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Growth Characteristics of Seven Hydrocarbon-Degrading Active Bacteria Isolated from Oil Contaminated Soil

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ABSTRACT

The growth characteristics of seven strains isolated from oil contaminated soil, as well as their respective degradation efficiency for various hydrocarbons were investigated. Factors that can impact biological oil degradation efficiency were revealed in a series of experiments. The results indicate that isolated strains could rapidly degrade crude oil, showing high activity in the first 13 h of bioremediation. These strains could grow in paraffin wax, which indicates that these strains could degrade long chain hydrocarbons. Some of them (SY_{22}, SY_{23}, SY_{24}, SY_{42}, SY_{43}) were able to use short chain hydrocarbons and aromatic hydrocarbons as substrate, so these five strains are the preferred ones for the bioremediation of oil contaminated soil. Suitable pH for the growth of these five strains was in the range from 7 to 9. NH_4NO_3 and oil concentrations should range from 1000 mg/L to 1500 mg/L in order to achieve optimum conditions for petroleum hydrocarbon degradation. Adding organic matter such as starch and glucose accelerated oil and PAH degradation capability of the SY_{22}, SY_{42} and SY_{23} strains. The presence of metal ions, such as Ni^{2+} and Co^{2+} in soil decreased the crude oil degradation efficiency of these strains, while metal ions, such as Fe^{2+} and Mn^{2+} did not affect the oil degradation activities.

INTRODUCTION

Eight million tons (Mt) of petroleum is spilled into the environment every year worldwide. In China, 0.6 Mts of petroleum enters into soil, groundwater, rivers and ocean every year. Oils are ubiquitous in environment and may be present at high concentrations at industrial sites associated with petroleum, coal tar, gas production and wood preservation industries (Wattiau 2002). The presence of petroleum oils in the environment causes serious health hazard because of their mutagenic and carcinogenic properties (Kastner 1998). Oil contamination is a severe threat for our environment and thereby attracts general concern. Hence, there is an increasing interest to remediate the sites contaminated with petroleum to minimize their threats to human health.

As compared to physico-chemical treatments, use of microbial technology to clean up oil-contaminated sites has been found an efficient, economical, eco-friendly and adaptable choice. Besides, it has potential advantages over physico-chemical methods such as complete degradation of pollutants, greater safety and less soil disturbance (Habe 2003). Bioremediation has become one of the most promising technologies for oil contaminated soil remediation. Microorganisms used for bioremediation are usually grouped as indigenous and exogenous microbes. The addition of nutrients increases the activity of native microorganisms; however, bioremediation is boosted with the addition of exogenous bacteria. The application of bioremediation using indigenous microbes is restricted because native microbes need a long time to domesticate, and thereby show low growth rates and low metabolic activity, which make decontamination slow and ineffective. However, a few bacteria who are able to grow on the four ring PAHs, specifically, fluoranthene (Rehmann 1999, Luepromchai 2007) and pyrene (Rehmann 1998, Churchill 1999) such as *Mycobacterium, Rhodococcus, Alcaligenes* and *Sphingomonas* have studied PAH degradation in soil. The application of addition of exogenous active bacteria in field experiments has increased. A study on the diversity of PAH degrading bacteria shows that *Sphingomonas* species are generally found to be fluoranthene degrading, while *Pseudomonas* strains were commonly associated with phenantherene degradation (Muller 1997). Therefore, the screening of hydrocarbon-degrading active bacteria to remediate oil polluted soil is a necessary task.

In the present investigation, hydrocarbon degradation was studied in minerial medium using hydrocarbons as a sole source of carbon and energy by seven bacterial strains isolated from oil contaminated soil in the north region of the Shaanxi province in order to find out the highest hydrocarbon degrading bacterial strain to be used further in the consortium for crude oil degradation in the field conditions. The effect of pH, nutrition (nitrogen and phosphorus), and pollution intensity on the oil degradation efficiency of the isolated strains were investigated. Furthermore, based on the

complexity of the soil systems, the degradation capacity of the isolated bacteria of different kinds of petroleum hydrocarbons and the effect of adding organic co-substrate and metal ions on the bioremediation were studied.

