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Carbon content reduction in a model reluctant clayey soil: Slurry phase n-hexadecane bioremediation

Ali Partovinia, Fereshteh Naeimpoor*, Parisa Hejazi

Biotechnology Research Laboratory, School of Chemical Engineering, Iran University of Science and Technology, P.O. Box 16846-13114, Tehran, Iran

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ABSTRACT

Clayey soils contaminated with organic pollutants are nowadays one of the important environmental issues as they are highly reluctant to conventional bioremediation techniques. In this study, biodegradability of n-hexadecane as a model contaminant in oil polluted clayey soil by an indigenous bacterium was investigated. Maximal bacterial growth was achieved at 8% (v/v) n-hexadecane as sole carbon and energy sources in aqueous phase. The predominant n-hexadecane uptake mechanism was identified to be biosurfactant-mediated using bacterial adhesion to hydrocarbon (BATH) test and surface tension measurements. The effect of n-hexadecane concentration, soil to water ratio, inoculum concentration and pH on total organic carbon (TOC) reduction from kaolin soil in slurry phase was investigated at two levels in shake flasks using full factorial experimental design method where 10,000 (mg n-hexadecane)(kg soil)$^{-1}$, soil–water ratio of 1:3, 10% (v/w) inoculum and pH of 7 resulted in the highest TOC reduction of 70% within 20 days. Additionally, slurry bioreactor experiments were performed to study the effect of various aeration rates on n-hexadecane biodegradation during 9 days where 2.5 vvm was found as an appropriate aeration rate leading to 54% TOC reduction. Slurry phase bioremediation is shown to be a successful method for remediation of clayey reluctant soils.

1. Introduction

Organic compound polluted soils are recognized as an important environmental issue due to their detrimental effects on human and various ecosystems. Diverse physical and chemical methods as well as bioremediation have been proposed and applied to remediate these contaminated soils, among which soil washing is recognized as a quick method compared to other methods [1]. However, due to the adaptability of microorganisms and other biological system, biological remediation is sought by many researchers to remediate environmental hazards [2–7]. Soil and groundwater can be remediated under in situ conditions, known as in situ bioremediation. This involves bioventing (supplying air and nutrients to stimulate the indigenous bacteria to degrade organic contaminants in soil), biosparging (injection of air to increase the oxygen concentrations in underground water as means of enhancing the rate of biological degradation) and bioaugmentation (addition of microorganisms to the contaminated sites) [8–10]. Ex situ bioremediation techniques require excavation of contaminated soils or pumping of water and include landfarming (spreading soil over an organized bed and stimulating the aerobic degradation of contaminants by indigenous microorganisms using periodical cultivating), composting (combining contaminated soil with nonhazardous organic such as fertilizer and agricultural wastes) and biopiles (combination of landfarming and composting techniques) [8,9,11,12]. The main advantage of bioremediation is its reduced cost compared to conventional techniques. Moreover it may lead to complete mineralization of the pollutant into inorganic minerals, H_2O, CO_2 (aerobic) or CH_4 (anaerobic). Bioremediation can deal with lower concentration of contaminants where the cleanup by physical or chemical methods would not be feasible [5,10].

Recently, slurry phase bioremediation as a simple and economical ex situ method has drawn the attention of some researchers [13–16]. In slurry bioreactors, a three-phase mixing condition is provided by addition of water to excavated soil in a containment vessel to enhance bioremediation rate [9]. Oxygen is supplied using compressed air diffusers or surface agitators. Biodegradation of contaminants in a slurry phase (SP) yields higher degradation rate compared to other biological treatments mentioned above [9,16,17]. In slurry phase, the increase in soil moisture results in a larger amount of solubilized contaminant and hence an elevated bioavailability [18]. Moreover, soil slurry bioreactors can be exploited to optimize and control the abiotic conditions of biodegradation [19,20].

Despite many researches on bioremediation of contaminated soils, removing organic compounds from finer particles such as

* Corresponding author. Tel.: +98 21 77240496; fax: +98 21 77240495.
E-mail address: fnaeim@iust.ac.ir (F. Naeimpoor).

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clay (mostly a particle size of nearly 5 μm) remains challenging due to their colloidal nature [15,21,22]. Clay particles are usually carriers of net negative surface charge and are good absorbents of water and ions. As a result of their high specific surface area, they strongly adsorb certain classes of compounds and are reluctant to conventional remediation techniques. Therefore, efficient removal of contaminants from clayey soils which can make up significant fraction of most soils (about 20–50%) [15,22] is demanding.

