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Abstract

The main purpose of study was conducted to isolate PAHs-degraders strain from Donan river mangroves ecosystem and to investigate the ability of isolated pure culture to degrade PAHs. The potential use of these marine bacteria as the environment clean-up agents was conducted by sublimizing with the 6 different Polyaromatic hydrocarbons (PAHs) compounds as a contaminant model such as phenothiazine, fluorene, fluoranthene, dibenzothiophene, phenathrene, and pyrene. The 16S rDNA amplification using primer 9F and 1510R has been applied and purification was made on the agarose (1 %). Sequenced results were obtained by comparing to NCBI Blast. Three rods shape Gram-positive endospore-forming bacteria were isolated from a mangrove site which is chronically contaminated from petroleum hydrocarbons. On the basis of phenotypic and phylogenetic data, three strains should be placed in the genus *Bacillus* as three distinct species, for which the names *Bacillus aquimaris, Bacillus megaterium*, and *Bacillus pumilis* are proposed. The other three strains were *Flexibactereae bacterium, Halobacilus trueperi*, and *Rhodobacteraceae bacterium.*

Keywords : *marine pollution, crude oil, persistent, hydrocarbonoclastic marine bacteria*

INTRODUCTION

In marine ecosystem, when oil spilled, thousands compound from petroleum hydrocarbons would be naturally dispersed and degraded in a few years. The aromatic compounds from a petroleum complex mixture, however, especialy the polycyclic aromatic hydrocarbons (PAHs) is recognized as an intermediate biodegradable compound, but these are most concerned due to their toxicity and tendency to persist in the environment (Sutherland *et al.*, 1995). In nature, biodegradation is a promising process for responding to contamination by petroleum hydrocarbon. The ability of microorganisms to degrade hydrocarbons and facilitate their mineralization by forming more labile organic compound through the breakdown of intra-moleculars bonds, has been extensively studied (Madsen, 1991). As a result microorganisme has contributed to the development of different bioremediation technologies. The main purpose of this research is to isolate PAHs -degraders strain from mangrove ecosystems and to investigate ability of a pure culture of selected bacterie i.e. *Bacillus megaterium*) to degrade PAHs.

METHODOLOGY

Bacterial isolation and culture conditions.

The whole bacteria were isolated from chronically contaminated sediment by petroleum hydrocarbons of a mangrove's tidal flat, Cilacap coastal. Isolation was conducted by the dilution plating technique on marine agar (MA) (Difco). The cell biomass of bacteria for DNA extraction analyses was obtained from marine broth (MB) (Difco) cultures at 30 ^{0}C. The cultures were agitated on a horizontal shaker at 150 r.p.m. and broth cultures were checked for purity by microscopic examination before being harvested by centrifugation. The isolated bacteria from sediment are purified; these pure cultures are then morphologically and physiologically verified. For non fastidious gram negative rods not belonging to the *Enterobacteriaciae*, the pure are then re-subculture on marine agar. After 3 x 24 hours the pure culture are then tested using 8 conventional test and 12 assimilation test.

Morphological and physiological characterization.

Cell morphology was examined by light microscopy. The cells were negatively stained with 1% (w/v) phosphotungstic acid and, after air drying, the grids were examined using a model CM-20 TEM (Philips). The Gram reaction was determined using the bioMe'rieux Gram stain kit according to the manufacturer's instructions. Nitrate reduction was determined as described by Lanyi (1987), using potatium nitrate as a substrate. Indole production was investigated with aid of tryptophane as substrate.