MATERIALS AND METHODS

The Source of samples: The tested crude oil and oil polluted soil were collected at oil wells in the northern region of the Shaanxi province. The strains separated from the oil contaminated soil were SY_{21}, SY_{22}, SY_{23}, SY_{24}, SY_{42}, SY_{43}, and SY_{44}.

Culture medium: The recipes of the different culture media used in this work are provided as follows.

Recipe of liquid or solid beef grease and peptone cultivation medium: 10g peptone, 3g beef grease, 5g NaCl, 1000mL distilled water, pH 7.0. The medium can be solidified using 20g of agar (Shen 1996).

The composition of mineral medium used in this work was as follows: 2g NH_4NO_3, 1.5g K_2HPO_4, 3g KH_2PO_4, 0.1g $MgSO_4 \cdot 7H_2O$, 0.01g anhydrous $CaCl_2$, 0.01g $Na_2EDTA \cdot 2H_2O$, 1000mL distilled water, with a pH ranging from 7.2 to 7.4 (Liang et al. 2004).

Crude oil culture medium: Addition of crude oil into liquid mineral salts culture medium. The medium above were all sterilized for 30 min under 121°C.

Isolating, screening and identification of bacterial strains: Enrichment cultures were prepared by addition 10g of fresh contaminated soils mixed with four soil samples into 100 mL sterilized mineral salts broth contained in screw-capped 250 mL Erlenmeyer flasks. The cultures were incubated at 30°C in a THZ-82 shaker, manufactured by Changzhou Guohua Electronic Appliance Ltd, China, at a speed of 180 r/min for 7 days. Then, fifty millilitres (50 mL) of the enriched cultures were transferred into a 100 mL of fresh sterile mineral broth (250 mL flask) containing 1 mL of the sterile crude oil and were shaken again at a speed of 180 r/min during 7 days at 30°C for the second enrichment. After such four successive weekly transfers, the cultures with a series of concentration gradient were inoculated on the mineral salts agar containing quantity crude oil to get the enriched consortium and separated petroleum degrading microorganism with a clearing zone around the inoculated region. The isolation and purification of the bacterial consortium were carried out on nutrient agar plates by conventional spread plate techniques. Plates were incubated at 37°C for 48 h after which isolated colonies were selected for further identification. All isolates were stored below 20°C as liquid cultures containing 20% glycerol (v/v) (Zhao et al. 2009).

The procedure of secondary screening for hydrocarbon degradation was as follows. Isolates able to grow on the mineral salts agar containing crude oil were further subjected to secondary screening in 100 mL cultures contained in 250 mL screw-capped Erlenmeyer flasks containing crude oil as the sole carbon and energy source. The secondary screening was to provide quantitative data on degradation of crude oil that form basis for selection of isolates. The procedure for crude oil degradation was as described by Obuekwe and Al-Zarban (Obuekwe 1998). The inocula were 0.1 mL aliquots of overnight nutrient broth cultures, washed twice in physiological saline (0.87% NaCl, pH 7.2) and suspended in the same to optical density of 0.1 (OD_{600}). The crude oil cultures were incubated at 30°C, in a THZ-82 shaker, manufactured by Changzhou Guohua Electronic Appliance Ltd, China, at a speed of 180 r/min for up to 10 days uninoculated flasks constituted the controls, accounting for abiotic losses.

The preparation of the bacterium suspension was carried out by inoculating the strains into liquid beef grease and peptone medium, which was pre-sterilized under 121°C for 30 min. The mixture of medium and bacteria was shaken for 36 h (180 r/min) under 30°C. Later, the mixture was centrifuged (180 r/min) and the resulting suspension was discharged while the residual sediment was washed 3 times using phosphate buffer. Finally, the washed sediments were diluted using phosphate buffer in order to adjust the number of the cells in bacterium suspension to be 1×10^8.

