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〈Original article〉

Characterization of Diesel Degrading *Enterobacter cancerogenus* DA1 from Contaminated Soil

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Abstract - The petroleum industry is an important part of the world economy. However, the massive exposure of petroleum in nature is a major cause of environmental pollution. Therefore, the microbial mediated biodegradation of petroleum residues is an emerging scientific approach used to resolve these problem. Through the screening of diesel contaminated soil we isolated a rapid phenanthrene and a diesel degrading bacterium identified as *Enterobacter cancerogenus* DA1 strain through 16S rRNA gene sequence analysis. The strain was registered in NCBI with an accession number MG270576. The optimal growth condition of the DA1 strain was determined at pH 8 and 35°C, and the highest degradation rate of the diesel was achieved at this condition. At the optimal condition, growth of the strain on the medium containing 0.05% phenanthrene and 0.1% of diesel-fuel was highest at 45 h and 60 h respectively after the incubation period. Biofilm formation was found significantly higher at 35°C as compared to 30°C and 40°C. Likewise, the lipase activity was found significantly higher at 48 h after the incubation compared to 24 h and 72 h. These results suggest that the *Enterobacter cancerogenus* DA1 could be an efficient candidate, for application through ecofriendly scientific approach, for the biodegradation of petroleum products like diesel.

Keywords : phenanthrene, *Enterobacter cancerogenus*, biodegradation, diesel, biofilm, lipase

INTRODUCTION

Environmental pollution is one of the major problems arising in the global economic world. One of the main causes of pollution is exposure to huge amounts of petroleum products (Deng *et al*. 2014). There is estimated to be seepage of 600,000 metric tons of natural crude oil per year worldwide (Kvenvolden and Cooper 2003). The persistence of hydrocarbon in the soil may cause the accumulation of pollutants in plants and animal tissue that may lead to mutation or even death (Alvarez and Vogel 1991). In this context, microorganism-oriented biodegradation of petroleum hydrocarbons has drawn a substantial amount of interest in the scientific field.

Several studies have reported on hydrocarbon degradation by microorganisms. Several genera of microorganisms such as *Pseudomonas aeruginosa*, *Stenotrophomonas acidaminiphila*, *Bacillus cibi*, *Bacillus megaterium*, and *Bacillus cereus* have shown oily sludge degradation (Cerqueira *et al*. 2011). Likewise, microorganisms such as *Bacillus pumilus*, *Bacillus megaterium*, *Stenotrophomonas maltophilia*, and *Pseudomonas aeruginosa* have been shown to promote biodiesel degradation (Meyer *et al*. 2012). It has also been reported that (*Agrobacterium*, *Pseudomonas*, *Bacillus*, *Burkholderia*, and *Sphingomonas*) strains oxidized phenanthrene (Aitken *et al*. 1998).

The biodegradation of petroleum hydrocarbons through microorganism application has attracted a great deal of interest due to the difficult biodegradation procedure (Deng

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et al. 2014). The process of biodegradation by microorganisms to remove the petroleum and other hydrocarbons is more economically viable than other remediation technologies (Das and Chandran 2011). Since the beginning of industrialization, huge amounts of organic compounds from petroleum have leaked into the environment, causing a serious environmental impact. For example, petroleum hydrocarbon compounds, which are important the energy sources used for industrialization, are exposed to the environment during the transportation of raw materials (Wei *et al*. 2005). Microbial application is considered to be the more practical approach for sweeping the petroleum hydrocarbons in the polluted areas (Cappello *et al*. 2007; Kubota *et al*. 2008). The most common environmental pollutants of petroleum hydrocarbons include aromatic hydrocarbon, n-alkane and cycloalkane (Deng *et al*. 2014). Bioremediation has been considered as one of the important strategies for the speedy elimination of pollutants. The mechanism for biodegradation involves mostly aerobic conditions and enzymatic reactions (Das and Chandran 2011). It has been previously reported that the bacteria that are able to degrade long-chain alkanes could be efficiently employed in the alleviation of environmental pollution (Wentzel *et al*. 2007).

