 8, and 16 days of treatment. To measure Optical density determination, the optical density of the fermented broth from each fungal isolated was determined after 0, 4, 8, and 16 days of treatment at 620 nm using a spectrophotometer. To measure CO_2 estimation, one mL of the fermented broth was taken after 4, 8, 12 and 16 days of treatment and titrated against 0.05 N NaOH solution. Two to three drops of phenolphthalein were used as the indicator and appearance of stable pink color was considered as the endpoint. The following formula can be used to calculate the amount of free CO_2. [9]

Free CO_2 (g L-1) :

$$= \frac{Titre\ value\ x\ Normality\ of\ NaOH\ x\ 1000\ x\ 44}{Volume\ of\ sample}$$

To measure the percentage of biodegradation by crude oil, the extracted crude oil was analysed using gravimetric analysis.[10] After 16 days of time interval, all flasks were taken out and microbial activities were stopped by adding 1% 1N HCl. Culture broth was mixed with 10% (v/v) hexane in separating funnel and was shaken. The extracted oil was passed through anhydrous sodium sulphate to remove moisture. The hexane was evaporated on 600C water bath using rotary evaporator. The following formula

can be used to calculate the percentage degradation of crude oil.[11]

$$\%\ Biodegadation = \frac{Wa - Wb}{Wa} x\ 100\%$$

Where Wa : is weight of crude oil (initial) and Wb : is weight of crude oil (after treatment).

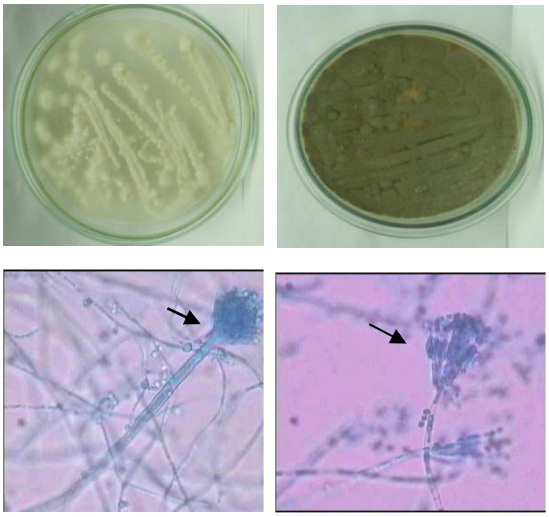
RESULTS

Two fungi strains were isolated from crude oil contaminated soil in siak Riau. The fungi were identified as *Aspergillus* sp LBKURCC151 (figure-1A) and *Penicillium* sp LBKURCC153 (figure-1B) based on the microscopic and macroscopic observation. The fungi were grown on PDA, and their cultural and morphological characteristics are summarized in Table 1.

Table 1. Cultural and morphological characteristics of the isolates on Potato Dextrose Agar (PDA)

Observation		Identified Fungi
Cultural Characteristics (Macroscopic)	**Mycellial and sporulation morphology (Microscopic)**	
Rapidly growing, cottony, white colony	Septate, sterigma arising from conidiophores terminated	*Aspergillus, sp* LBKURCC151
Rapidly growing, greenish yellow colony	Branching, septate, conidiophores, sterigma arising from conidiophore metulae	*Penicillium, sp* LBKURCC153

The two strains that displayed the highest diameter growth in previous experiment were studied for their capability to degrade crude oil in a liquid medium. The ability of the two selected fungal isolates in degrading crude oil were evaluated through three parameters: 1) the change in pH of fermented medium (Figure 2); 2) the growth of fungi as detected by optical density (Figure 3); 3) CO_2 released during degradation assay (Figure 4). Furthermore, percentage of biodegradation was determined by gravimetric analysis in final fermented day (Figure 5). Discoloration of fermented media showed in figure 6.



(A) (B)

Figure 1. Colony surface and microscopic observation of *Aspergilus sp* LBKURCC151 on PDA**(A)** *Penicillium sp* LBKURCC153 on PDA**(B).**

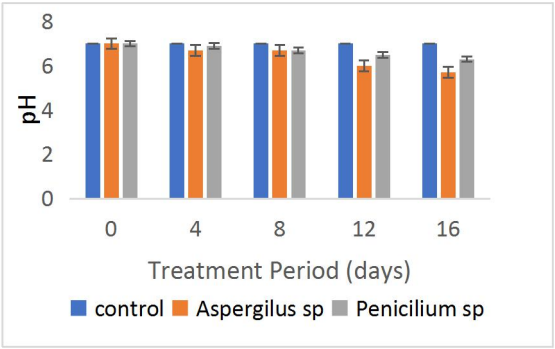


Figure 2. Changes in pH of the medium during the degradation of petrol in 16 days, Data are shown as mean ± standard deviation, n=3".