Study on the growth characteristics of the strains: Under sterile conditions, strains were inoculated in 200 mL liquid beef grease and peptone medium, which had been previously sterilized. Then the mixture of bacteria and culture medium was shaken at 30°C and 180 r/min. Afterwards, optical density (OD_{600}) of the bacterium liquid using light (600 nm wavelength) was measured at regular intervals.

Extraction and analysis of petroleum hydrocarbons: This sample was shaken and the pH adjusted below 3. Then, the sample was placed into funnels, shook and the total volume brought to 100 mL after adding 20 mL of carbon tetrachloride in order to extract the hydrocarbons present in it. This mixture was kept static for segregation to take place (layered). The under layer was filtered and dried using anhydrous sodium sulphate and then placed into a volumetric flask (50 mL). The upper-layer was extracted using carbon tetrachloride twice, then filtered, and placed into the volumetric flask. The concentration of total petroleum hydrocarbons was determined using a non dispersive infrared oil analyser and the biodegradation of total petroleum hydrocarbon h was determined using the following equation (1).

$$h = \frac{c_0 - c_x}{c_0} \times 100\% \quad ...(1)$$

In this equation (1), c_0 and c_x represent the residual concentration of petroleum hydrocarbons in blank samples and the test samples in mg/L, respectively.

Hydrocarbon degradation efficiency tests of strains: Six common hydrocarbon compounds which included normal octane, paraffin wax, benzene, methyl benzene, phenol and naphthalene were added to the mineral medium that was previously sterilized (121°C for 30 min) using high pressure steam. Then, 5 mL of bacterium suspension with 0.1 of OD_{600} was added to the medium. This mixture was shaken for 36 hours under 30°C and 180 r/min. To note here for phenol and naphthalene, the bacterium suspension was added after all the phenol was evaporated from the mixture. Naphthalene firstly mixed with acetone was also added into the bacterium suspension after all the acetone was evaporated from the mixture. Finally, the optical density D_{600} of the culture solution under 600nm was measured and the concentration of total petroleum hydrocarbons was determined at regular intervals.

Evaluation of the factors influencing the activity of hydrocarbon-degrading bacteria: Crude oil samples were mixed with petroleum ether to prepare a solution having a concentration of 60 g of crude oil per litre of solution. Then, the mixture was filtered using a 0.25 mm filter membrane. The filtrate was placed into a flask and the petroleum ether was completely evaporated from the flask. Then, pre-sterilized mineral medium and 5 mL of bacterial suspension were added into the flask. Using this oil-contaminated soil samples as the starting point, several petroleum degradation experiments were performed at different conditions of pH, co organic matter, nitrogen source, carbon source and metal ions.

RESULTS AND DISCUSSION

Growth Characteristic of Hydrocarbon-Degrading Active Bacteria

Growth characteristic in hydrocarbon medium: Table 1 shows bacterial growth, bacterial density, and the rate of oil biodegradation reached by each strain after 7 days of cultivation. The strains grew well in oil media and emulsified crude oil. The density of bacteria after 7 days of cultivation was observed to range between 1×10^7/mL and 1×10^9/mL. These results indicate that the strains used petroleum as the carbon source. The biodegradability (*h*) after 7 days of cultivation was between 43.8% and 58.9%, which exceeded the biodegradability of formerly reported petroleum-degrading bacteria B01(25.8%-32.8%)(Lin et al. 1997) and was close to that of O-8-3 *Pseudomonas*, marine bacteria SJ-06W, SJ-6, and SJ-16A-2 as previously reported (Ding et al. 2001, Liang et al. 2004).

These strains of petroleum-degrading active bacteria were all Gram-negative bacteria. The strains SY_{21}, SY_{22}, SY_{23}, SY_{24}, SY_{42}, SY_{43} and SY_{44} were identified as *Acinetobacter*, *Neisseria*, *Plesiomonas*, *Xanthomonas*, *Zoogloea*, *Flavobacterium* and *Pseudomonas*, respectively. Previous research has shown that Gram-negative bacterium dominate in microbes that can degrade petroleum hydrocarbon (Chen et al. 2002). The *Xanthomonas*, *Zoogloea*, *Flavobacterium* and *Pseudomonas* strains have been extensively studied and used.