On the other side, one of the major environmental problems today is hydrocarbon contamination resulting from petroleum and petrochemical industries. In this group, the oil-polluted soils containing mostly aliphatic hydrocarbons namely alkanes, alkenes and alkynes, can be pointed out. Amongst alkanes, the mid length n-alkanes are identified as the most significant soil contaminants [23,24]. Belonging to this category, n-hexadecane ($C_{16}H_{34}$) has been used by many researchers as a model contaminant with low aqueous solubility (0.9 μg L^{-1} in distilled water at 25 °C) [24–27]. Bioremediation has been applied to degrade diverse oil components by bacteria [19,21,25–33] and fungi [24]. Biodegradation of n-hexadecane as a model contaminant has also been the subject of many studies focusing on hexadecane mineralization in diesel fuel contaminated soil, effect of biosurfactants, and hexadecane biochemical degradation pathways and its oxidation intermediates [24–31,34–38]. Among the microorganisms used for n-hexadecane biodegradation, *Pseudomonas* seems to be one of the best known bacteria capable of degrading n-hexadecane as sole carbon and energy sources [26–28,30,35,38].

Although slurry phase bioremediation of organic compound contaminated soils has been successfully used by some researchers, there have only been a few studies carried out on bioremediation of reluctant soils containing fine particles such as clayey soils [15,21]. Therefore, in this study, we use a model system consisting of kaolin particles with adsorbed n-hexadecane and the total organic carbon (TOC) measurement [39] is used to examine biodegradation of n-hexadecane and its immediate oxidation products which can be released in part by hexadecane-utilizers such as *Pseudomonas* during growth and/or after cell death [35]. Biodegradation capability of an indigenous bacterium and its properties is preliminary examined in aqueous phase. Subsequently, the influence of various factors on carbon content reduction in a clayey soil in slurry shake flask experiments is investigated. Furthermore, slurry phase bioreactor experiments are carried out to look into the effect of aeration rate as an important factor on bioremediation process.

2. Materials and methods

2.1. Clay soil preparation

Kaolin (SZWNK1 type) was obtained from Iran China Clay Industries Corporation (ICCIC) as a clayey soil containing (%, w/w): Al_2O_3, 24–25; SiO_2, 61–62; Fe_2O_3, 0.45–0.65; TiO_2, 0.04; CaO, 1.2–1.5; MgO, 0.6; Na_2O, 0.3 and K_2O, 0.4 having a LOI value (loss of weight on ignition) of 9.5–10 (%, w/w). Soil was sieved to obtain particles with sizes smaller than 150 μm (mesh size 100) and these particles were used in all slurry phase experiments. Soil matrix was then artificially contaminated by addition of 1000 or 3000 mg n-hexadecane dissolved in 100 mL n-hexane to 100 g soil. This is followed by homogenization of soil-contaminant-solvent mixture using ultrasonic homogenizer (Bandelin Electronic UW 2070) for 10 min and soil drying at 60 °C for 24 h. The soil is then aged for 7 days and finally sterilized. The treated soil will therefore contain 10,000 or 30,000 (mg n-hexadecane)(kg dry soil)$^{-1}$ as the original soil lacks organic carbon.

Table 1
Selected variable levels for experimental design in slurry phase shake flask experiments.

Levels	Variables			
	pH (A)	SWR[a] (B)	Inoculum (C) (%, v/w)	*n*-$C_{16}H_{34}$ (D) (mg HXD) (kg dry soil)$^{-1}$
Low (−1)	5.5	1:5	5	10,000
High (+1)	7.0	1:3	10	30,000

[a] Soil to water ratio.

2.2. Bacterial inoculum

The microorganism used in the study, an indigenous *Pseudomonas* sp., was subcultured on nutrient agar plates. A loop of cells from a fresh subculture (24 h) was transferred into 250-mL flasks containing 50 mL of nutrient broth (NB) and incubated at 30 °C and 200 rpm until the early stationary phase. The culture was then centrifuged at 4300 rpm for 20 min and cells were resuspended in a saline solution (8.5 g L^{-1} NaCl) to reach an OD_{600} of about 1 to obtain a uniform bacterial population for inoculation. A 10% v/v (v/w) bacterial inoculum was used in aqueous phase (slurry bioreactor) experiments. The amount of bacterial inoculum in slurry phase shake flask experiments was variable as shown in Table 1.

2.3. Culture media

The mineral salts medium (MSM) consisted of (in g L^{-1}): NH_4Cl, 4.0; KH_2PO_4, 2.5; NaCl, 0.5; $MgSO_4$, 0.3; $FeCl_3{\cdot}6H_2O$, 0.03; $CaCl_2$, 0.01 and $MnCl_2{\cdot}4H_2O$, 0.01 in distilled water. The pH of MSM medium was adjusted to 7 by addition of 2N NaOH before inoculation [27]. The MSM solution was used in all slurry phase experiments whilst it was supplemented with 0–10% (v/v) n-hexadecane as carbon and energy sources in all aqueous phase experiments.

