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Isolation of crude oil degrading bacteria and classification by fluorescence *in situ* hybridization

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Abstract : Crude oil contamination occurs during petroleum production. It affects the surrounding area of the operating sites. High level of crude oil contamination could affect the consumer's health. This study focuses on isolation and classification of bacteria in crude oil contaminated soil that show a potential in bioremediation and can be used in the future. Crude oil contaminated soil at the study site was collected. One gram of soil samples was used for isolation by enriching in Luria broth. Selective media, Bushnell Haas, with 1.0% crude oil were used to isolate crude oil degrading bacteria at 37°C in aerobic condition. Forty-eight isolates of crude oil degrading bacteria were isolated which could be divided into 27 Gram positive and 21 Gram negative bacteria. Bacterial cells were fixed on slides before adding 4 different DNA probes; HGC69a LGC354afor Gram positive and BET42a GAM42a for Gram negative bacteria. The hybridization was conducted at 46°Cfor 90 min and analysed under the fluorescence microscope.The result found that the highest number of bacteria from the isolation belong to phylum Proteobacteria and class *Gammaproteobacteria* for 33.33% and the rest of them belong to phylum Actinobacteria Firmicutes and others.

Keywords : crude oil, degradation, hybridization, Proteobacteria

Introduction

Microorganisms that have potential for hydrocarbon degrading are bacteria such as *Pseudomonas* and *Bacillus.* In the recent research, *Pseudomonas* is recorded that can degrade 49.93% of crude oil in 20 days (Panda *et al*., 2013). *Bacillus* is the effective genus which shows the capacity of crude oil degradation and found in high concentration of crude oil contaminated soil (Ijah and Antai, 2003). In addition, *Corynebacterium, Flavobacterium, Micrococcus*, *Rhodococcus, Acinetobacter* and *Burkholderia* (Sathishkumar *et al*., 2008 and Tanase *et al*., 2013)were also found the crude oil degradation ability. Beilen *et al*., (2006) gave the explanation about this ability which is the consequence of alkane monooxygenase, enzyme that can degrade alkane compounds in aerobic condition. Moreover, fungi, for example, *Fusarium* also can degrade poly cyclic aromatic hydrocarbon which is one of the components of crude oil (Olivier *et al*., 2004).

The knowledge about bacteria in natural environments is limited, and studying microbial diversity in nature is not an easy task. In natural ecosystems, microorganisms exist in high numbers despite the fact that there are several thousands of microbial species that have not yet been described. One gram of soil or sediment may contain $l0^{10}$ bacteria as counted by fluorescent microscopy after staining with a fluorescent dye. In pure sea water, the number of bacteria is approximately 10^6 per millilitre (Torsvik *et al*., 1990). Nucleic acid hybridization using specific probes is an important qualitative and quantitative tool in molecular bacterial ecology (Guo *et al*., 1997). These hybridization techniques can be done on extracted DNA or RNA, or *in situ*. (Theron and Cloete, 2000). One of the most popular DNA hybridization methods is FISH (Fluorescent *in situ* hybridization). Spatial distribution of bacterial communities in different environments such as biofilms can be determined using FISH (Schramm *et al*., 1996).

This study focuses on isolation and classification of bacteria in crude oil contaminated soil that shows a potential in bioremediation and can be used in the future.

Methodology

1. Enrichment

One gram of crude oil contaminated soil was mixed in distilled water and 5ml of distilled water was taken into Luria Bertani broth with 1% of crude oil, then incubated at 37°C for 48hr. Cultures were centrifuged at 5,000 rpm for 10 min and washed with 0.1M phosphate buffer solution (Modified from Mittal and Singh, 2009).

2. Isolation

Bacterial cells were inoculated in Bushnell Haas broth with 1% of crude oil which is the carbon source for bacteria and incubated at 37°C for 48hr. The medium composition is as follows: $MgSO_4$ (0.2 g/L), K_2HPO_4 (1.0 g/L), KH_2PO_4 (1.0 g/L), $FeCl_3$ (0.05 g/L), NH_4NO_3 (1.0 g/L) and $CaCl_2$ (0.02 g/L) with pH 7.2. Agar (20 g/L) was added for BH agar. Cultures were spreaded on Bushnell Haas agar and incubated at 37°C for 7 days after that cultures were kept in Nutrient agar slant at 4°C (Modified from Mittal and Singh, 2009 and Islam *et al*., 2013).