The growth trend in oil liquid medium: Fig. 1 shows bacterial growth as a function of time. The curves in Fig. 1 indicate that bacterial growth rates were low during the first 13 hours, after which the bacterial growth rates followed a logarithm growth period during the next 13~23 hours; and then turned into a slow down growth period during the following 23~40 hours. Finally, bacteria began to die after 40 hours of activity. Thus, the strains showed the highest activity during the 13 to 23 hours of life.

Growth trend in oil agar medium: Fig. 2 shows the growth trend of the same 7 strains in agar media and the variation of the diameter of colony forming of the different strains as a function of time. The colonies of SY_{21} were formed after 4 hours of activity. After 9.2 hours, the diameter reached 4 mm. This colony expanded continuously in the first 20 hours during which the average growth rate was 12.84 mm/d. In addition, the colony growth was circular having an ivory-opaque colour with an arid and disordered surface. The SY_{22} strain formed a circular ivory and semitransparent colony after 4 hours of inoculation. The surface of the colony was wet and orderly with a diameter of 3 mm after 9.2 hour. Similar to the previous case, the colony expanded continuously within the first 20 hours with an average growth rate of 8.41 mm/d. The colony formed by the strain SY_{23} was ivory and opaque with an arid and disordered surface. The SY_{23} colony was formed after 9.2 hours reaching a diameter of 2 mm after 15 hours. This colony also expanded continuously with an average growth rate of 2.49 mm/d. The SY_{24} formed an opaque and creamy yellow colony after 4 hours of inoculation. Its surface was flat and disordered, and the colony expanded continuously during the first 37 hours at a growth rate of 8.43 mm/d. After inoculation, the strain SY_{42} formed a white transparent and circular colony. The surface of the colony was wet and orderly. The average growth rate of this colony was 5.30 mm/d. The colony made up by the SY_{43} strain was white-transparent and disordered with a wet and smooth surface. The average growth rate of this colony was 5.30 mm/d. The SY_{44} strain formed an ivory semitransparent circular colony. Its surface was wet, smooth and orderly, and reaching a diameter of 4.2 mm after 4 hours. The colony expanded continuously during the first 15 hours with an

Table 1: Growth and identification of the isolated strains cultivated in hydrocarbon medium after 7 days of cultivation.

Strain	Growth and emulsification	Bacteria ($CFU.mL^{-1}$)	quantity h (%)	Identification
SY_{21}	Complete emulsification and dense liquid	5.3×10^7	43.8	*Acinetobacter*
SY_{22}	Forming oil film and flock	2.4×10^7	46.7	*Neisseria*
SY_{23}	Complete emulsification and dense liquid	3.6×10^9	58.9	*Plesiomonas*
SY_{24}	Complete emulsification and forming flock	1.2×10^7	45.0	*Xanthomonas*
SY_{42}	Complete emulsification and dense liquid	3.2×10^8	47.6	*Azotobacter*
SY_{43}	Forming oil film and flock	6.7×10^8	53.3	*Flavobacterium*

Table 2: Growth tendency and degradation efficiency of the seven isolated strains in different hydrocarbon media.

Hydrocarbon medium	OD_{600}							h (%)						
	SY_{21}	SY_{22}	SY_{23}	SY_{24}	SY_{42}	SY_{43}	SY_{44}	SY_{21}	SY_{22}	SY_{23}	SY_{24}	SY_{42}	SY_{43}	SY_{44}
C_8H_{18}	0.103	0.013	0.011	0.116	0.017	0.249	0.015	35.2	12.8	12.0	54.4	21.6	56.8	20.8
Paraffin wax	0.300	0.322	0.132	0.117	0.320	0.409	0.450	43.7	60.1	47.3	47.3	66.6	81.3	62.8
Benzene	0.023	0.011	0.120	0.08	0.036	0.056	0.035	21.0	10.0	90.5	80.9	46.0	71.2	63.9
Naphthalene	0.073	0.034	0.032	0.048	0.030	0.030	0.040	44.7	42.6	35.0	34.4	40.8	43.5	42.6
Phenol	0.033	0.017	0.112	0.104	0.058	0.085	0.067	21.0	10.0	90.5	80.9	46.0	71.2	63.9
Xylene	0.014	0.052	0.075	0.090	0.023	0.007	0.007	8.3	11.1	84.7	93.8	9.7	6.9	4.2