2.4. Experiments

2.4.1. Aqueous phase experiments

Aqueous phase experiments were carried out in 250-mL flasks containing 50 mL medium. After inoculation, cultures were incubated at 30 °C on a rotary shaker (200 rpm) for 10 days. Biodegradation of n-hexadecane was assessed by measuring bacterial growth. n-Hexadecane at a concentration of 8% (v/v), leading to the maximum growth (see Section 3.1), was used in further experiments performed for 20 days to study the cell growth and bacterial adhesion ability to hydrocarbon.

2.4.2. Clayey soil slurry phase shake flask experiments

All slurry phase shake flask experiments were carried out in 250-mL Erlenmeyer flasks each containing 50 g slurry, incubated at 30 °C and 200 rpm for 20 days. The effects of four variables (see Table 1): pH (A), soil to water ratio (B), inoculum % (C) and n-hexadecane concentration (D) on biodegradation of n-hexadecane in clayey soil were investigated at two levels by performing 2^4 experiments based on full factorial design method as shown in Table 2. All designed experiments as well as control experiments for assessment of non-biological degradation were performed in duplicate. To examine hexadecane biodegradation, the soil total organic carbon (TOC) measurement was used. Viable bacterial count was also performed to indirectly measure hexadecane biodegradation. In addition, surface tension was measured as a criterion to investigate biosurfactant production. Minitab software V. 15 was used for statistical analyses.

Table 2
Full factorial experimental design for slurry phase shake flask experiments and the three responses.

No of exp.	X_A[a]	X_B	X_C	X_D	TOC reduction (%)	Surface tension ($mN\,m^{-1}$)	Viable bacterial count ($CFU \times 10^{-8}\,(mL\,slurry)^{-1}$)
1	−1	+1	−1	−1	39.6	46.3	90
2	+1	+1	−1	−1	51.5	37.8	120
3	−1	+1	+1	−1	45.7	40.0	102
4	+1	+1	+1	−1	71.2	29.3	202
5	−1	−1	−1	−1	28.3	52.0	69.5
6	+1	−1	−1	−1	62.8	37.7	165
7	−1	−1	+1	−1	34.2	54.4	106
8	+1	−1	+1	−1	44.2	47.9	120
9	−1	+1	−1	+1	17.7	57.7	60
10	+1	+1	−1	+1	18.4	54.2	89
11	−1	+1	+1	+1	19.7	52.3	85
12	+1	+1	+1	+1	16.3	59.6	67
13	−1	−1	−1	+1	11.8	53.6	58
14	+1	−1	−1	+1	42.6	43.8	190
15	−1	−1	+1	+1	13.6	49.7	109.5
16	+1	−1	+1	+1	49.6	40.9	177.5

[a] X_A to X_D are the coded values of variables *A* to *D*.

2.4.3. Clayey soil slurry phase bioreactor experiments

A 1 L laboratory glass bioreactor as shown in Fig. 1 was utilized to examine the effect of aeration rate (0, 2.5, 5 vvm) on n-hexadecane biodegradation in slurry phase. Into the bioreactor were added 100 g of contaminated soil, 260 mL of MSM solution and 40 mL of bacterial inoculum (soil to water ratio of 1:3). All experiments were performed at room temperature ($29 \pm 1\,^{\circ}C$) with initial pH of 7 for 9 days. One control experiment without inoculation at the highest aeration rate of 5 vvm was performed to examine the physicochemical effects.

Bioreactor was sterilized at 121 °C for 20 min in an autoclave. Air was sterilized via a 0.2 μm filter and passed through a humidifier before entering the bioreactor to avoid culture evaporation. Bioreactor off-gas was passed through a trap before leaving the system. A slurry sample of 2 mL was taken daily via a glass tube fixed into the bioreactor using a syringe and filter system allowing sterile air to purge the tube for the next sampling. Hexadecane biodegradation was assessed only by TOC measurement in bioreactor experiments.

