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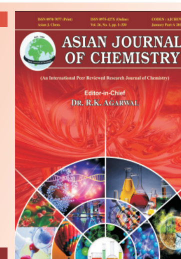

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Factors Influencing Crude Oil Biodegradation by *Pseudomonas* sp. DG17

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Pseudomonas is unique in the soil with the ability to degrade petroleum susbstrate. In the present work, effect of environmental factors including, crude oil concentration, salinity, pH and nutrient on the biodegradation of crude oil as well as the biodegradability of different alkanes by *Pseudomonas* sp. DG17 were investigated. The results showed that inoculum cell content had positive and crude oil concentration, salinity, pH and biosurfactant had negative effect. *Pseudomonas* sp. DG17 requires an optimal substrate concentration was 500 mg L^{-1}, the optimum inoculation cell content (OD_{600}) was 1, the optimum salinity was 1 %, the optimum pH range was 6-8 and the optimum biosurfactant additon was 10 mg L^{-1}. In additon, high concentraton of biosurfactant had toxice effect on the biodegradation of crude oil by DG17. Moreover, the biodegradability of C_{12} to C_{19} of saturable alkanes and cell growh of *Pseudomonas* sp. DG17 decreased in order of increasing molecular weight.

Keywords: *Pseudomonas*, Crude oil, Biodegradation, Factors, Alkanes.

INTRODUCTION

Biodegradation by natural microorganisms in the soil represents one of the primary mechanisms by which petroleum and other hydrocarbon pollutants can be removed from the environment[1]. Most of the microorganisms, including bacteria and fungi, that living in the soil have the ability to biodegrade the organic pollutant. However, they must adapt to the toxic environment primarily. Many factors can influence the bioremediation process and should be monitored. These factors include substrate concentration, type and concentration of the pollutant(s), salinity, pH, nutrient and oxygen availabilities and the concentration of microorganisms in the impacted site[2-5]. The biodegradation of hydrocarbons has been intensively studied in laboratary conditions[2,5-7]. On the other hand, the low water solubility of petroleum components caused them to separate with the high hydrocarbon biodegradable micro-organisms that living in the soils, thus, the bioremediation of petroleum contaminated soils is greatly limited by the poor bioavailability of hydrophobic contaminants[8,9]. Biosurfactant secreted by microorganisms can help, by solubilization or emulsification, to release hydrocarbons sorbed to soil organic matter and increasing the aqueous concentrations of hydro-phobic compounds, resulting in higher mass transfer rates[10-12].

The aim of this study was to investigation the effect of physical environmental factors on the *trans*-membrane transport of octadecane by an isolated bacterial *Pseudomonas* sp. DG17 from crude oil polluted soil. The factors analyzed were inoculum cell content, crude oil concentration, salinity, pH and biosurfactant addition. The results of this study could offer some new discovery on the relationship between crude oil biodegradation and biosurfactant addtion.

EXPERIMENTAL

Pseudomonas sp.DG17 (CGMCC: NO. 5052; NCBI accession No.: JN 216878), used in this study, was isolated from petroleum contaminated soil according to Hua and Wang[13]. Cells of DG17 were inoculated in mineral salt medium (MSM) sterilized by autoclaving at 121 °C for 20 min, containing (per liter at pH 7) 0.4 g Na_2HPO_4, 0.15 g KH_2PO_4, 0.1 g NH_4Cl, 0.05 g $MgSO_4{\cdot}7H_2O$, 0.0015 g $CaCl_2$, 0.1 g $NaNO_3$, 1 mL trace medium (per 100 mL solution containing 0.5 mg $CuSO_4{\cdot}5H_2O$, 1.0 mg H_3BO_3, 1 mg $MnSO_4{\cdot}5H_2O$, 7.0 mg $ZnSO_4$). Cultures were maintained at 4 °C on crude oil solid medium. Crude oil dissolved in dichloromethane was injected into the flask filtered by 0.2 μm filter membrane, then the flask was put in the table concentrator in order to remove dichloromethane. After that, cells of DG17 were inoculated in mineral salt medium to begin the biodegradation of crude oil.

For cell proliferation, *Pseudomonas* sp.DG17 was inocu-lated into LB medium (5 % yeast extract, 10 % peptone and 5 % sodium chloride, pH 7.0) at 10 ºC for 48 h. Cells were centri-fuged, washed with sterilized MSM and transferred to 50 mL centrifuge tube containing mineral salt medium.