Acid production from carbohydrates was determined as described by Leifson (1963) and using the API 50CH system (bioMerieux) *i.e.* glucose. Urease activity was determined as described by Cowan & Steel (1965). Hydrolysis of aesculine and gelatin were determined as described by Cowan & Steel (1965). Assimilations test were conducted for several subtrates such as glucose, arabinose, mannose, mannitol, n-acetylglucosamine, maltose, gluconate, caprate, adipate, malate, cyctate, and phenyl-acetate. The cell mass of microbiawas suspended in 2 ml artificial sea water which contained mineral salt medium (MSM) was composed of 23 g/L of NaCl, 0.75 g/L KCl, 5 g/L of Tris (hydroxymethyl) aminomethane, 1 g/L NH_4Cl, 3.9 g/L $MgSO_4$, 5 g/L $MgCl_2$, 1.5 g/L $CaCl_2$, 0.12 g/L K_2HPO_4, 0.002 g/L $FeSO_4$, $7H_2O$ (Syakti *et al.*, 2004).

16S rDNA sequencing and phylogenetic analysis.

Chromosomal DNA was isolated and purified according to Yoon et al. (1996). 16S rDNA was amplified by PCR using two universal primers 9F and 1510R. The PCR product was purified with a QIAquick PCR purification kit (Qiagen). The sequencing of the purified 16S rDNA was performed using an ABI PRISM BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems) as recommended by the manufacturer. The purified sequencing reaction mixtures were electrophoresed automatically using an Applied Biosystems model 377 automatic DNA sequencer. Alignment of sequences was carried out using CLUSTAL W software (Thompson *et al.*, 1994). Gaps at the 59 and 39 ends of the alignment were omitted from further analysis.

PAHs sublimation

Some PAHs were used as model organic contaminants to study the effects of petroleum hydrocarbons on marine sedimentary bacterial compartment. The culture were previously maintained in Marine agar, and then transferred to ONR7 media. Strain grown on ONR7 media are then sublimized with the following PAHs: Phenothiazine, Fluorene, Fluoranthen, Dibenzothiophene, Phenanthre and Pyrene. The procedure for sublimation follows Harayama *et al.* (2004).

RESULTS AND DISCUSSION

Microbial identification

The results of the physiological character and identification are presented in Table 1, The isolated microorganisms was identified through Gram staining, and 16S rDNA analyses. The physiological characteristic of isolated strain is further analyses with the API 2O NE,. The later method is used to rapidly verifying the physiological and enzymatic characters of isolated strain. API 20 NE provides information on physiology and enzymatic activities of those isolates which is very important for understanding the ecological role of those isolate in nature., Our finding result is dealing with other results from producing company (Biomeriux). For this reason, we conducted the analyses of 16S rDNA sequence from tnose sediments. Sequenced results showed six rods shape Gram-positive endospore-forming bacteria (Figure 1 and appendix). The culturable PAHs degraders belonged to four genera. On the basis of phenotypic and phylogenetic data, three strains should be placed in the genus *Bacillus* as three distinct species, for which the names *Bacillus aquimaris, Bacillus megaterium*, and *Bacillus pumilis* are proposed respectively for the isolated microorganisms from sediment S2, S3, and S6. 16S rDNA analysis placed three other interesting species were belong to genus of *Flexibacteraceae bacterium*, *Halobacillus trueperi*, and *Rhodobacteraceae bacterium* respectively S1, S4, and S5. The isolates in the similarity group had identical DNA bands based on the positions of the bands on the gels. The isolate with identical bands are generally assumed to be similar at the species or subspecies level (Gürtler and Stanisich, 1996; Jensen *et al.*, 1993). When growing on 10% TSA, the isolated groups of Bacillus (S2, S3, and S6) formed white pale colonies, while three other genera formed small orange, yellow, and opac colonies respectively for S1, S4 and S5.

Table 1. Summary result of characterization of the 6 culturable, PAHs-degrading bacterial isolated from surface sediment samples of Donan river mangrove swamps

Strain number	Taxon*	Differentiating characters
S1	*Flexibacteraceae bacterium*	Esculin, and p-nitrophenyl-β-D galactopyranoside are positive
	AY264841, 98 %	Glucose, mannose, maltose are weekly assimilated
S2	*Bacillus aquimaris* EU372864 99 %	Aesculin, hypoxanthine, tyrosine and xanthine are not hydrolysed.
S3	*Bacillus megaterium* EU869261 99 %	Arabinose is positive, Maltosa negative
S4	*Halobacillus trueperi* EU 624433 99%	Esculin and maltose are positive
S5	*Rhodobacteraceae bacterium* AJ871951 100%	Esculin, gelatin, p-nitrophenyl-β Dgalactopyranoside are positive
S6	*Bacillus pumilis* EU869282 99%	Arabinose is negative, urea and esculin are positive