2.5. Analyses

2.5.1. Aqueous phase experiments

2.5.1.1. Bacterial growth. To monitor the bacterial growth in aqueous phase either optical density or direct biomass measurements was used. Samples were first refrigerated at 4 °C to solidify the remaining n-hexadecane and the optical density of aqueous phase at 600 nm [25,27,28] was then measured with spectrophotometer (Metertech model SP8001). Eq. (1) was found to correlate the cell dry weight concentration (CDW in $g\,L^{-1}$) and the OD_{600} as an indirect measurement of bacterial growth in the linear range ($OD_{600} < 1$).

$$CDW\ (g\,L^{-1}) = (0.565)OD_{600}, \quad R^2 = 0.98 \tag{1}$$

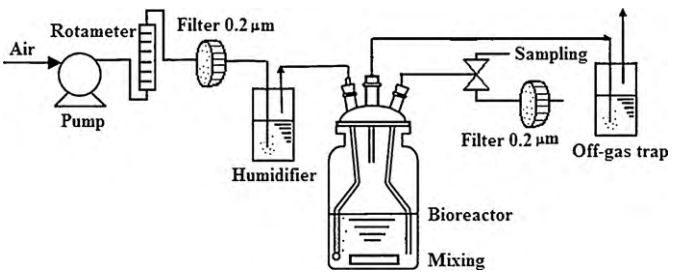

Fig. 1. Schematic diagram of the lab-scale slurry phase bioreactor.

2.5.1.2. Bacterial adhesion to hydrocarbon (BATH). Uptake of hydrophobic compounds is postulated to occur via interfacial accession and/or biosurfactant-mediated mechanisms [25,34]. Therefore, BATH test was performed according to Rosenberg et al. [40] to investigate the mechanism of n-hexadecane uptake by the selected bacterium. Bacteria were grown in MSM with 8% (v/v) n-hexadecane as the sole carbon source. At early stationary phase, the cell pellets were washed with phosphate buffer (pH = 7) and re-suspended in the same buffer to reach an optical density of 0.6 at 600 nm. Seven test tubes each containing 3 mL of this cell suspension and 0.15 mL of n-hexadecane were prepared, vortexed for 0, 10, 20, 30, 40, 50, 60 s and left for 10 min to separate the aqueous and organic phases. Finally, the optical density of the aqueous phase was measured at 600 nm for each mixing time. The percentage of bacterial adhesion to hydrocarbons: BATH%= $100 \times (1 - (OD_{600}$ after mixing/OD_{600} before mixing)) is reported versus mixing time [25,30,40].

2.5.1.3. Surface tension analysis. Samples were centrifuged at 4300 rpm for 20 min. The organic phase was extracted (by solidification) from the supernatant and the remaining aqueous phase was used for surface tension measurement via a digital tensiometer (Sigma, KSV 701) equipped with a 6 cm De Nuoy platinum ring. An average of triplicates reading was used in this study.

2.5.2. Clayey soil slurry phase experiments

For total organic carbon measurement, samples taken from either shake flasks or bioreactor were first centrifuged at 4000 rpm for 20 min. The solid phase was then dried at 40 °C for 24 h and about 0.25 g of this dried soil was used for TOC analysis. For shake flask experiments, the aqueous phase was refrigerated at 4 °C for 24 h to extract possible existing n-hexadecane by solidification and the remaining liquor was used for surface tension measurement.

2.5.2.1. Total organic carbon measurement. To estimate soil organic matter, carbon content determination is widely employed by using various methods such as dry combustion for total carbon and chromic acid oxidation for easily oxidizable carbon measurements. Since our contaminated clayey soil has n-hexadecane and its possible oxidation products, which contain only easily oxidizable carbon, we have used chromic acid oxidation method of Walkley and Black based on titration method for TOC measurement [21,41,42]. In this method, dichromate reacts with soil organic carbon as shown in Eq. (2):

$$2Cr_2O_7^{2-} + 3C + 16H^+ \rightarrow 4Cr^{3+} + 3CO_2 + 8H_2O \tag{2}$$

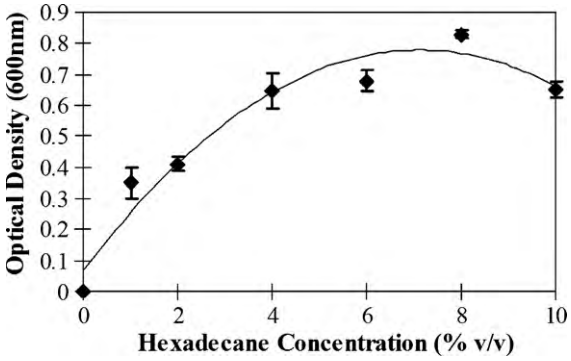

Fig. 2. Effect of initial n-hexadecane concentration on bacterial growth in aqueous phase.