Environment factors that influence biodegradation of crude oil: To study the influence of environmental factors on the crude oil biodegradation processed by *Pseudomonas* sp. DG17, the crude oil concentration, salinity, pH, nutrient addition and biosurfactant addition varied according to experimental design described below. In parallel, control experiments were performed to analyze the oil lost by evaporation and sampling. Meanwhile, after incubation for 35 days, three different samples were conducted simultaneously for standard deviation analysis.

Different crude oil concentrations: Different amount of crude oil stock solution (20000 mg L^{-1}) dissolved in dichloromethane were filtered by 0.2 mm filter membrane into the flasks containing 150 mL of MSM (pH 7.0-7.2) and the final crude oil concentration were 100, 200, 500, 800, 1000, 2000 mg L^{-1}, respectively. After dichloromethane in the medium was removed through volatile, cells of DG17 were inoculated into the flasks and the final cell density (OD_{600}) was 0.40. The flasks were incubated at 150 rpm and 10 °C.

Initial cell concentration: Stock solution of crude oil (20000 mg L^{-1}) dissolved in dichloromethane was filtered by 0.2 mm filter membrane into the flasks containing 150 mL of MSM (pH 7.0-7.2) and the final crude oil concentration was 500 mg L^{-1}. After dichloromethane in the medium was removed through volatile, cells of DG17 stock solution were inoculated into the flasks and the final cell density (OD_{600}) were 0.1, 0.2, 0.5, 0.8, 1.0, 1.5, 2.0, respectively. The flasks were incubated at 150 rpm and 10 °C.

Salinity and pH: Salinity of culture medium was adjusted to 0.5, 1, 2, 3, and 5 % using NaCl. Stock solution of crude oil (20000 mg L^{-1}) dissolved in dichloromethane was filtered by 0.2 mm filter membrane into the flasks containing 150 mL of MSM (pH 7.0-7.2) and the final crude oil concentration was 500 mg L^{-1}. After dichloromethane in the medium was removed through volatile, cells of DG17 were inoculated into the flasks and the final cell density (OD_{600}) was 0.40. To test effect of pH on the biodegradation of crude oil, culture medium was adjusted to 4, 5, 6, 7, 9, 10 using 2 mol L^{-1} of HCl or 2 mol L^{-1} of NaOH. The final crude oil concentration was also controlled at 500 mg L^{-1}. Cells of DG24 were inoculated into the flasks and the final cell density (OD_{600}) was 0.45. The flasks were incubated at 150 rpm and 10 °C.

Preparation of biosurfactant addition: Biosurfactant stock solution (10000 mg L^{-1}) dissolved in deionized water was added in the flasks containing 150 mL of MSM (pH 7.0-7.2). The final biosurfactant concentration were 0, 10, 20, 40, 80, 100 mg L^{-1}, respectively. Stock solution of crude oil (20000 mg L^{-1}) dissolved in dichloromethane was filtered by 0.2 mm filter membrane into the flasks containing 150 mL of MSM (pH 7.0-7.2) and the final crude oil concentration was 500 mg L^{-1}. Cells of DG17 were inoculated into the flasks and the final cell density (OD_{600}) was 0.45. The flasks were incubated at 150 rpm and 10 °C.

Analysis methods: After incubation for 35 days, the residual crude oil was extracted from the flasks. The liquid in the flasks was transfer into 500 mL separating funnel. Then, washing the flasks by 150 mL of dichloromethane and acetone (V:V = 3:1) for three times in order to collect the whole crude oil. All the cleanout liquid was transfered into the separating funnel. Then the organic phase was ultrasonic extracted (KQ-100DE, Kunshan ultrasonic instrument company, China) for 10 min for three times. The organic phase was transferred into a beaker (treated by nitric acid) in order to volatilize dichloromethane and acetone. The crude oil biodegradation was calculated by gravimetric method as follows: 100 × (1-crude oil that in the beaker/initial crude oil that supplemented in the flask).

The GC instrument was equipped with split injector and a VF-5 column was used for separation (30 m, 0.25 mm id, 0.25 μm film thickness). The split ratio was 10:1 and sample amount was1 ul. The temperature program started at 60 °C and was held for 5 min. Then the splitter was opened and the oven was heated to 290 °C at a rate of 10 °C/min and hold for 15 min. The solvent delay time was set to 6 min. The transfer line temperature was set to 250 °C. Mass spectra were recorded at 1 scan/s under electron impact at 70 eV, mass range 40-650 amu. Standard sample which contains different carbon chain length from C_{10}-C30 (Sigma, America) was measured at the same time.