Lipase has a ubiquitous distribution around the world's flora and fauna (Akram Kashmiri *et al*. 2006). The lipases are involved in the hydrolysis of ester bonds (Boczar *et al*. 2001) and fats (Langrand *et al*. 1990). Numerous species of yeasts, bacteria, and moulds produce lipases with various enzymological specificities and properties (Akram Kashmiri *et al*. 2006). Similarly, there have been numerous reports that have cited common lipase-producing bacterial strains such as *Pseudomonas fluorescens*, *P. aeruginosa*, *Bacillus cereus*, *B. coagulans*, *Staphylococcus aureus*, and *S. hyicus* (Simons *et al*. 1998). Lipase is reported to become involved in order to catalyse the esterification of carboxylic acid with the alcohols (Nielsen *et al*. 2008). Lipases are used as additives in the formulation of detergent, in waste treatment cocktails for downstream industrial processes, in cleaning solutions, and also for domestic use (Leonov 2010).

Lipases have been extracted from microorganisms such as bacteria, fungi, and yeasts (Saeed *et al*. 2005). The majority of lipases are mostly isolated from eukaryotic microorganisms of the genera of the fungi *Thermomyces*, *Mucor*, *Rhizopus*, and *Candida*, including some prokaryotes such as *Burkholderia* or *Pseudomonas* (Mrozik *et al*. 2008). Lipase regulates various metabolic processes such as those involved in lipid and lipoprotein metabolism, including fat digestion, reconstitution, and absorption in eukaryotes. In the case of plants, lipase resides in energy reserve tissues. Numerous species of yeasts, moulds, and bacteria produce lipases with different enzymological properties and specificities (Kashmiri *et al*. 2006). Lipases are categorized as the class of enzymes that catalyse the hydrolysis of long chain triglycerides. The microbial-oriented lipase has attracted much attention in the speedy development of the enzyme technology (Hasan *et al*. 2006). Lipase has been considered for years to be an important enzyme for the biodegradation of petroleum products (Resnick 1998; Riffaldi *et al*. 2006; Adekunle and Adebambo 2007).

Environmental pollution due to petroleum products is an emerging problem. In this context, the microorganism-mediated bioremediation tools are considered to be some of the most ecofriendly methods available for mitigating these problems. Taking this into consideration, we describe here the characteristics of such a novel microbial candidate that can efficiently boost the biodegradation of diesel.

MATERIALS AND METHODS

1. Isolation of diesel degrading bacteria in crude oil

10 g of oil-contaminated soil samples were shaken in 100 mL distilled water for 24 hours at 30°C and 150 rpm, and the supernatant was inoculated at 1°C in Minimal Salt Medium (MSM) medium supplemented with 1% diesel at 30°C and cultured for weeks. Then, 100 μL of the medium was plated on a solid medium (MSM broth, diesel 1%, agar 1.5%) containing 1% diesel, and cultured until a colony was formed. Table 1 below shows the culture medium used for culturing the colony formed by the pure separation of platinum. The sub-cultured culture medium was mixed with sterilized glycerol at a final concentration of 15−30% and stored at −80°C (Choi *et al*. 2010).

2. Identification of Bacteria

In order to amplify the 16S rRNA region of the strain, 518f primer (5′-CCA GCA GCC GCG GTA ATA C-3′) and

Table 1. Compositions of minimal salt medium (MSM) for bacteria culture.