The excess $Cr_2O_7^{2-}$ is then back titrated with standard Fe^{2+} solution (see Eq. (3)) to determine the amount of reacted dichromate.

$$6Fe^{2+} + Cr_2O_7^{2-} + 14H^+ \rightarrow 2Cr^{3+} + 6Fe^{3+} + 7H_2O \quad (3)$$

The easily oxidizable organic carbon in soil (%) is calculated as given in Eq. (4):

$$\%C = \frac{(B - S) \times M \text{ of } Fe^{2+} \times 12}{\text{g of Soil} \times 4000} \times 100 \quad (4)$$

where: B = mL of Fe^{2+} solution used to titrate blank. S = mL of Fe^{2+} solution used to titrate sample. M = molarity of Fe^{2+} solution. 12/4000 = milliequivalent weight of C in g.

To assess the accuracy of the measured TOC values (Eq. (4)), 7 standard polluted soil samples at 0, 5000, 10,000, 15,000, 20,000, 25,000, 30,000 (mg n-hexadecane)(kg dry soil)$^{-1}$ were prepared and their TOC were measured. A correction factor of 1.013 was obtained and Eq. (5) was used to report the TOC of the unknown samples.

$$TOC = \text{Correction Factor} \times (TOC)_{measured} \quad (5)$$

2.5.2.2. Surface tension analysis. Surface tension of the aqueous phase separated from the soil slurry was measured as described in Section 2.5.1.

2.5.2.3. Microbial analysis. From each slurry phase shake flask, 1 mL slurry sample was taken at sterile condition for enumeration of bacteria. The sample was serially diluted from 10^{-4} to 10^{-7} in distilled water. 0.1 mL of diluted samples was spread on nutrient agar plates (duplicated plates). Plates were incubated at 30 °C for 48 h. The colony forming units per milliliter of slurry (CFU mL^{-1}) was determined.

3. Results and discussion

3.1. Biodegradation of n-hexadecane in aqueous phase

Biodegradability of n-hexadecane at concentrations from 0 to 10% (v/v) (0–77 g L^{-1}) was investigated in aqueous phase. Fig. 2 illustrates the variation of culture optical density (OD_{600}) with n-hexadecane concentration, showing an increase in growth up to 8% (v/v) (61.6 g L^{-1}) n-hexadecane where a maximum growth is attained. Comparison of this high n-hexadecane concentration with the maximum value of 20 g L^{-1} used in many previous reports [26,28,29,34] shows that 8% (v/v) n-hexadecane utilized in this work is the highest n-hexadecane concentration used so far in aqueous phase shake flask experiments.

Time course of bacterial growth at 8% (v/v) n-hexadecane is shown in Fig. 3a. The culture experiences a 12-day delay since

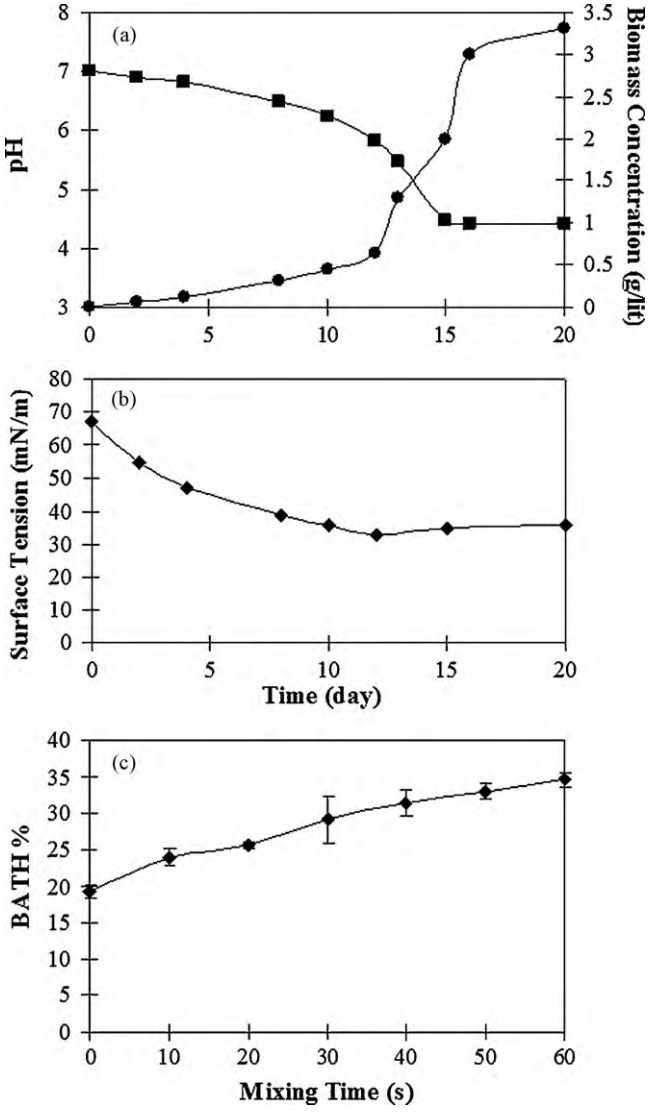

Fig. 3. Time course of batch culture and the effect of mixing time on bacterial adhesion to n-hexadecane (at early stationary phase) on mineral salt medium supplemented with 8% (v/v) n-hexadecane as sole carbon source in aqueous phase. Biomass concentration (●) and pH (■) (a), surface tension (b) and cell hydrophobicity (c).