Biodegradability analysis of different alkanes: Biodegradation of saturable alkanes, including dodecane ($C_{12}H_{26}$), tetradecane ($C_{14}H_{30}$), hexadecane ($C_{16}H_{34}$), octadecane ($C_{18}H_{38}$), nonadecane ($C_{19}H_{40}$) by *Pseudomonas* sp. DG17 as the sole carbon and energy source were studied. Stock solution of alkanes (1000 mg L^{-1}) that dissolved in hexane were injected into 250 mL Erlenmeyer flasks filtered by 0.2 μm filter membrane. The final alkane concentration in the MSM was 400 mg L^{-1}. After hexane in the medium was removed through volatile, cells of DG17 were inoculated into the flasks. The inoculum of DG17 was 5 % (v/v) and cell density at OD_{600} was about 0.40.

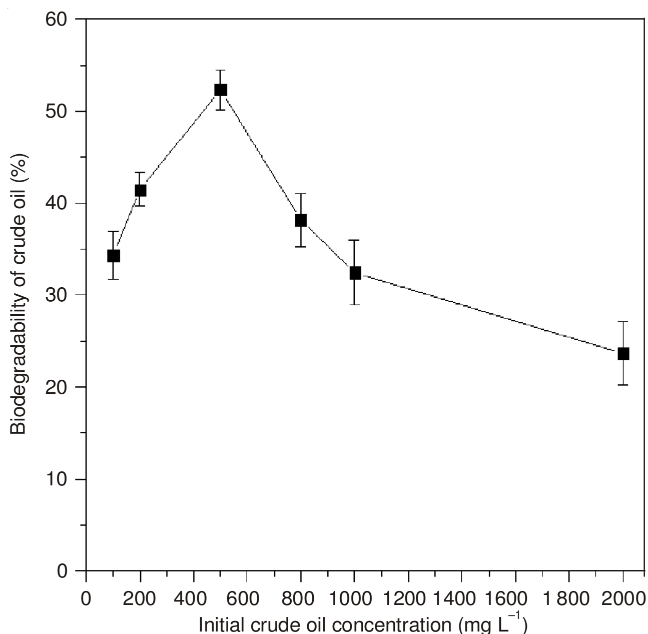
Then, the flasks were incubated at 150 rpm and 10 °C. After incubation for 8 days, residual alkanes in the medium was extracted and analyzed. The control group that not supplemented with cells was used for abiotic loss analysis of alkanes. The whole culture medium was transferred into centrifuge tube and cell pellets were collected by centrifugation at 4,000 rpm.

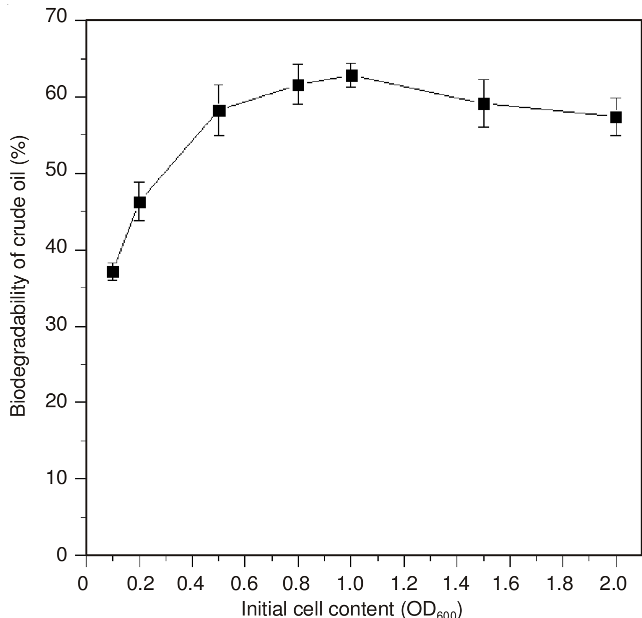
The supernatant was transferred into 250 mL separating funnel containing equal volume of *n*-hexane. Then, the organic phase was ultrasonic extracted for three times and transferred into a beaker (treated by nitric acid) in order to volatilize hexane. Finally, 2 mL of hexane was used to dissolve alkanes for GC-MS analysis. Take octadecane as an example, standard curve of concentration was as follows: $Ai = 7 \times 10^7X$, in which Ai was peak area and X was alkane concentration (mg L^{-1}). GC-MS condition was described as above. The biodegradability of alkanes was calculated as follows: 100 × (1-residual alkanes that in the beaker/alkanes that in the control group flask). Three different samples were conducted simultaneously for standard deviation analysis.