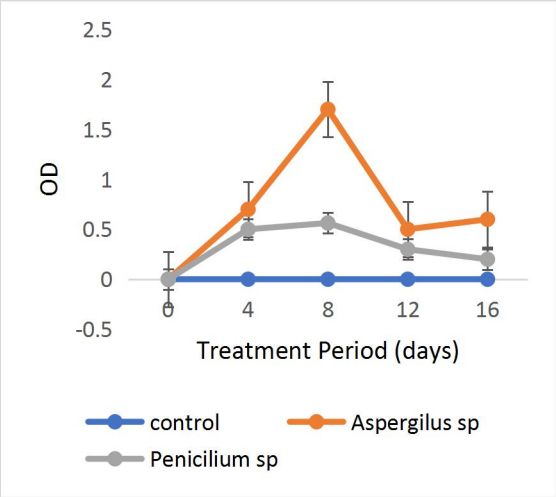


Figure 3. Changes in optical density during the degradation of crude oil at 620 nm. Data are shown as mean ± standard deviation, n=3".

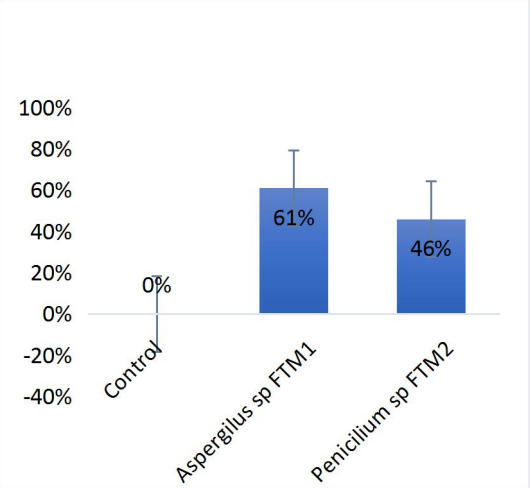


Figure 5. Crude oil degradation percentage of *Aspergillus* sp LBKURCC151, *Penicillium,* sp LBKURCC153. Data are shown as mean ± standard deviation, n=3".

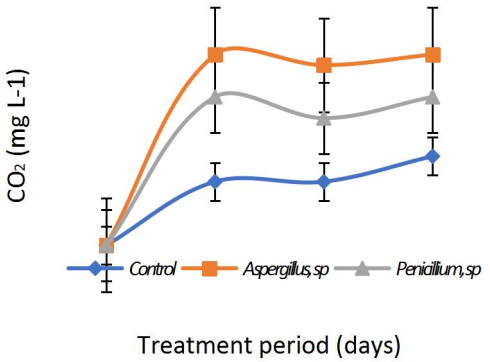


Figure 4. Carbon dioxide released (mgL^{-1}) during the degradation of crude oil. Data are shown as mean ± standard deviation, n=3".

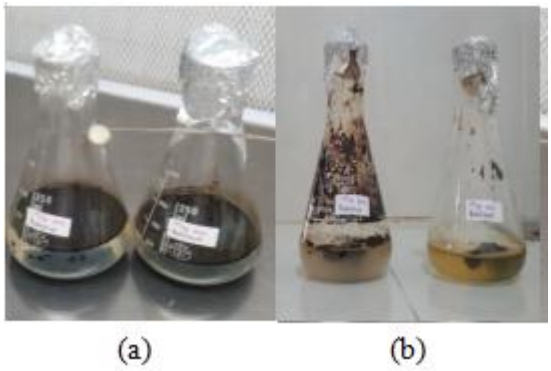


Figure 6. Biodegradation of crude oil by *Aspergillus* sp LBKURCC151 and *Penicillium* sp LBKURCC153 (a) 0 day incubation (b) after 16 day incubation.

DISCUSSION

Aspergillus and *Penicillium* have been frequently reported as filamentous fungi which can remove hydrocarbons.[12] These fungi were able to grow and display highest diameter growths in Bushnell-Haas agar medium containing 5% (v/v) crude oil as sole carbon source, but with different efficiency compared to controls. These fungi isolates formed a fine and uniform mycelium compared to their growth in BHM agar without addition of 5% (v/v) crude oil.