no easily accessible carbon source is available. A sharp increase occurs thereafter in bacterial growth until the day 16 where it starts leveling off and finally it reaches 3.3 g dry weight of cells per liter. The culture pH decreases gradually from 7 to 6 and during the fast growth phase further decreases to about 4.5 and remains constant at stationary phase. It should be mentioned that reports exist on means of lessening the long lag phase such as addition of supplementary material (EDTA and yeast extract) [28] or using an adapted inoculum [34]. However, we preferred not to do so in order to make this study more useful for natural environment bioremediation purposes. Biosurfactant production was also examined as n-hexadecane degrading strains commonly produce biosurfactant [25,27,29]. Fig. 3b shows that the culture surface tension reduces from an initial value of about 68 mN m^{-1} to a minimum value of 32.8 mN m^{-1}. Given that biosurfactant-producers have the ability to reduce the surface tension below 40 mN m^{-1} [43] and since we had a pure culture with no initial surfactant in culture media, we conclude that biosurfactant was generated by this bacterium and it can be considered as a good biosurfactant producer. Interestingly, the onset of fast growth is

synchronized with the time (day 12) when surface tension first reaches its minimum value. This confirms that production of biosurfactant, preferably above CMC (critical micelle concentration), helps solubilizing and/or emulsifying hydrophobic compounds such as n-hexadecane into the growth medium and hence facilitating their biodegradation [33,44]. Similarly, Whang and co-workers have reported that addition of biosurfactant to diesel-water system at concentrations above CMC enhances diesel emulsification leading to improved diesel biodegradation [44].

With regard to the mechanism of hexadecane uptake by this bacterium, results obtained via BATH test, see Fig. 3c, show that the percentage of bacterial adhesion to n-hexadecane equals to 19% without mixing and this increases up to 35% by increasing the mixing time to 60 s. Previous studies have shown that biosurfactants produced by hydrophobic cells (high BATH value) had only a minor role in n-hexadecane degradation and hence interfacial accession is the predominant mechanism for n-hexadecane uptake [25,30,34]. On the contrary, biosurfactants produced by hydrophilic cells (low BATH value) were essential for growth on n-hexadecane [25,34]. Obtaining a relatively low BATH value for this strain showing significant surface tension reduction, reveals that biosurfactant-mediated uptake is the major mechanism for n-hexadecane uptake. One can then conclude that biosurfactants support growth of this strain via pseudosolubilization of n-hexadecane. Bouchez-Naitali and co-workers [25,34] have also reported biosurfactant-mediated mode of substrate uptake for two *Pseudomonas aeruginosa* strains (GL1 and Au1) grown on hexadecane.

3.2. Bioremediation of clayey soil in slurry phase shake flasks

The effects of four important variables each at two levels, as described in Table 2, on TOC reduction, surface tension and CFU

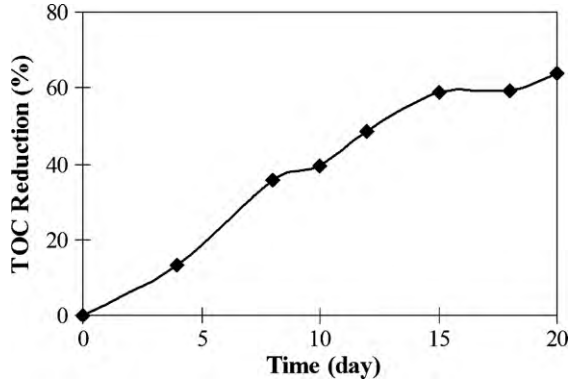

Fig. 4. Time course of TOC reduction for slurry shake flask experiment No. 4.

measurements (as a criterion for growth and substrate uptake) after 20 days are given in the last three columns of Table 2. To assure the suitability of the chosen time (20 days) for comparison purposes, the time course of n-hexadecane biodegradation is illustrated in Fig. 4 at the same conditions as experiment No. 4 (Table 2) leading to the highest n-hexadecane degradation. Fig. 4 confirms that at optimal conditions the maximum degradation of hexadecane is achieved within 20 days and hence this time a quite suitable for comparison purposes.