Components	Concentration ($g\,L^{-1}$)	Components of trace element solution	Concentration ($g\,L^{-1}$)
Na_2HPO_4	0.86	$ZnSO_4 \cdot 7H_2O$	2.32
$NaNO_3$	0.85	$MnSO_4 \cdot 7H_2O$	1.78
KH_2PO_4	0.56	$CuSO_4 \cdot 5H_2O$	1.0
$MgSO_4 \cdot 7H_2O$	0.37	KI	0.66
K_2SO_4	0.17	H_3BO_3	0.56
$CaCl_2 \cdot H_2O$	0.007	$Na_2MoO_4 \cdot 2H_2O$	0.39
Fe(III) EDTA	0.004	EDTA	1.0
Trace element solution	0.25 mL	$FeSO_4 \cdot 7H_2O$	0.4
		$NiCl \cdot 6H_2O$	0.004

805r primer (5′-GAC TAC CAG GGT ATC TAA TC-3′) were used as templates for PCR, and amplified products were obtained in order to determine the base sequence. The determined nucleotide sequence was identified by homology searching through BLAST run by NCBI MEGA 6 version, and the phylogenetic tree was constructed using maximum parsimony 1000 bootstrap.

3. Characterization of phenanthrene degrading strains

We characterized the growth kinetics of the isolate using different concentrations of phenanthrene as the sole carbon source in MSM media and used different concentrations of phenanthrene to compare with *Escherichia coli*. Likewise, we also characterized the growth kinetics of the isolate using 0.1% diesel-fuel as the sole carbon source in MSM media and compared with *E. coli*.

4. Optimal test conditions for phenanthrene degradation

The strain was cultured overnight in MSM media, centrifuged at 7,000 rpm, and washed twice with 25 mM phosphate buffer. The strain was then diluted to 1 : 1,000 into fresh MSM media where sterile diesel-fuels of 0.05% and 0.1% were used as the sole carbon sources. Cultures were then grown at 35°C with agitation for 72 h, and samples were taken after every 12 h then serially diluted and plated in order to determine viable colony forming units (CFU). *E. coli* was used as a negative control (Gran-Scheuch *et al*. 2017).

5. Phenanthrene quantification

The procedure mentioned by Alarcon *et al*. (2013) was followed in order to quantify the phenanthrene concentrations using excitation-emission fluorescence spectroscopy with multivariate data analysis. Briefly, the two volumes of hexane were vigorously mixed for 60 s and used in culture media for the extraction of non-polar compounds. The extraction was repeated three times, the excitation-emission spectra were measured in a Varian Cary-Eclipse luminescence spectrometer (Mulgrave, Australia), and the phenanthrene concentration was determined through second order calibration by using PARAFAC and U-PLS/RBL algorithms. All the data computation was implemented in Matlab v.7.6 (Mathworks, Natwick, MA).

6. Biofilm formation assays

The Biofilm formation was assessed using a protocol outlined in (O'Toole 2011). The bacteria isolates of an optical density (OD) 0.1 were diluted to 1 : 100 in M9 media into the 96 well polystyrene plates. The growth was measured after 24 h of incubation by the absorbance at 600 nm (UV/VIS spectrophotometer). The media was further aspirated, washed thoroughly and stained with a crystal violet solution. The extract was obtained through the ethanol-acetone solution and the absorbance was measured at 590 nm. The absorbance of the crystal violet was then normalized to the optical density of each culture (Gran-Scheuch *et al*. 2017).

7. Lipase production

The bacteria was cultured in an optimal condition at pH 8 and 35°C for 24 h. The bacteria culture media consisted of (w/v): yeast extract (0.15%), sodium chloride (1.0%), peptone (0.5%), and crude oil (0.5%). After culturing, 5% of seed culture was inoculated in a 50 mL medium (w/v) containing dihydrogen orthophosphate 0.1%, potassium pep-

tone 0.5%, magnesium sulfate 0.01%, and sodium chloride 1%. The culture media was incubated at 35°C at 150 rpm for 12 hours. Finally, the culture media was centrifuged at 1,000 rpm for 10 minutes, and the obtained supernatant was used to determine the lipase activity (Mohan *et al*. 2008).