average growth rate of 24.32 mm/d. The average growth rate of the 7 isolated strains ranged from 2.49 to 32.4 mm/d.

The Degradation Ability of Petroleum-Degrading Active Bacteria Toward Different Types of Hydrocarbons

The majority of petroleum-degrading bacteria can degrade only few kinds of hydrocarbons (Wang et al. 1990, Chen & Liu 2002). The middle-chain and long-chain normal alkane can be degraded by most petroleum-degrading bacteria. However, the short-chain hydrocarbons and aromatic hydrocarbons can only be degraded by few petroleum-degrading bacteria. For the majority of bacteria it is difficult to digest short-chain and aromatic hydrocarbons, which can even be toxic.

In this work, the hydrocarbon degradation capability of the 7 strains was evaluated using the following hydrocarbon compounds: octane, paraffin wax, benzene, methyl benzene, phenol and naphthalene. The initial concentrations of these hydrocarbon compounds were 125 mg/L, 64800 mg/L, 200 mg/L, 14.4 mg/L, 200 mg/L, and 330 mg/L, respectively. During these tests the temperature and pH were set at 30°C and 7, respectively.

Table 2 shows that the seven strains grew in the paraffin wax media (The optical density D_{600} measured range from 0.117 to 0.450). The degradation efficiency of paraffin wax shown by the SY_{43} strain was 81.3%, which was the highest degradation efficiency observed, while the strain SY_{21} showed the lowest efficiency at 43.7% degradation. The degradation efficiencies of the other strains were between SY_{43} and SY_{21}. All of strains showed a high degradation capability toward middle and long-chain alkane, as the 90% of paraffin wax consisted of C_{18}~C_{61} normal and isomeric alkanes (Zhao et al. 1996). The degradation efficiencies of naphthalene by the seven strains were about 40%. The SY_{23} and SY_{24} strains showed high ability to degrade benzene, methyl benzene and phenol as the degradation efficiency reached from 80% to 90%. The majority of these strains showed low degrading efficiency toward normal octane, with the exception of strains SY_{24} and SY_{43}, which showed degradation rates of 54.4% and 56.8% respectively. These observations indicate that the strains SY_{22}, SY_{23}, SY_{24}, SY_{42}, and SY_{43} are capable of degrading more than one hydrocarbon, which makes them potential candidate strains for the bioremediation of petroleum contaminated soil.

Factors Influencing the Hydrocarbon Degradation Efficiency of Petroleum-Degrading Active Bacteria

The Effect of pH: In microorganisms, biochemical reactions are catalysed by enzymes. It is well known that enzymatic reactions occur within a suitable pH range and microorganisms are sensitive to the alteration in pH. Thus, it is necessary to determine the optimum pH value suitable for petroleum degradation by bacteria. The pH value of soil normally ranges between 2.5 and 11.0. Thus, before the inoculation of strains into the crude oil media (petroleum

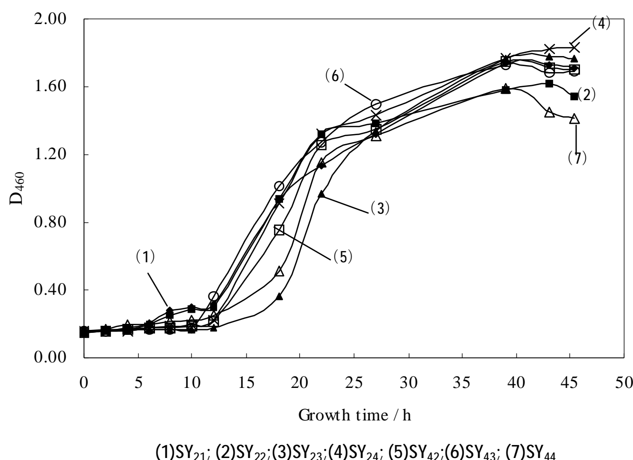

Fig. 1 Growth trend of the seven isolated strains in liquid cultivation medium.