*. Closest relative based on partial 16S rDNA sequence

PAHs degraders

PAH-degrading bacteria was isolated through shaken aqueous enrichment providing the PAHs as source of carbon and energy. The potential use of these marine bacteria as the environment clean-up agents was applied by sublimizing with the 6 different Polyaromatic hydrocarbons (PAHs) compounds as a contaminant model. The cultures were able to utilize phenothiazine, fluorene, fluoranthen, dibenzothiophene, phenanthre and pyrene. Positive degradation is shown by formation of clearing zone around colony or change of colony's color due to transformation of PAHs into other substances. The formation of clear zone is dependent on culture maintenance condition. The culture should be kept on PAHs containing media. Based on our experience the culture will lose their ability to degrade PAHs when the cultures are conserved in rich complex C-medium such as marine agar.

Bacillus megaterium

Our particular interset was to *Bacillus megaterium* which is potentially capable to use and then degrade four different PAHs such as phenothiazine, fluorene, fluoranthene, and e: phenanthrene. Little known that *B. megaterium* is PAH degrader. *B. megaterium* has low activity for the oxidation of the PAHs phenanthrene, fluoranthene and pyrene but protein engineering has proven increased PAHs oxidation (Carmichael *et al.*, 2001). M. megaterium is reported to degrade pyrene in a slurry phase (Gaskin and Bentham, 2005). There is no published information concerning *B. megaterium* able to degrade dibenzothiophene.

Table 2. PAHs sublimation test of six PAHs degraders

Identified Strains	PAHs					
	A	B	C	D	E	F
Flexibacteraceae bacterium	+	-	+	-	-	+
Bacillus aquimaris	+	-	-	-	-	-
Bacillus megaterium	+	+	+	-	+	-
Halobacillus trueperi	-	-	+	-	-	-
Rhodobacteraceae bacterium	+	+	-	-	+	-
Bacillus pumilis	+	-	-	-	+	-

Note : A: Phenothiazine, B: Fluorene, C: Fluoranthene, D: Dibenzothiophene, E: Phenenthrene, F: Pyrene

Potential extra-cellular product of *B. megaterium*

Hemolytic assay was performed in order to determine qualitatively the capacity of B. Megaterium to produce biosurfactant. The result showed formation of clearance zone encycle the colonies. B. megaterium can grow at 29°C -37°C but grew optimally at 37°C. The B. Megaterium was cultivated on a horizontal shaker with pH media setted up at 8 and 30‰ of salinity. During 7 days of course time, Biomass of B. megaterium culture was grown optimally at day of 5, where interfacial tension measurement reached its minimal value of 32.82 mN/m. This extent indicates potential biosurfactant production from Bacillus species (Cooper and Goldenberg, 1997). This result, even preliminary but promising in regard with the capability of B. megaterium produce an extracellular agents, which is useful to reduce interfacial tension. Recent study conducted by Thavasi *et a.l* (2007) stated the biosurfactant produced by B. megaterium was classified as a glycolipid with carbohydrate and lipid combination of 28:70%.