8. Lipase assay

Lipase assay was determined through spectrophotometry. The reaction was induced in the reaction mixture that contained 50 µL of substrate solution (1 mM p-NPP containing 1% Triton X-100), 100 µL of 50 mM Tris buffer (pH: 7.0), and 350 µL of H_2O by the addition of 100 µL of enzyme solution. Following 10 min of incubation, 1 mL of 2% sodium dodecyl sulfate (SDS) solution was added in order to stop the reaction. Measurement was carried on the absorbance at 420 nm using a UV/VIS-spectrophotometer. One unit of lipase activity was considered to be the amount of enzyme releasing 1 µmol of p-nitrophenol per minute (Tan *et al*. 2003).

9. Effect of incubation time and temperature on lipase activity

The effects of incubation time and temperature on lipase activity were investigated at 24, 48, and 72 hours, in medium and incubated at 35°C. The production time of 24 h was kept constant for the consecutive experiments (Ghaima *et al*. 2014).

10. Statistical analysis

The experiment was independently repeated three times and comprised of three replications per treatment. The data were statistically analyzed using SAS 9.4 software (SAS Institute, Cary NC, USA). The data from these experiments were pooled together and subjected to Duncan's multiple range test: $p \leq 0.05$.

RESULTS AND DISCUSSION

1. Isolation, identification and characterization of oil-degrading microorganisms

The diesel degrading bacteria was isolated from the oil-contaminated soils, and this microorganism was named DA1. As a result of the analysis of the 16S rRNA gene sequence of the selected strain, the homology of BLAST was found, and DA1 was subsequently identified as *Enterobacter cancerogenus* sp. The phylogenetic tree was constructed by aligning similar sequences using the Clustal W. MEGA ver 6.0. 1000 Bootstrap replication was used for robust statistical support in each node of the phylogenetic tree (Fig. 1).

2. Determination of optimal stage of degradation

The successful biodegradation of oil depends on one's ability to establish favorable conditions in the contaminated environment (Das and Chandran 2011). The biodegradation rate depends on various factors like the state, composi-

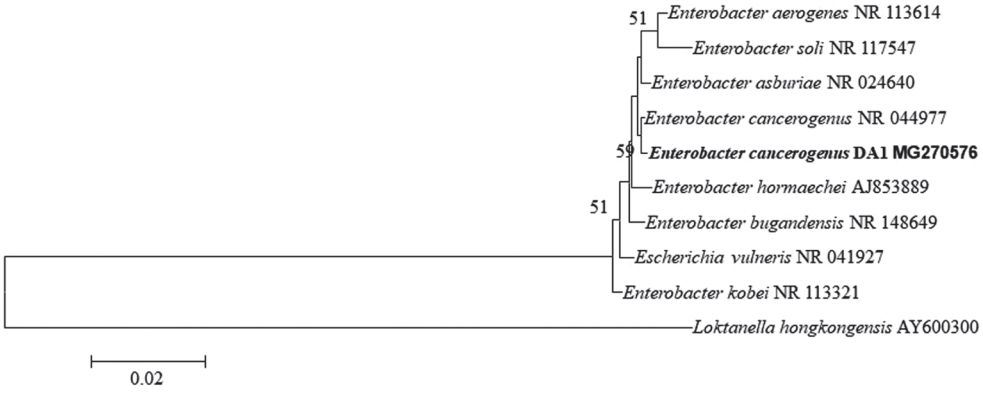

Fig. 1. Phylogenetic tree constructed by the maximum parsimony method using 16S rRNA gene sequence analysis of the isolate using the primer pair 518f (5′-CCA GCA GCC GCG GTA ATA C-3′) and 805r (5′-GAC TAC CAG GGT ATC TAA TC-3′), which formed a clade with the *Enterobacter cancerogenus* species, identified after a BLAST search in the NCBI Gene bank database using MEGA version 5 (100% bootstrap support).