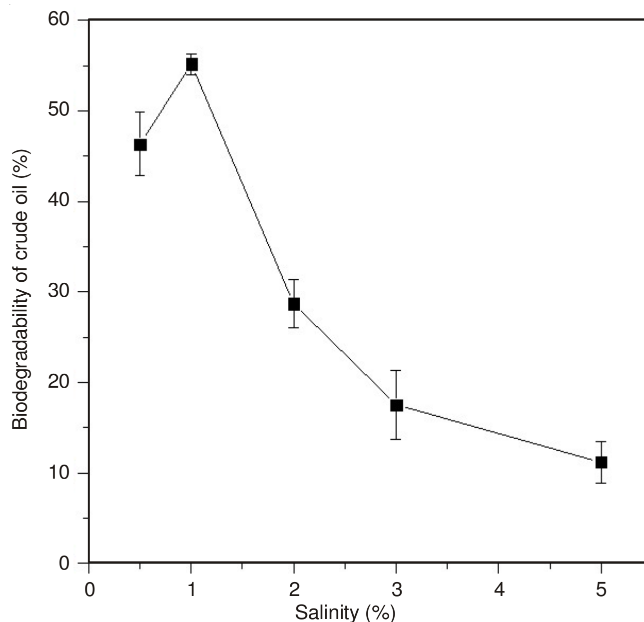
Cell growth on alkanes: During the biodegradation assays of alkanes, cell density at OD_{600} was monitored at different time intervals. For each point, 10 mL of broth was analyzed by spectrophotometer. This method provided only qualitative results because only the optical density of the aqueous phase was measured even though some of cells migrated into the organic phase.

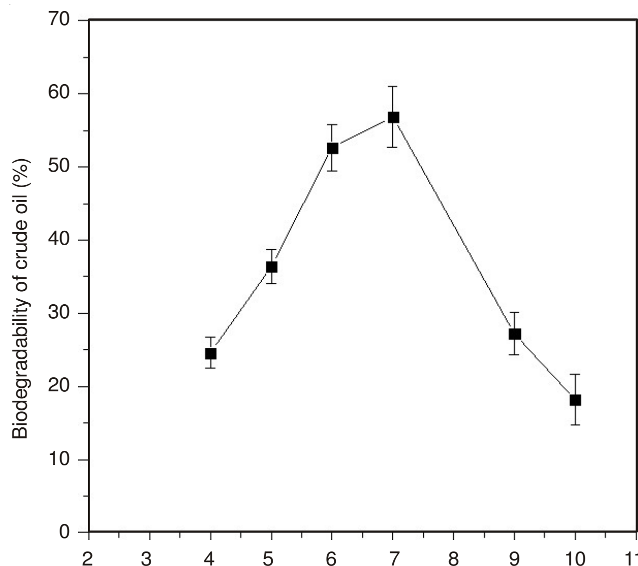
RESULTS AND DISCUSSION

Effect of substrate concentration, cell content, salinity, pH and biosurfactant on the biodegradation of crude oil: The experimental design results are presented in Fig. 1. Under the condition tested, the crude oil removal percentage ranged from 67.62 ± 3.37 % to 11.16 ± 2.26 %. When initial crude oil concentration was 500 mg L^{-1}, crude oil biodegradability achieved to 52.31 ± 4.18 %. While, the removal percentage decreased along with the increase of the initial oil concentration. Meanwhile, it was found that high inoculum content of *Pseudomonas* sp. DG17 resulted in high crude oil removal percentage. Biodegradability of crude oil was 62.82 ± 1.55 % when initial cell content value was 1.0 (OD_{600}). However, removal percentage did not increased along with the increase of cell content and crude oil biodegradability was maintained at stable level when cell content was higher than 1.0. Thus, it was inferred that biodegradability of crude oil was both related with initial oil concentration and cell inoculum content.