3. Cell fixation

Bacterial isolates were inoculated in nutrient broth at 37°C for 24 hr. Two milliliter of culture was taken for fixation. Ice-cold 99% ethanol 0.6 ml and 1xPBS 0.5 ml were added, then stored at -20°C for 1-2 hr for gram positive bacteria. Paraformaldehyde 1.5 ml and 1xPBS 0.5 ml were added and incubated at 4°C for 3hr. Then centrifuged at 10,000 rpm for 5 min and removed supernatant after that 1 ml of 1xPBS was added (repeat 1-2 times). Ice-cold 99% ethanol 0.6 ml and 1xPBS 0.5 ml were added, then stored at -20°C for Gram negative bacteria (Lee, 2013).

4. Fluorescence in situ hybridization

Teflon coated slides were washed with 70% ethanol and 1% HCl and dried. Fixed cells 5 µl were dropped on Teflon coated slides. Slides were dried at room temperature. Hybridization buffer (5M NaCl; 1M Tris/HCl; 10% (w/v) SDS; Deionize water) was mixed with 4 different DNA probes; HGC69a, LGC354a for Actinobacteria and firmicutes, respectively (gram positive bacteria) and BET42a, GAM42a for *Betaproteobacteria* and *Gammaproteobacteria*, respectively (gram negative bacteria). Master mix of hybridization buffer and DNA probes were added on slides 10 µl per well, then incubated at 46°C for 90 min. Slides were analysed under the fluorescence microscope and protected from directly light (Lee, 2013).

Results and Discussion

1. Isolation

Forty-eight of bacteria were isolated from crude oil contaminated soil by selective media, Bushnell Haas broth which indicated that forty-eight of bacteria could use crude oil as carbon source for growth (Bushnell and Haas, 1941).

In addition, this can be assumed that bacteria will have the ability of crude oil degradation and can be applied for bioremediation. After gram stain, bacterial isolates could be divided into 27 Gram positive and 21 Gram negative bacteria. Some characteristics were showed in Table 1.

2. Fluorescence in situ hybridization

The highest number of bacteria from the isolation which are 16 isolations belong to phylum proteobacteria and class *Gammaproteobacteria* for 33.33% and the rest of them belong to phylum Actinobacteria 18.75%, Firmicutes 2.08% and others. The examples of pictures from fluorescence microscope were shown below. (Figure 1 and Figure 2) *Gammaproteobacteria* were found the most from the isolation because plenty genera of this bacterial class were often found the ability for hydrocarbon degradation and also were used in bioremediation such as *Pseudomonas*, *Acinetobacter* and *Enterobacter* (Korda *et al*., 1997; Islam *et al*., 2013). *Gammaproteobacteria* were also found several in sludge wastewater. As *Gammaproteobacteria* are of substantial ecological significance. Therefore, GAM42a is a widely used and routinely applied FISH probe (Siyambalapitiya and Blackall, 2005).

However, there are the isolates that cannot be detected by used probe which can be caused by several reasons. First, non-specific probe cannot hybridize with the bacterial isolates. Another reason is lack of sensitivity of hybridization of nucleic acids which is the most notable limitation of nucleic acid hybridization methods. If sequences do not present in high copy number, such as those from dominant species, probability of detection is low (Fakruddin and Manna, 2013).

Conclusion

Forty-eight of bacteria were isolated from crude oil contaminated soil which could be divided into 27 Gram positive and 21 Gram negative bacteria. The highest number of bacteria from the isolation which are 16 isolations belong to phylum Proteobacteria and class *Gammaproteobacteria* for 33.33% and the rest of them belong to phylum Actinobacteria 18.75%, Firmicutes 2.08% and others.

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Table 1. Colony morphology, cell shape and Gram stain of bacterial isolation