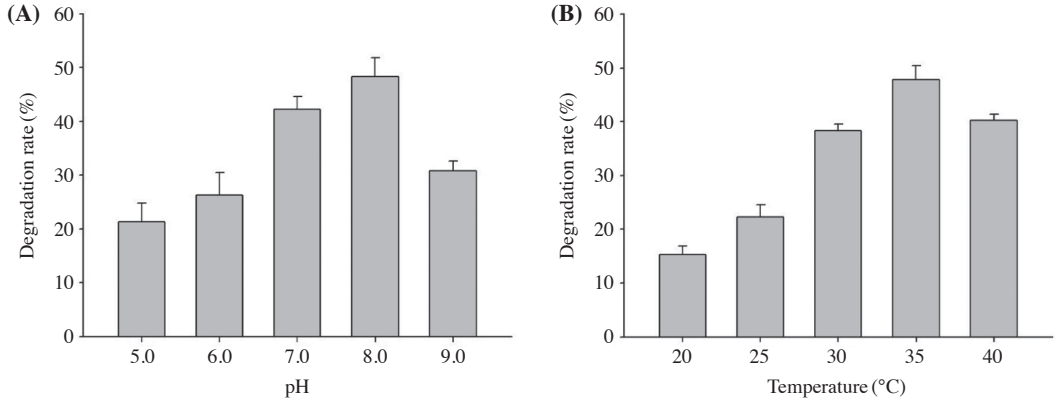

Fig. 2. (A) Effects of pH and (B) temperature on the degradation of diesel oil by strain *Enterobacter cancerogenus* DA1. Bars represent means ± SD ($n = 3$).

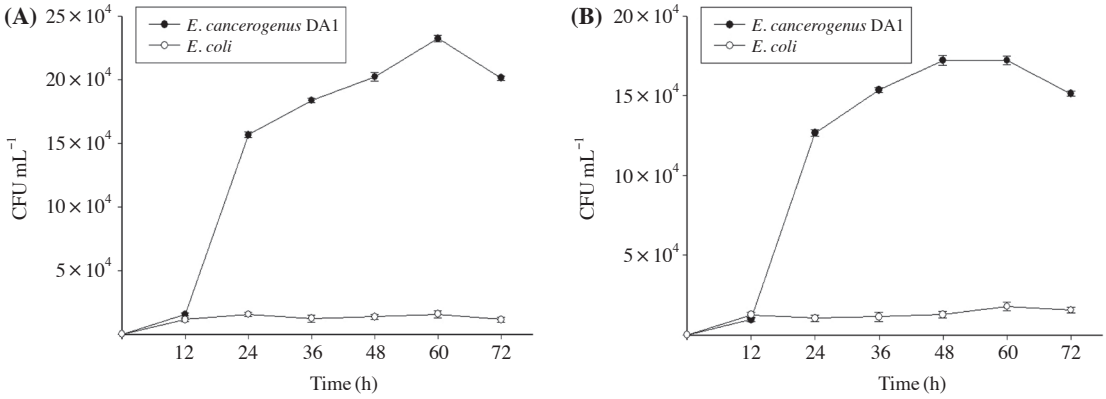

Fig. 3. Growth performance of strains mediated with (A) 0.05% phenanthrene and (B) 0.1% diesel-fuel as the sole carbon source in MSM.

tion, and concentration of the oil or hydrocarbons (Leahy and Colwell 1990). In our test it was found that the diesel degradation rate was significantly higher at pH 8 than at any other pH, followed by pH 7 > 9 > 6 > 5. Likewise, the highest degradation rate was achieved at 35°C temperature, followed by at (40°C > 30°C > 25°C > 30°C) (Fig. 2). It has been reported that the efficiency of the biodegradation of soil bacteria is 50% (Pinholt *et al*. 1979).