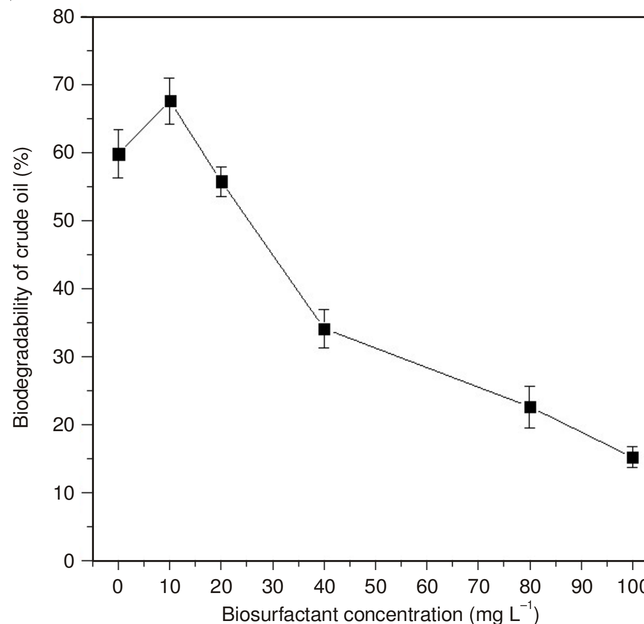


Fig. 1. Effect of factors on the crude oil biodegradability by *Pseudomonas* sp. DG17

Salinity and pH are two most important factors that influence the biodegradability of hydrocarbons by most kinds of microorganisms. In the studies, it was found that pH and salinity have negative effect on the removal of crude oil. The highest removal percentage of crude oil was 55.16 ± 1.17 % when salinity of the medium was 1 % and this value declined along with the increase of salinity. Similarly, when pH of the medium was lower than 6 or higher than 9, the removal percentage of crude oil was also limited. For example, the oil biodegradability was only 18.14 ± 3.47 % when pH value was 10. Severe changes of pH, such as strong acid or alkali, damage the biodegradation function of *Pseudomonas* sp. DG17.

Biosurfactants increase the oil surface area and that amount of oil is actually available for bacteria to utilize hydrocarbons. In this case, biosurfactant was also considered as a factor that could influence biodegradation of crude oil. In our studies, rhamnolipids biosurfactant was supplemented in the medium to assess oil removal ability by *Pseudomonas* sp. DG17. The results showed that the optimum addition content was 10 mg L^{-1} with the oil removal percentage at 67.62 ± 3.37 %. In addition, as along with the increase of rhamnolipids, the biodegradability of DG17 was damaged obviously.

Biodegradability of different alkanes: As shown in Fig. 2, after incubation for 8 days, the biodegradability of dodecane, tetradecane, hexadecane, octadecane, nonadecane by *Pseudomonas* sp. DG17 was 71.52 ± 3.86 %, 58.44 ± 3.15 %, 46.28 ± 5.16 %, 41.57 ± 4.82 % and 38.13 ± 3.64 %, respectively. Previous study have shown that *Pseudomonas* sp. DG17 preferentially utilized C_{12} to C_{28} of *n*-alkanes[14]. Maximal degradation of dodecane was obtained and high carbon number alkanes were degraded to less extent.