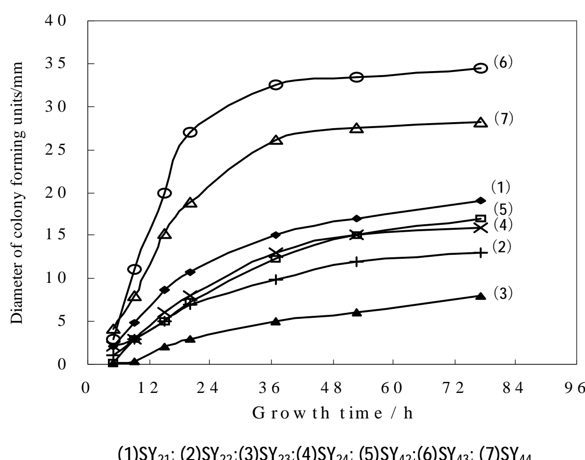

Fig. 2: Growth tendency of seven isolated strains in agar cultivation medium.

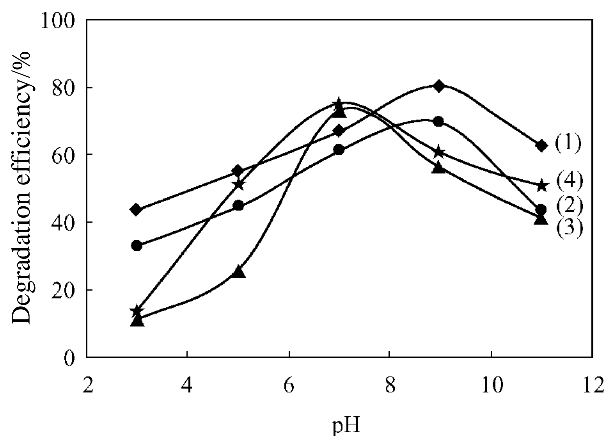

Fig. 3: Effect of pH value on the hydrocarbon degradation efficiency of some of the isolated strains.
(1) SY_{22}; (2) SY_{23}; (3) SY_{24}; (4) SY_{42}

concentration was fixed at 600 mg/L), the pH value was adjusted to 3, 5, 7, 9, and 11 for each medium. The experiments were carried out at a rotation speed of 180 r/min for 96 hours at 30°C, after which the concentration of petroleum hydrocarbon was determined and the degradation efficiency was calculated. Fig. 3 shows the degradation efficiency of four strains.

Fig. 3 indicates that SY_{22} and SY_{23} strains could degrade oil at a pH 9.0 with degradation efficiencies of 80% and 69.4%, respectively. Meanwhile, strains SY_{24} and SY_{42} had the ability of degrading oil at a pH value of 7.0 with degradation efficiencies of 73.1% and 74.9%, respectively.

Effect of petroleum hydrocarbon concentration: Five (5) mL of bacterium suspension was inoculated in the crude oil media. The concentration of hydrocarbon was varied as follows: 200, 600, 1000, 1500, 3000 mg/L. The Bioremediation tests were conducted at 30°C and the pH was adjusted to a value of 8.0. The rotation speed was set at 180r/min for 96 hours. Table 3 summarizes the degradation efficiencies of the strains. The observations in Table 3 indicate that the hydrocarbon degradation efficiency shown by these strains exceeded 60% when the mass concentration of petroleum hydrocarbon was 1000 mg/L. The hydrocarbon degradation efficiency shown by the SY_{24} strain was reduced when the mass concentration of petroleum hydrocarbon increased to 1500 mg/L. The hydrocarbon degradation efficiency of all the strains was reduced when the mass concentration of petroleum hydrocarbon was increased to 3000 mg/L. These results indicate that excessive concentration of petroleum hydrocarbon restricted the growth of the strains and consequently reduced the hydrocarbon degradation efficiency. The SY_{23} strain showed the highest TPH degradation efficiency at all TPH concentrations, which indicates its endurance to the TPH toxicity.