In our study, the growth of the bacterial strain exhibited a long lag phase, and after 12 h started rapidly proliferating, and finally reached a maximum growth at 48 h in the 0.05% phenanthrene mediated media (Fig. 3A). Likewise, the strain exhibited the same duration of growth lag phase in the 0.1% diesel fuel mediated and rapidly proliferated after 12 h, reaching a peak growth at 60 h after incubation (Fig. 3B). The strain *E. coli*, included as a control, was unable to use phenanthrene as an energy source and did not proliferate growth under these conditions. Our results are consistent with those of Wongsa *et al*. (2004) in which 90–95% of the total diesel oil added to MSM media was degraded by *Pseudomonas aeruginosa* within two to three weeks and *Serratia marcescens* species could degrade around 50–60% of diesel. The temperature affects the solubility of the hydrocarbons (Foght *et al*. 1996). The addition of nutrients enhances biodegradation (Cooney 1984). It has been reported that the species like *Arthrobacter sulphureus* RKJ4, *Brevibacterium* sp. HL4, *Acidovorax delafieldii* P4-1, and *Pseudomonas* sp. are involved in phenanthrene degradation (Samanta *et al*. 1999). Likewise, species such as *Alcaligenes faecalis* AFK2, *Beijerinckia* Bwt, and *Pseudomonas* SPM64 have also been reported to degrade the phenanthrene (Kiyohara *et al*. 1982). Similarly, Boldrin *et al*. (1993) reported that *Mycobacterium* sp. strain BB1 successfully degraded the phenathrene used as the sole carbon source. It has been

reported that the maximum biodegradation rate occurred at temperature range between 30−40°C (Bartha and Bossert, 1984; Das and Chandran, 2011) and at pH range between 6−9 (Das and Chandran, 2011), our results also indicate that the optimal conditions for the maximum growth of the strain are at pH 8 and temperature 35°C.

3. Biofilm production

Biofilm has several benefits in an organism, such as protection against environmental assaults and insults (Morikawa 2006). In our study, the adhered biomass of the strain was quantified through staining with crystal violet. *E. coli* was used as a control. The value of phenanthrene degrading bacteria was compared to *E. coli* in order to establish statistical differences. After 24 h of incubation, biofilm formation was quantified through staining with crystal violet and it was found that the biofilm formation was significantly higher at 35°C than at 30 and 40°C (Fig. 4). Biofilm was formed by strain in the Nunc-TSP lid system. It has been reported that the strain *Mycobacterium frederiksbergense* LB501T could form biofilm on anthracene crystal (Wick *et al*. 2002). Polycyclic aromatic hydrocarbon (PAHs)-degrading bacteria forms biofilms through the production of extracellular polymeric substances (Johnsen and Karlson 2004). Biofilm formation becomes involved in the mass transfer of PAHs to the bacterial cell. It has been reported that the majority

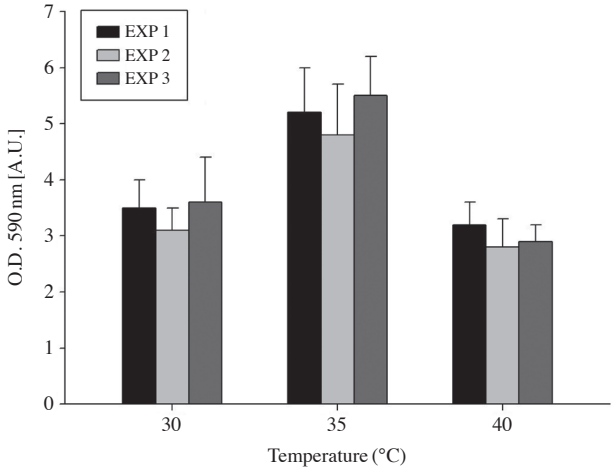

Fig. 4. Biofilm production on polystyrene microtiter plates at different temperatures. Bars represent means ± SD ($n = 3$). Three parallel bars represent the average absorbance at OD 590 nm for three replicates at three different temperatures in 24 hours (Experiment 1, Experiment 2, and Experiment 3).