Statistical analyses of the three responses are depicted in Fig. 5. Our targets are to attain high TOC reduction, high CFU and low amount of surface tension (or high surface tension reduction). For all three responses, pH and n-hexadecane concentration were found to be effective variables albeit to different extents and trends. High level of pH and low level of n-hexadecane are in favor of TOC

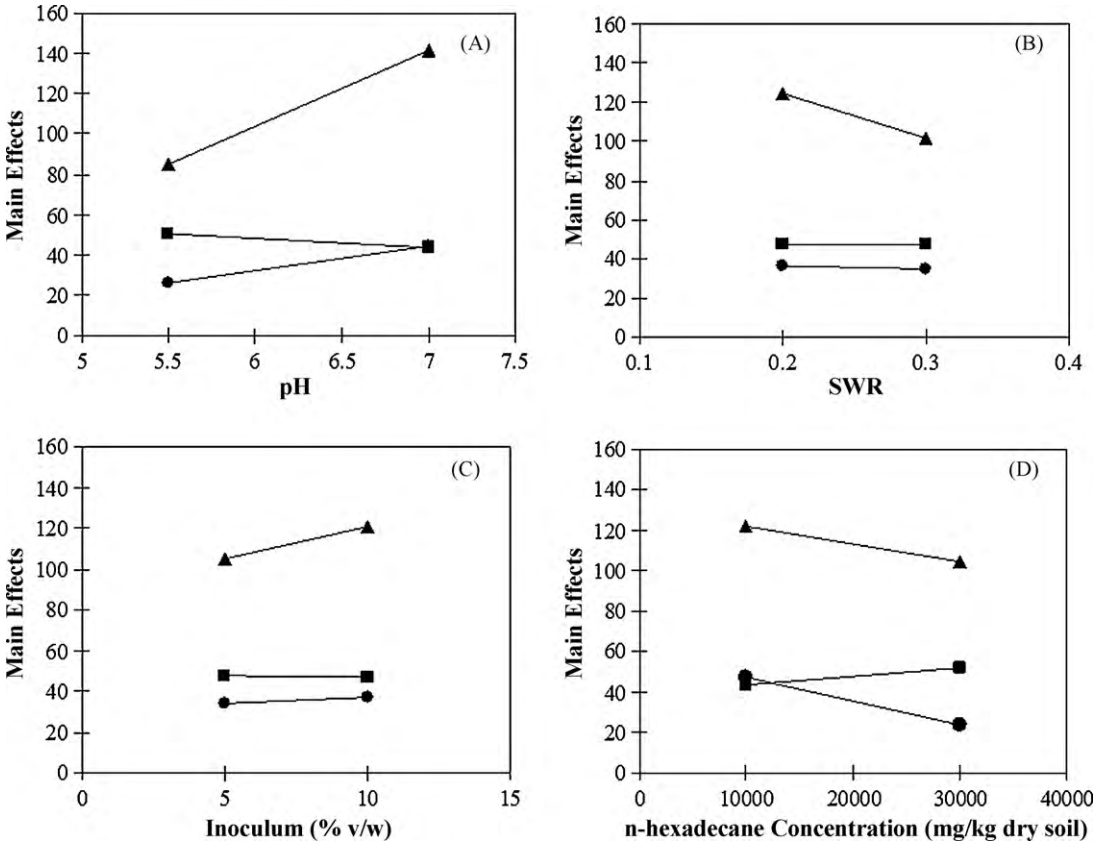

Fig. 5. Main effects chart of four variables (A) initial pH; (B) soil to water ratio; (C) inocolum % and (D) n-hexadecane concentration on the three responses in slurry phase shake flask experiments. TOC reduction (●); ST (■) and CFU (▲).

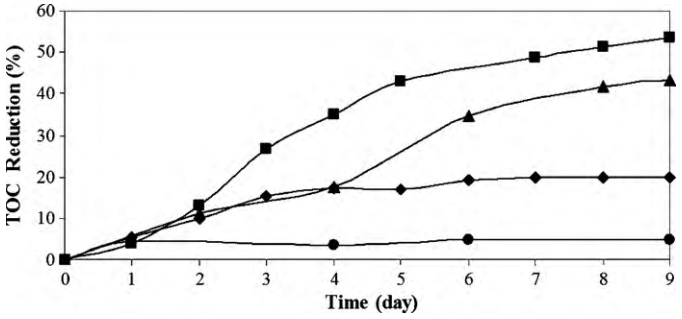

Fig. 6. Bioremediation of n-hexadecane polluted soil in slurry phase bioreactor. Without inoculation and 5 vvm aeration rate (●); zero aeration rate (▲); 2.5 vvm aeration rate (■) and 5 vvm aeration rate (♦).

and surface tension reductions. Inoculum (%) and soil to water ratio are recognized to have insignificant effects on the amount of TOC reduction and surface tension. Similar to our results, Nano and co-workers [14] have reported that 10, 20 and 40% soil concentration in slurry have insignificant effect on the final contaminant concentration. Noticeably, only CFU amount was influenced by all four variables examined in our work. High levels of pH and inoculum (%) and low levels of SWR and n-hexadecane support higher CFU amounts.