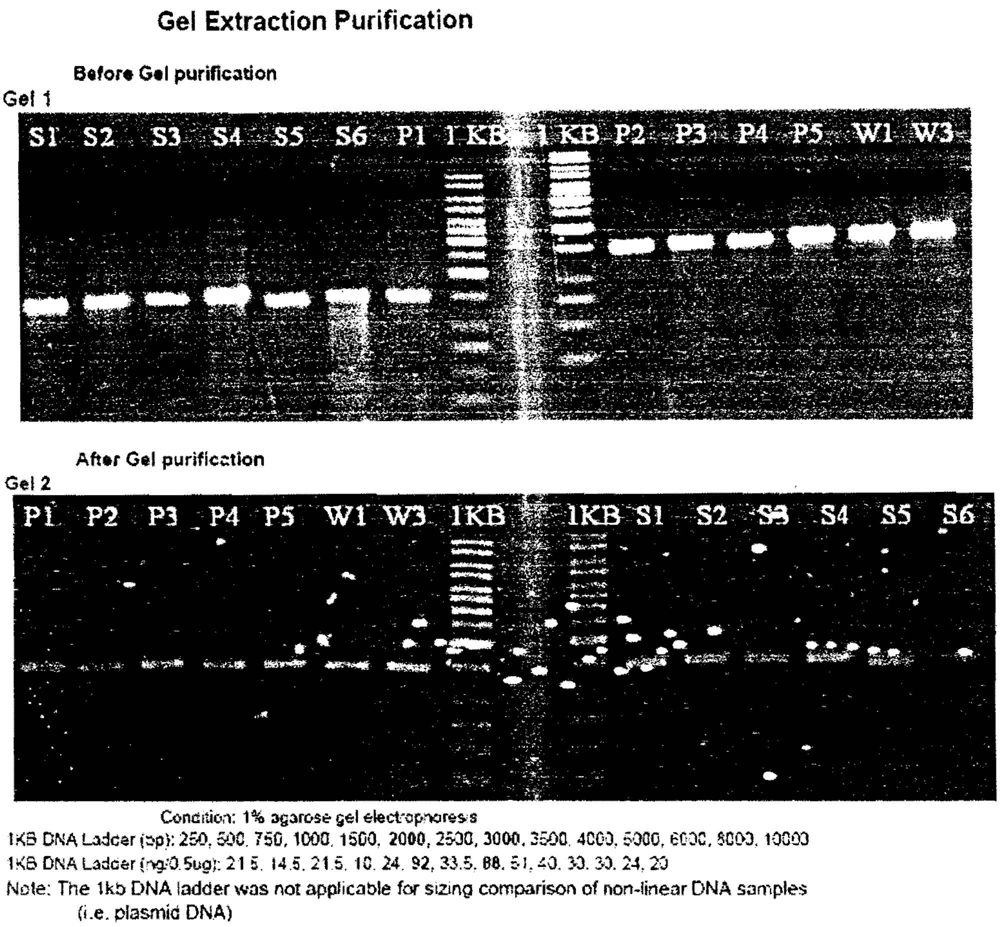

Figure 3. Gel extraction purification.

For the application of bioremediation, it is possible from this result is to enhance biological contamination of mangrove sediment at Donan river which is chronically contaminated by petroleum hydrocarbons, by adding an amount of massive culture of microbial consortia as well as their prospective production of biosurfactant. Thus, bioremediation is normally achieved by stimulating the indigenous microbiota from a contaminated site after culturing or naturally occurring microorganisms. Stimulation is achieved by the addition of growth substrates, nutrients, terminal electron acceptor, electron donors, or some combination therein, resulting in an increase in contaminant biodegradation and biotransformation (Harayama *et al.*, 1999).

Generally speaking, an extra-cellular microbial product has been shown to play key roles in optimalization into the overall clean-up process a contaminated sites leading to cleaner, faster, cheaper by bioremediation efforts. We have to mention here that the region where the research conducted is near from many heavy industries (*e.g.* raffinery, fertilizer, food, cement), therefore, the conducted research is one step ahead toward mitigation of environmental pollution.

CONCLUSION

We obtained six isolates of potentially PAHs-degraders from mangrove sediment of Donan river, Cilacap. The whole species were able to grow and has potential capacity to use PAHs as their sole of carbon and energy. Our particullar interest for *Bacillus megaterium* decided as regards their capacity to perform positive results of sublimation tests of PAHs (*i.e.* phenothiazine, fluorene, fluoranthene, and phenenthrene). Further studies will be performed to elucidate the fate of PAHs and structural characterization B. megaterium's produced biosurfactant.

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