Effect of different nitrogen sources: In order to determine the impact of the nitrogen source on the strain degradation efficiency, a series of different nitrogen sources were used in addition to NH_4NO_3 (200 mg/L) in the inorganic media. The concentration of the different nitrogen sources was set as 350 mg/L. The rotation speed was set at 180r/min for 96 hours at 30°C and at pH of 8.0. After 96 hours of inoculation the concentration of the residual petroleum was determined. Table 4 presents the hydrocarbon degradation efficiency as a function of nitrogen source. Table 4 indicates that all the strains showed the highest degradation efficiency when NH_4NO_3 was used as the nitrogen source, while the lowest degradation efficiencies were observed when $NaNO_3$

Table 3: Effect of petroleum hydrocarbon concentration on the strains hydrocarbon degradation efficiency.

TPH concentration (mg/L)	h (%)			
	SY_{22}	SY_{23}	SY_{24}	SY_{42}
200	35.3	61.5	57.5	49.2
600	57.8	62.0	60.5	60.6
1000	61.6	64.8	63.5	63.5
1500	63.2	66.8	50.2	65.8
3000	46.0	56.2	43.0	37.4

Table 4: Effect of nitrogen sources on the petroleum hydrocarbon degradation efficiency.

Nitrogen source	h (%)			
	SY_{22}	SY_{23}	SY_{24}	SY_{42}
NH_4NO_3	46.7	48.5	52.5	42.6
$(NH_4)_2SO_4$	20.2	47.1	30.0	30.9
$NaNO_3$	13.3	10.9	28.6	21.2

Table 5: Effect of carbon sources on the strains' petroleum hydrocarbon degradation efficiency.

Carbon source	h (%)			
	SY_{22}	SY_{23}	SY_{24}	SY_{42}
Oil	43.8	36.9	35.0	17.6
Oil + Starch	71.5	46.7	41.5	70.2
Oil + Glucose	58.2	60.8	30.7	35.6

Table 6: Effect of metal ions on the strains degradation efficiency.

Metal ion	h (%)		
	SY_{21}	SY_{23}	SY_{43}
-	58.2	38. 9	53.3
Fe^{2+}	64.1	46.7	40.2
Mn^{2+}	54.3	50.6	54.6
Ni^{2+}	33.3	20.0	10.9
Co^{2+}	42.5	43.0	45.1

was used as the nitrogen source. This indicates that NH_4NO_3 is the best nitrogen source for the strains under evaluation, which is in agreement with the results presented by other researchers (Wang et al. 2006). It is important to mention that the SY_{23} strain showed a high degradation efficiency when $(NH_4)_2SO_4$ and urea were used as nitrogen sources, which points out that SY_{23} could be used for remediation situations where these nitrogen sources are readily available.

Effect of different carbon sources: Glucose and starch were used as cosubstrate in the mineral medium in which crude oil concentration was fixed at 1000 mg/L. These experiments were carried out at a rotation speed of 180 r/min for 96 hours at a temperature and pH of 30°C and 8.0, respectively.