Run 4 with 10,000 mg n-hexadecane per kg soil, soil to water ratio of 1:3, inoculum concentration of 10% (v/w) and pH of 7 resulted in the highest n-hexadecane removal efficiency of 71.2 alongside the lowest surface tension of 29.3 mN m^{-1}. The second highest n-hexadecane removal rate of 62.8% was obtained at run 6 with 10,000 mg n-hexadecane per kg soil, soil to water ratio of 1:5, inoculum concentration of 5% (v/w) and pH of 7 which resulted in a surface tension of 37.7 mN m^{-1}. This confirms that biosurfactant production facilitates n-hexadecane biodegradation [29,33,44]. With respect to CFU, the highest amount is experienced at Run 4. Therefore, the conditions of run 4 i.e., pH = 7, n-hexadecane concentration of 10,000 mg (kg dry soil)$^{-1}$, SWR = 1:3 and 10% inoculum are in line with our targets and hence are the preferred values of the four examined variables. These conditions are used in all slurry phase bioreactor experiments.

3.3. Bioremediation of clayey soil in slurry phase bioreactor

To investigate the influence of aeration rate on n-hexadecane biodegradation, slurry phase bioreactor experiments were carried out for 9 days at 0, 2.5 and 5 vvm aeration rates while agitation rate remained constant. Results in Fig. 6 show a TOC reduction of 43% without aeration while aeration at a rate of 2.5 vvm increases the TOC reduction to 54%. Obviously, increasing the air flow rate can increase the contact between n-hexadecane, biomass and oxygen and thus enhancing the mass transfer and most likely biodegradation rate [32]. However, further increase in air flow to 5 vvm had negative effect on TOC reduction. This can be attributed to the inhibitory effect of oxygen at this level on n-hexadecane biodegradation.

The superiority of slurry bioreactor for n-hexadecane biodegradation compared to slurry shake flasks can be perceived by 54% TOC reduction in slurry bioreactor at aeration rate of 2.5 vvm within 9 days (see Fig. 6) whereas in shake flask experiment run 4 only about 37% TOC reduction was obtained (see Fig. 4) within the same period of time. This means that TOC reduction of about 6% per day is achievable in bioreactor for a period of 9 days, while that is about 4 for shake flask experiments. The increase observed in biodegradation rate in slurry bioreactor compared to shake flasks implies that much lower biodegradation time is needed to attain the same TOC reduction using slurry bioreactor [15,32]. Table 3 compares the time required for 43–44% TOC reduction in slurry shake flask and bioreactor experiments. It can be seen that the time required for this amount of TOC reduction in shake flask is nearly two folds higher than that of slurry bioreactor with 2.5 vvm aeration rate.

Table 3
Comparison of biodegradation time in slurry shake flask and bioreactor experiments for 43–44% TOC reduction.

Experiments	Rate of aeration (vvm)	TOC reduction (%)	Time (day)
Shake flask (run 4)	–	43.91	11
Bioreactor	0.0	43.35	9
	2.5	43.10	5

To determine the contribution of non-biological factors on TOC reduction, a bioreactor experiment at the highest examined aeration rate of 5 vvm was performed as control. Results showed a TOC reduction of only about 5% which could be attributed to physico-chemical effects.

4. Conclusion

The indigenous *Pseudomonas* sp. used in this work was well capable of biodegrading n-hexadecane at as high concentration as 10% (v/v) in aqueous phase as sole carbon and energy sources. However, inhibitory effect of n-hexadecane was observed above 8% (v/v). Biosurfactant-mediated uptake was found to be the dominant n-hexadecane uptake mechanism. Slurry phase bioremediation of the reluctant clayey soil used in this work resulted in a significant TOC reduction of about 70% in shake flask experiments. Hexadecane concentration and pH were found to be the effective parameters on TOC reduction in hexadecane biodegradation. For our reluctant clay contaminated soil, using properly aerated slurry bioreactor increased biodegradation rate and hence the biodegradation time reduced markedly compared to slurry shake flask.

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