Figure 2 depicts the variations in the pH of medium during the fermented period and pH was found to be decreasing gradually from 7 – 5.7 and 7- 6.3 for *Aspergillus* sp LBKURCC151 and *Penicillium* sp LBKURCC153, respectively. The acidic environment might indicate the degradation of crude oil and a higher catabolic activity causes the pH to decrease within 16-day incubation period.[13] *Aspergillus* sp LBKURCC151 grew better in acidic condition than *Penicillium* sp LBKURCC153. Previous study also reported that several fungal isolates such *Aspergillus*

niger, *Trichoderma viride* and *Fusarium solani* showed good growth in culture medium at pH 5.5.[14] Many species can metabolize over a wide pH range from highly acidic to extreme alkaline. Fungi have higher insensitivity to high hydrogen ion concentration and narrow pH range than bacteria. Decreasing pH levels probably is caused by organic acids production and other metabolic products by microbial degradation of hydrocarbon.

Figure 3 shows the variations in the optical density which seems to be fluctuating change in optical density at 620 nm were recorded after 4, 8, 12 and 16 fermented days. The growth rate of each fungus had different maximum growth peaks. *Aspergillus* sp. LBKURCC151 had a maximum growth on the 8th day at 1.701 while *Penicillium* sp. LBKURCC153 had a maximum growth at 0.563. There were wide fluctuations in the growth of each fungus. The change in optical density could be attributed to the fact that they used the hydrocarbons as substrates for growth by probably releasing extracellular enzymes and acids which are capable of breaking down the recalcitrant hydrocarbon molecules, dismantling the long chains of hydrogen and carbon, thereby, converting petroleum into simpler forms or products that can be absorbed for the growth and nutrition of the fungi. This result was similar to previous research which shows that growth pattern of fungi in minimal salt had fluctuations.[15]

Figure 4 shows an increase in CO_2 released during the degradation of crude oil by these fungi. Release of carbon dioxide during the degradation can be used as an indication for activity of fungi in the growth media. *Aspergillus* sp LBKURCC151 showed more CO_2 production than *Penicillium* sp LBKURCC153. In the previous study, *Penicillium* sp could decompose the substrate hydrocarbon and release more CO_2 because of enzymes that were produced at a faster rate. Therefore this fungus can be utilized effectively as an agent of petrol degradation.[8] A large amount of CO_2 was liberated in BHM with isolates than in control medium. Respiration of microorganisms occur very rapidly during the initial period of incubation when the lighter and more readily degraded fractions are degraded but slow down as the residue becomes more difficult to degrade on account of the increase of heavier fractions eg. aromatic compounds.

The ability to analyses crude oil compounds into its components leads to oxidation of the carbon source in the crude oil components. Figure 5. *Aspergillus* sp LBKURCC151 and *Penicillium* sp LBKURCC153 were the fungi isolates demonstrating active ability to biodegrade crude oil in this study. There was a great difference between the fungi isolates that were inoculated in Bushnell-Haas broth media amended with 5% (v/v) crude oil compared to those inoculated in Bushnell-Haas broth media without 5% (v/v). The highest percentage of crude oil concentration decrease was by culture *Aspergillus* sp LBKURCC151 with 61% while *Penicillium* sp LBKURCC153, with 46%, had the least ability to degrade after 16 days under agitation condition of treatment. This result was similar with previous research which showed that *A.niger*, *A.fumigatus*, *P.funiculosom* exhibited biodegradation of hydrocarbons higher than other strains when inoculated in mineral salt media with 2% crude oil, percentage of loss of concentration of crude oil reached 95%, 75%, and 65%, respectively.[4] In a similar study, *Aspergillus niger* recorded the highest weight losses of 8.6%

and *Penicillium documbens* (7.9%) after 3 weeks of incubation.[7] In earlier study also showed the same results with *Aspergillus niger* had highest percentage of hydrocarbons degradation.[11] *Aspergillus* species are the most efficient for utilizing hydrocarbons in crude oil compared to different genera.[9]

In this study, two fungi isolates were able to grow in 5% (v/v) and have capability to degrade crude oil which showed discoloration of fermented media (figure 6). Their capacity to remove hydrocarbon could be due to adaptation of these fungi to the pollutant composition, as well as to the enzymatic systems of the fungi.[16]

CONCLUSION

The data contained in this study show that two indigenous fungi isolates of crude oil contaminated soil have the potential in degrading petroleum hydrocarbon pollutants. This results confirmed by gravimetric analysis that have biodegradation percentage of *Aspergillus* sp LBKURCC151 and *Penicillium* sp. LBKURCC153 isolates reached 61% and 46%, respectively. To implement the isolates or their products, e.g., enzymes as bioremediating agents, through studies on optimal growth conditions, degradation mechanism and microorganisms consortium are necessary.

Conflict of Interest

The authors declare that there is no conflict of interest degrading the publication of this paper.

Acknowledgment

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