Isolate	Colony morphology			Shape	Gram stain
	Form	Margin	Elevation		
C01	circular	entire	convex	rod	+
C02	circular	entire	convex	rod	+
M01	circular	undulate	raised	rod	+
M02	circular	entire	raised	rod	+
M03	spindle	undulate	flat	rod	-
M04	irregular	undulate	flat	rod	-
M05	irregular	undulate	flat	rod	+
M06	circular	entire	convex	rod	+
M07	Circular	undulate	flat	rod	-
M08	circular	entire	flat	rod	-
M09	circular	entire	flat	rod	+
M10	circular	undulate	flat	rod	-
M11	circular	undulate	flat	rod	+
M12	spindle	undulate	flat	rod	+
M13	circular	entire	flat	rod	-
M14	circular	entire	flat	rod	+
M15	circular	entire	flat	rod	+
M16	circular	entire	flat	rod	-
M17	circular	entire	flat	rod	+
M18	circular	entire	flat	rod	+
M19	spindle	entire	flat	rod	-
M20	circular	entire	flat	cocci	-
M21	circular	undulate	flat	rod	+
M22	circular	undulate	flat	rod	-
M23	spindle	undulate	flat	rod	+
S101	circular	entire	flat	rod	+
S102	spindle	undulate	flat	rod	-
S104	circular	undulate	flat	rod	+
S105	circular	undulate	flat	rod	+
S106	spindle	undulate	flat	cocci	+
S107	circular	entire	flat	rod	+
S108	circular	entire	flat	rod	-
S111	circular	erose	flat	rod	+
S112	spindle	erose	flat	rod	+
S113	circular	entire	flat	cocci	+
S114	spindle	erose	flat	rod	+
S115	circular	undulate	flat	rod	+
S116	circular	entire	convex	rod	+

Table 1. Colony morphology, cell shape and Gram stain of bacterial isolation (continued)

Isolate	Colony morphology			Shape	Gram stain
	Form	Margin	Elevation		
S117	circular	entire	flat	rod	-
S118	circular	entire	convex	rod	-
S119	circular	entire	convex	rod	-
S120	circular	erose	flat	rod	-
S121	circular	erose	flat	rod	+
S122	circular	erose	flat	rod	+
S301	circular	entire	raised	rod	+
S303	circular	undulate	flat	rod	+
S304	circular	entire	flat	rod	+
S302	circular	entire	raised	rod	-

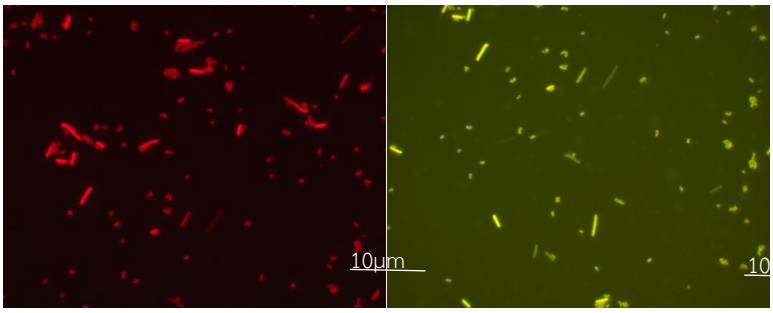

Figure 1. Positive control of Firmicutes (left) and Actinobacteria (right)

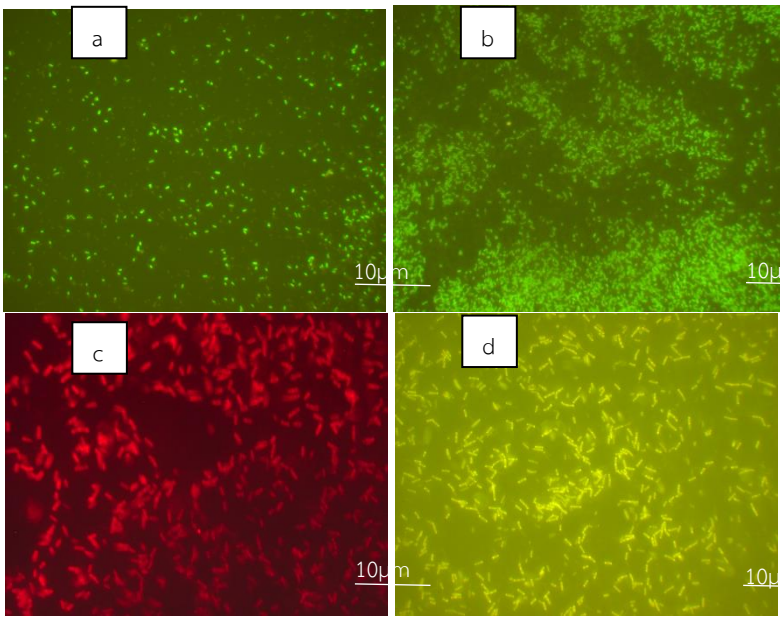

Figure 2. Bacteria of isolation show fluorescence color of Gammaproteobacteria (a,b), Firmicutes (c) and Actinobacteria (d)