Table 5 showed the effect of dosing cosubstrate on the removal efficiency of petroleum. Table 5 shows that the degradation efficiency of crude oil by the SY_{22} and SY_{23} strains increased from 43.8% and 17.6% to 71.5% and 70.2%, respectively. These results show that the strains degradation efficiency was enhanced by the use of glucose and starch as carbon sources. The explanation is that glucose and starch can be used as co-metabolism medium during crude oil degradation process (Shen et al. 2005). The SY_{22} and SY_{42} strains had high degradation efficiency of naphthalene (42.6% and 40.8% as showed in Table 2), these efficiencies were improved after adding glucose and starch. The SY_{23} strain also showed high degradation efficiency of benzene, methyl benzene, and phenol with corresponding efficiencies of 90.5%, 84.7% and 90.5%. These observations indicate that the SY_{23} strain can degrade PAH in crude oil and that the degradation efficiency can be increased to a large extent after adding starch. Thus, bacterial activity can be improved by adding the appropriate carbon sources, in this case glucose and starch. It has been previously reported that using glucose as carbon source improves the degradation efficiency of PAH and if glucose is fed intermittently the abilities of bacteria to degrade crude oil could be maximized (Kishore et al. 2007).

Effect of different metal ions: The concentration of metal ions increased in oil fields due to the aging and mineralization of soil during the weathering process of petroleum contaminated soil (Alex et al. 2008). In order to find the impact of the presence of metal ions on the biological removal of petroleum, metal ions of Fe^{2+}, Mn^{2+}, Ni^{2+} and Co^{2+} were added into the mineral salt liquid media in which crude oil concentration was 1000 mg/L.

The experiment was carried out at rotation speed of 180 r/min for 96 hours at 30°C and a pH of 8.0. Table 6 shows the calculated oil removal efficiencies, and it clearly shows that the degradation efficiency of the strains declined significantly after adding Ni^{2+}. It seems that a high concentration of Ni^{2+} restricted the activities of the microorganisms. For instance, the degradation efficiency of SY_{43} strain was decreased from 53.3% to 10.9%. In contrast, adding Fe^{2+} improved the degradation efficiency of crude oil (SY_{21} and SY_{23} strain). However, the activity of SY_{43} strain was restricted. After adding Mn^{2+} the degradation efficiency of oil by SY_{23} strain was enhanced by 12% while the degradation efficiency of SY_{21} and SY_{43} strain was not affected. The degradation efficiency of oil achieved by the SY_{21} and SY_{43} strains decreased 16% and 12% respectively after adding the metal ions of Co^{2+}. These results point out that the addition of metal ions such as Fe^{2+} and Mn^{2+} has a favourable influence on the oil degradation efficiency. On the contrary, the

degradation efficiency of petroleum hydrocarbons was decreased after adding metal ions such Ni^{2+} and Co^{2+}.

CONCLUSIONS

1. Seven strains were isolated from oil contaminated soil. The degradation efficiency of oil after seven days of cultivation ranged from 43.8% to 58.9%, which indicates high strains activity. These strains show a logarithm growth trend after 12 to 13 hours of inoculation with an average growth rate of the respective colonies between 2.49 and 2.4 mm/d. The strains SY_{21}, SY_{22}, SY_{23}, SY_{24}, SY_{42}, $SY_{43,}$ and SY_{44} were categorized as *Acinetobacter, Neisseria, Plesiomonas, Xanthomonas, Zoogloea, Flavobacterium* and *Pseudomonas* respectively.
2. These strains are capable of using normal octane, paraffin wax, benzene, methyl benzene, phenol and naphthalene as the sole carbon source. Five of these strains: SY_{22}, SY_{23}, SY_{24}, SY_{42} and SY_{43} show the ability of degrading more than one hydrocarbon, which make them potential candidates for the bioremediation of petroleum contaminated soil.
3. A pH value of 7.0 was optimum for the growth of strains SY_{21} and SY_{42} while a pH value of 9.0 was optimum for the development of strains SY_{22} and SY_{23}. The strains show an optimum degradation of crude oil when NH_4NO_3 was used as a nitrogen source in contaminated soil containing oil concentrations ranging from 1000 mg/L to 1500mg/L. The oil degradation efficiency of strains SY_{22}, SY_{42} and SY_{23} is significantly enhanced by the addition of starch and glucose.
4. The presence of metal ions such as Ni^{2+} and Co^{2+} in the oil contaminated soil decreases the strains degradation efficiency of oil, while the presence of Fe^{2+} and Mn^{2+} does not affect the oil degradation by the strains, on the contrary might improve it.

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