PAHs-degrading bacteria forms biofilms in microtiter wells coated with PAHs crystals (Johnsen and Karlson 2004). The microbial polymer regulates the transport of phenanthrene by performing as an actor to carry the phenanthren sorbents (Dohse and Lion 1994). It has been previously reported that the *Sphingomonads* that are able to degrade PAHs secrete exopolysaccharides grown as biofilm (Johnsen *et al*. 2000). Similarly, strains like *Rhodococcus corynebacterioides* can form biofilm on flakes incubated with 0.25% kerosene (Gentili *et al*. 2006). Biofilm formation is an important phenomenon adapted by microorganisms for their survival (Davey and O'toole 2000). Biofilms serve several benefits in an organism, including protection against environmental assaults and insults (Morikawa 2006). The biofilm structure serves a protective function for strain survival and in promoting a long shelf life in crude oil degrading activity (Gentili *et al*. 2006). The biofilm formation of the *Enterobacter cancerogenus* DA1 might also play a similar role to fight against the environmental stress and enhance the oil degradation process.

4. Determination of lipase production

Lipase enzymes activities are considered to be the most efficient parameter for detecting hydrocarbon degradation in soil (Riffaldi *et al*. 2006). In our study, the lipase production of the strain DA1 was found to be significantly higher at 48 h than at 24 and 72 h. Similarly, the lipase production was found to be higher at 72 h than at 24 h (Fig. 5). It has previously been reported that the biodegradability of the lipid content in biodiesel is regulated by enzymes like lipase (Meyer *et al*. 2012). Lipase is primarily involved in the hydrolysis of ester bonds (Boczar *et al*. 2001; Meyer *et al*. 2012). In petroleum products such as biodiesel and diesel blends, it is reported that the enzymes such as lipase, oxygenase, and esterase are involved in the oxidation of fatty acid methyl-ester chains and hydrocarbons which are transformed into the fatty acid and through the β-oxidation and get incorporated in the cell (Meyer *et al*. 2012). The significant level of oil and lipid conjugates enters into the soil in the form of triacylglycerols, which are the primary storage fat in animal and plant tissue. The degradation of this lipid is induced by lipases through acting on the caboxylester bonds present in the acylglycerols liberating the glycerol

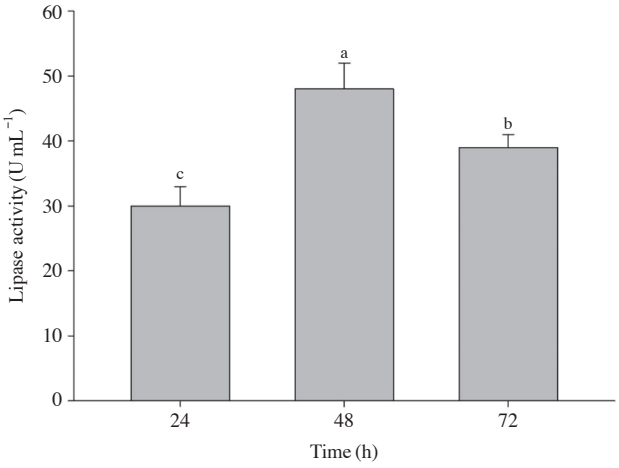

Fig. 5. Effect of incubation time on lipase production by *Enterobacter cancerogenus* DA1. Bars represent means ± SD ($n = 3$). Means followed by a different letter indicates a significant difference determined by DMRT ($p \leq 0.05$).

and fatty acid (Margesin *et al*. 2002). It is likely that the lipase production of the *Enterobacter cancerogenus* DA1 interacts in a similar manner as for diesel degradation.

CONCLUSION

An isolation of a highly efficient diesel and phenanthrene degrading bacterial strain through screening from the diesel contaminated soil was identified as *Enterobacter cancerogenus* DA1. The favorable growth conditions for the strain growth and the optimal conditions required by the strain were well elucidated in this research. Besides these findings, we were also able to acquire knowledge about the ability of the strain to promote the degree of biodegradation by considering different factors like time, temperature, pH, the amount of concentration that is degraded in different growth conditions, including physiological mechanisms like biofilm formation and lipase production. The isolation of such a novel strain that degrades petroleum products like diesel may be an efficient tool for replenishing the natural soil and minimizing the pollution caused by the excessive release of petroleum products through anthropogenic activities.

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