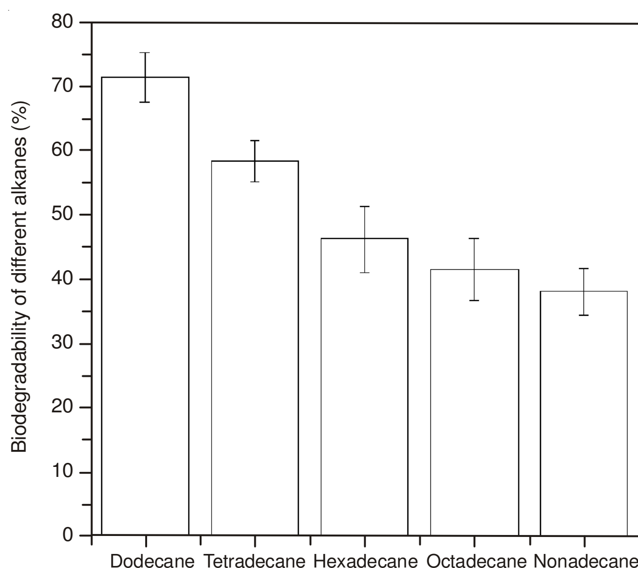


Fig. 2. Biodegradability of different alkanes by *Pseudomonas* sp. DG17

Cell growth on saturable alkanes: The growth potentials of the isolate on different alkanes was shown in Fig. 3. Biomass of *Pseudomonas* sp. DG17 increased along with the decrease of carbon chain length. After an initial slow growth or lag phase, the population of DG17 increased along with the incubation time. In addition, DG17 showed obviously exponential growth under the condition assessment. When dodecane served as the sole carbon and energy source, cell content increased to 0.39 to 1.68 after incubation for 8 days. Meanwhile, it was found that the bacterium also displayed brief lag phase of about 3 days when grown on hexadecane or octadecane. On octadecane, the highest population density (OD_{600}) of 0.88 was achieved on day 7 and cell growth entered into stationary phase. The results indicated that low molecular saturable alkanes were easier to be utilized and stimulate the growth of the isolate.

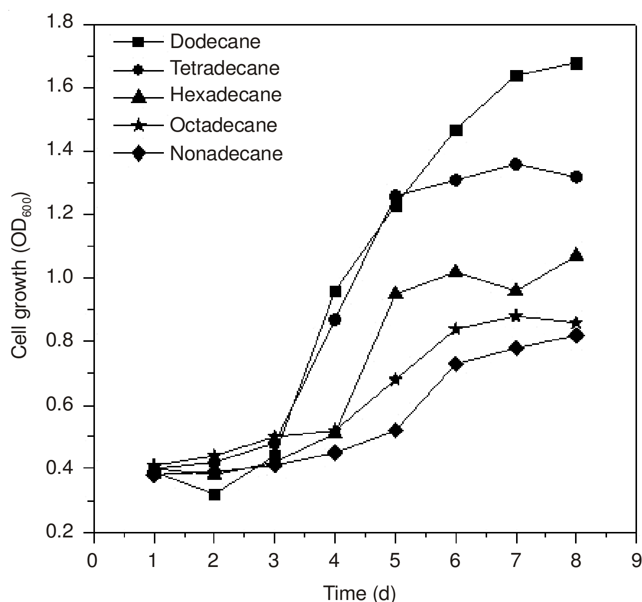


Fig. 3. Growth of *Pseudomonas* sp. DG17 on saturable alkanes

The usefulness of microorganisms for bioremediation must survive and be active under *in situ* conditions. The presence of extremophiles in polluted extreme habitats, which are adapted to the prevailing conditions and able to metabolize a wide range of hydrocarbons, indicates their usefulness for bioremediation[15]. In this case, it is necessary to know the optimal growth condition of microorganisms in order to obtain useful bioremediation results. Oil concentration and inoculum cell amount of microorganism are two major factors that influence the biodegradation effect. Some reports showed the negative effect of oil concentration on crude oil removal. It was found that cumulative extents of ^{14}C-phenyldodecane mineralization reduced with increasing oil concentrations when concentration of oil was higher than 0.1 % and were significantly decreased in soil spiked with 1 and 10 % cable oil concentrations. The results also inferred that this decrease in catabolic activity is proposed not to be linked to nutrient availability or toxicity effects since nutrient amendment did not increase rates or extents of ^{14}C-phenyldodecane mineralization and the addition of a specialized degraders without nutrient amendment did produce an increase in ^{14}C-phenyldodecane mineralization at high oil concentrations. In addition, low crude oil concentration also limited the oil removal since indigenous microbial did not have sufficient carbon source and biomass was limited[16]. On the other hand, a higher microbial population results in a greater oil biodegradation rate, especially under the conditions that were used for the experiment, that is, without the addition of nutrients to support

microbial growth. Therefore, the inoculum amount has an important role[2]. In this case, bioaugmentation was used as a technique to stimulate oil biodegradation[17,18]. For *Y. lipolytica* IMUFRJ 50682, a higher microbial population results in a greater oil biodegradation rate without the addition of nutrients to support microbial growth[2].

Many environments pressure that are characterized by acidic or alkaline pH, or high salt concentrations, could inhibit the biodegradation ability of microorganisms[19,20]. It is also shown that oil degradation has been inhibited by increased salinity or increased soil water salinity that inhibits the microbial hydrocarbon degradation[21]. For instance, increasing concentrations of NaCl (w/v) increased the lag time and decreased the rate and extent of mineralization of aliphatic and aromatic substrates[19,20] found that addition of NaCl to a petroleum-contaminated Arctic soil decreased hexadecane mineralization rates in the initial stages of bioremediation and increased lag times. High salt concentrations can inhibit the activity of microbes that are not adapted to salt. Possible reasons for this effect include direct inhibition of metabolic activity because of unfavorable high osmotic potential of the microbe's environment[22]. Thus, the removal of salt from oil-contaminated soils may reduce the time required for bioremediation[15]. In this study, when salt concentration was higher than 1 % (w/v), crude oil removal was obviously inhibited after incubation for 35 days. It was inferred that cells grew in high salinity could experience a long lag time before eventually beginning to mineralize the substrate at a rate similar to that of less stressed cells. This lag time may be interpreted as a period of adaptation of the community to the stressors, during which microbes able to endure the conditions and/or for gene exchange to occur. Hydrocarbon mineralization is favored by near neutral pH values[2]. For example, bacterium KL2-13 requires an optimum pH range of 6-8 to biodegrade oil. Severe changes of pH, such as strong acid or alkali, damage the normal functions of membrane channel proteins, transporters and signaling pathway protein, cause cells to lose the function of permselectivity. Some microorganisms can also degrade hydrocarbons in the acidic environment since genes that encode enzymes involved in biodegradation process[23].

Results showed that consortia could be generally divided into three categories, for which surfactants had positive, neutral, or negative effect on the biodegradation of diesel fuel[24] The increase in cell hydrophobicity can be induced in the presence of biosurfactant combined with slightly soluble substrate. Increased cell hydrophobicity promoted attachment of cells to hydrocarbon droplets, thus enhancing alkane degradation[25]. In some cases an increase in degradation rate was observed, whereas in other cases a decrease in degradation rates was noted after addition of surfactants. For example, added rhamnolipids above critical micellar concentration (CMC) enhanced the apparent aqueous solubility of hexadecane, enhanced biodegradation of hexadecane, octadecane, *n*-paraffins, creosotes and other hydrocarbon mixtures in soil and promoted bioremediation of petroleum sludges[26,27]. In this study, biodegradation of crude oil was stimulated when concentration of rhamnolipids was lower than 10 mg L^{-1}. However, the biodegradation ability of DG17 was limited obviously when rhamnolipids concentration was higher than 10 mg L^{-1}. Some studies demonstrated that biosurfactant that produced by microorganism could not initiate the degradation of hydrocarbons[28]. have shown that the presence of low concentration of biosurfactant PS (rhamnolipid + alginate) in culture media was neutral to the growth of Gram-positive *B. subtilis* and Gram-negative *P. aeruginosa*, but only detrimental to Gram-positive *B. subtilis* when the concentration of biosurfactant PS was greater than CMC. If the micelle concentration be too high, pollutant may become effectively diluted across a large number of micelles, resulting in decreased mass transport due to stronger partitioning into the micellar phase. In our study, CMC value of rhamnolipids was 70 mg L^{-1}. Crude oil biodegradability by *Pseudomonas* sp. DG17 was lower than 25 % when rhamnolipids concentration was above CMC value.

Previous studies have shown that *Pseudomonas* sp. DG17 could produce rhamnolipids type biosurfactant and uptake and mineralize alkanes C_{12} to C_{28}[14]. Under neutral conditions, in the addition of *Pseudomonas* sp. DG17, biodegradability of alkanes and biomass declined along with the increase of molecular. Many kinds of microorganism could utilize alkanes well during oil biodegradation process and similar results were also reported. Rhodococcus strains degrade hydrocarbons between *n*-C_{12} to *n*-C_{20} is well known[29,30] also reported degradation up to *n*-C_{30} by various strains of Rhodococcus. Thalassolituus oleivorans was found to obligately utilize long chain *n*-alkanes with a substrate range up to C_{20}[31]. In addition, Cladosporium resinae growing on alkane mixtures removed *n*-alkanes sequentially in order of increasing molecular weight, each at about the same rate as during growth on it as single alkane[32]. In the review of Wentzel *et al.*[33], two unrelated classes of enzymes for long-chain *n*-alkane oxidation were proposed: (1) the class of cytochrome-P450-related enzymes in both yeasts and bacteria, *e.g.*, bacterial CYP153 enzymes and (2) the class of bacterial particulate alkane hydroxylases (pAHs).

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